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**Document Name:** AOAC: Official Methods of Analysis (Volume 1)

**CFR Section(s):** 9 CFR 318.19(b)

**Standards Body:** AOAC International

*Official Incorporator:*
THE EXECUTIVE DIRECTOR
OFFICE OF THE FEDERAL REGISTER
WASHINGTON, D.C.

APPROVED

# OFFICIAL METHODS of ANALYSIS

*15th Edition, 1990*

# ASSOCIATION of OFFICIAL ANALYTICAL CHEMISTS

*Agricultural Chemicals; Contaminants; Drugs*

# VOLUME ONE

# OFFICIAL
# METHODS OF ANALYSIS

OF THE

## ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS

EDITED BY KENNETH HELRICH

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FIFTEENTH EDITION, 1990

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PUBLISHED BY THE
ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, INC.
SUITE 400
2200 WILSON BOULEVARD
ARLINGTON, VIRGINIA 22201 USA

Printed in the United States of America

Printed on acid-free paper

Library of Congress Catalog Card Number 85-647589
ISBN 0-935584-42-0
ISSN 0066-961X

A copy of the 15th edition of this publication is on file with the Office of the Federal Register. U.S. Government Agencies may apply to the Director of the Office of the Federal Register for approval to incorporate this edition by reference in their regulations. The procedures that Federal agencies must follow in applying for the Director's approval are in Title 1, Part 51, of the *Code of Federal Regulations*.

# Important Notices to Librarians and All Users of this Edition

## FREE SUPPLEMENTS

Purchasers of this volume will receive the annual supplements of *Changes in Official Methods of Analysis* without additional charge only by removing the card inserted after p. 40, filling in the specific name and address to which the supplements should be sent, and returning this card to the Association. Address must include postal code or delivery cannot be assured.

## SURPLUS METHODS

Neither the 10th, 11th, 12th, 13th, or 14th editions should be destroyed upon appearance of the 15th edition. They contain surplus methods which are not reprinted in the 15th edition. See page xvii for the definition of surplus methods. If methods marked with this symbol, ★, continue to be used and therefore should be retained in full, please notify the Association.

## USE OF METHODS

Analytical methods and procedures included in this volume are those which AOAC members have evaluated and validated through collaborative studies and appropriate similar techniques to give accurate and reproducible analytical results on the matrix and analyte to which the method is stated to apply, provided the analysis is conducted by a competent analyst as written. No warranty, implied or expressed, is made by the Association on methods described or products mentioned. The mention of commercial trade names does not imply endorsement by AOAC or its members over similar products that might be suitable. AOAC and its members who have aided in development and validation of methods enclosed assume no responsibility for any economic, personal injury, or other damage that may occur to individuals or organizations because of use of such methods.

## EQUIVALENT COMMERCIAL PRODUCTS

AOAC recognizes that the instrumentation and laboratory supplies stated in these published methods may have equivalents which will produce analytical results having the same statistical performance. Names of manufacturers and suppliers and trade names are furnished solely as a matter of identification and convenience within the context of the way each method was developed within the originator's laboratories, without implication of AOAC promotion, approval, endorsement, or certification. The same or equivalent products, instruments, supplies, apparatus, or reagents available from suppliers other than those named or other brands from other sources may serve equally well if proper validation indicates that their use is satisfactory.

## INQUIRIES

Inquiries regarding procedures published in this book should be directed to AOAC, Executive Director, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. Telephone (703) 522-3032. Facsimile (703) 522-5468.

Inquiries regarding purchase of *Official Methods of Analysis,* supplements ("Changes in Official Methods"), *Journal* of the AOAC, or other AOAC publications should be directed to AOAC, Fulfillment Coordinator, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. Telephone (703) 522-3032. Facsimile (703) 522-5468.

## COMMENTS ON METHODS

AOAC adopts methods that show by their performance data, obtained through the collaborative study, what can be expected of them. As analysts use AOAC methods, they generate additional information and data concerning the applicability, specificity, sensitivity, and accuracy of the methods. Analysts are requested to advise AOAC about their experiences with official methods published in this book. In particular, analysts should notify AOAC of problems in the performance of any method that indicate the method should be revised or restudied. Direct comments to AOAC, Assistant Executive Director, Technical Services and Development, 2200 Wilson Blvd, Arlington, VA 22201-3301 USA. Telephone (703) 522-3032. Facsimile (703) 522-5468.

# Preface to the 15th Edition

The most obvious change in this new edition of *Official Methods of Analysis of the AOAC* is the new format, splitting the original single volume into two volumes containing Agricultural Chemicals, Contaminants, and Drugs in Volume I, and Food Composition in Volume II. Extensive discussions, user polls, and committee deliberations regarding the most useful and desirable form for publication of the 15th Edition led to the decision to make this change. While this has necessitated the repetition of a few items such as the index and the safety chapter, the convenience of smaller volumes with a logical division of subject matter is a definite advantage. The two volume arrangement also allows for more manageable growth as the number of validated methods increases.

In actual content, the most striking change in this new edition is the assignment of permanent numbers to all official methods. This tedious and time consuming task was undertaken primarily because, as Editor William Horwitz stated in the preface to the Thirteenth Edition in 1980, "Users expressed a desire for a system that will keep the same reference number of a given method from edition to edition." There are significant advantages to a permanent numbering system. Since AOAC methods are cited worldwide in laws and regulations at every level of government, in definitions of standards of identity, and in public and private specifications and contracts, it is practical and highly desirable to have a single, unchanging number for any method. Permanent numbers will reduce citation errors and simplify citation by eliminating the necessity to specify editions in some instances. The publication of future editions will not be encumbered by the need to keep track of changing numbers for existing, unchanged methods. There is also the advantage of desirable consistency for electronic databases.

Permanent numbers are based on the year the method first appeared in "Changes in Official Methods of Analysis" in the *Journal*. The year determines the first three numbers with the next digits being simply the sequence in which the methods were adopted in a given year. For example, the first method adopted in 1988 and published in "Changes in Official Methods of Analysis" in 1989 would be given the number 989.01. The year of adoption was not researched for methods adopted before 1960. For those, the numbers are based on the date of the first reference, or, if that was not available, on the year the method first appeared in *Official Methods of Analysis of the AOAC*. An index to the new numbers is included to facilitate locating methods when only the method number is known. References for the more recent methods in the 15th Edition have been verified, corrected, and brought up to date so that the user can more readily find the original work that resulted in adoption of the method. The list of suppliers, as well as supplier references in each method, has also been revised and updated.

Method performance data appear at the beginning of methods adopted as part of "Changes in Official Methods of Analysis" in 1989. Previously published method performance data (14th Edition) have been deleted because of a change in the procedure for calculation. All future new methods will have the method performance section included, using the performance parameters that were adopted by the AOAC Board of Directors in 1988. Method performance data are generated from the collaborative study results.

The addition of about 150 new methods to the 15th Edition continues to respond to the AOAC mandate to keep pace with the practical needs of regulatory and research chemists and microbiologists. Some previously adopted methods have been expanded in scope; some have had efficiency or accuracy improved. Additional methods have been declared surplus and omitted from the present Edition in instances where they were no longer sufficiently used to warrant reprinting. Again, the user is asked to preserve previous editions for the rare instance when a surplus method may be needed.

Liquid chromatography (LC) and gas chromatography (GC) have continued to be the most popular and useful techniques of analysis. A variety of detectors are still being utilized, along with internal standards. In addition to sophisticated modern instrumentation, such classical techniques as gravimetric analysis, distillation and physical separations, and Kjeldahl nitrogen determinations are still yielding new, needed methods.

Among the most innovative techniques are the additions to the chapter on Microbiological Methods. Over twenty new methods have been added to this chapter. These include DNA colony hybridization, enzyme immunoassay, microbial receptor assay, and immunodiffusion methods. Many new methods utilize beads, pretreated pectin gel films, dry rehydratable films, and hydrophobic grid plates. Sources of the kits and pertinent information on components are provided. In some instances, generic substitutions are possible for kit components.

As always, thanks are due to the many individuals who worked so diligently and carefully to maintain the quality of the *Official Methods of Analysis of the AOAC*. These include the Associate Referees, General Referees, collaborators, and Methods Committee members who researched, perfected, validated, and reviewed each method. The General Referees, beyond the call of duty, contributed their services as Chapter Associate Editors, arranging and reviewing chapters where their expertise was an invaluable asset. The AOAC Official Methods Board, Editorial Board, and Board of Directors provided the guidance throughout the duration of this project. The AOAC Scientific Publications staff did a heroic job from the very beginning in editing methods, keeping the methods publication on schedule, handling the unending details of actual publication, and renumbering over 1,000 pages of methods with innumerable cross references.

Kenneth Helrich
Editor, *Official Methods of Analysis*

# Contents

## Volume I

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# About the Association

## PURPOSE AND FUNCTION

The primary objectives of the Association of Official Analytical Chemists (AOAC) are to obtain, improve, develop, test, and adopt uniform, precise, and accurate methods for the analysis of foods, drugs, feeds, fertilizers, pesticides, water, or any other substances affecting public health and safety, economic protection of the consumer, or quality of the environment; to promote uniformity and reliability in the statement of analytical results; to promote, conduct, and encourage research in the analytical sciences related to agriculture and public health and the regulatory control of commodities in these fields; and to afford opportunity for discussion of matters of interest to scientists engaged in relevant pursuits.

AOAC itself maintains no laboratories, conducts no analyses, performs no tests. The actual work of devising and testing methods is done by members of AOAC in their official and professional capacities as staff scientists of federal, state, provincial, and municipal regulatory agencies, experiment stations, colleges and universities, commercial firms, and consulting laboratories.

AOAC coordinates these scientific studies, receives and evaluates the results, gives official sanction to acceptable methods, and publishes and disseminates the methods.

The reliability of methods of analysis is more important than ever before. Regulatory agencies need reliable, reproducible, and practical methods to enforce laws and regulations. Industry needs reliable methods to meet compliance and quality control requirements. Few organizations in the world are devoted primarily to testing and validating analytical methods through interlaboratory collaborative studies—as is AOAC.

## MEETINGS

The Annual International Meeting is the focal point of AOAC's yearly work. Here, members have opportunities to exchange ideas with colleagues from all over the world, and to update their technical knowledge at scientific sessions and symposia, exhibits, and short courses.

The regional section program provides AOAC-affiliated local or regional scientific meetings, workshops, short courses, and other activities for laboratory analysts. Each regional section is organized by a local volunteer committee.

## COOPERATIVE ACTIVITIES

AOAC has established joint committees, liaison, and representation with numerous scientific organizations worldwide. Thus, methods are often developed in cooperation with other standards-setting bodies. AOAC liaison representatives for contact outside North America are the following: Derek Abbott, 33 Agates Ln, Ashtead, Surrey KT21 2ND, England; Lars Appelqvist, Swedish University of Agricultural Sciences, Dept of Food Hygiene, S 750 07 Uppsala, Sweden; and Margreet Lauwaars, PO Box 153, 6720 AD Bennekom, The Netherlands.

## AWARDS

The awards program of AOAC includes the following: The Scholarship Award is given each year to a student intending to do further study or work in an area important to public health or agriculture.

The Fellow of the AOAC Award is given to selected members in recognition of at least 10 years of meritorious service to the Association as referees and/or committee members.

The Harvey W. Wiley Award, honoring the "father" of the U.S. Pure Food and Drug Act and a founder of AOAC, is presented each year to a scientist or group of scientists who have made outstanding contributions to analytical methodology in an area of interest to AOAC. The $2500 award is supported by the Wiley Fund.

## PUBLICATIONS

*Official Methods of Analysis* includes full details of official methods but no descriptive or interpretative material or tables of data. However, AOAC publishes the *Journal of the AOAC*, which contains research articles and reports of the development, validation, and interpretation of analytical methods, and all collaborative study results. *Journal* contributors and its readers represent the worldwide analytical science community. The *Journal* is a forum for the exchange of information among methods researchers. The *Journal* also records the transactions of the Annual International Meeting, including committee and referee reports, lists of officers, referees, and committee members, and all official actions of the Association, including newly adopted methods. The Association publishes a variety of other books, manuals, video tapes, and symposium proceedings of interest to analytical scientists.

## MEMBERSHIP

The organization of AOAC consists of the members; the Board of Directors, a governing body concerned with administration and policy making; Official Methods Board; Editorial Board; special standing committees and other groups concerned with development of methods and general activities; and the headquarters staff which carries out the publications program and manages the Association.

The AOAC Bylaws provide for individual members and sustaining members. Chemists, microbiologists, and other scientists engaged in analysis or analytical research related to agriculture and public health, and employed by a college or university, any agency of a local, state, provincial, or national government, or firm or industry concerned with commodities or substances of interest to AOAC may be members. Sustaining members are government agencies or private industries that provide financial support to AOAC.

*The Referee*, published 12 times yearly, is sent free to all members, and contains news about methods, collaborative studies, meetings, publications, AOAC people, board and committee activities, regional sections, and other items of interest. All members also receive the membership directory, issued annually.

# GUIDE TO METHOD FORMAT*

**Unique number** identifies method by year of adoption or first appearance in *Official Methods of Analysis* (older methods). 980= first action 1980; .06= sequence of adoption in 1980.

**Method head** may include analyte and matrix, type of method, official status, cooperating organization.

**980.06 Captan in Pesticide Formulations**
**Liquid Chromatographic Method**
**First Action 1980**
**Final Action 1982**
***AOAC-CIPAC Method***

(Method is suitable for tech. captan and formulations with captan as only active ingredient.)

**Applicability statement**—limitations on use of method or other information.

**Chemical names** of pesticides and drugs are given at end of pertinent chapter.

***A. Principle***

Captan is extd from inerts with soln of diethyl phthalate in $CH_2Cl_2$. Soln is chromatgd on microparticulate silica gel column, using $CH_2Cl_2$ as mobile phase. Ratio of captan peak ht to diethyl phthalate peak ht is calcd from UV response and compared to std material for quantitation.

**Scientific basis** for method of analysis.

**Cautionary notes** refer to Safety Chapter.

(*Caution:* See safety notes on pipets and pesticides.)

***B. Apparatus and Reagents***

(**a**) *Liquid chromatograph.*—Able to generate over 1000 psi and measure $A$ at 254 nm.

**Specifications** for necessary laboratory apparatus and reagent preparations. See also "Definitions of Terms and Explanatory Notes."

**Addresses** for suppliers frequently cited throughout book are listed in "Definitions of Terms and Explanatory Notes."

(**d**) *Diethyl phthalate.*—EM Science No. 1295.
(**e**) *Reference std captan.*—Chevron Chemical Co., PO Box 4010, Richmond, CA 94804.

***C. Preparation of Standard***

**Method** may be divided into several descriptive sections.

(**a**) *Internal std soln.*—0.312 mg diethyl phthalate/mL. Weigh ca 156 mg diethyl phthalate and transfer to 500 mL vol. flask. Dil. to vol. with same $CH_2Cl_2$ to be used for mobile . . . within 20%.

**Letters** identify main sections for ease of citation and cross-referencing.

***D. Preparation of Sample***

Accurately weigh sample expected to contain 40 mg captan into glass bottle. Pipet in 50 mL internal std soln. Place on mech. shaker 15 min. Centrf. and filter supernate thru glass fiber paper. Prep. fresh sample daily.

***E. Determination***

Adjust operating parameters to cause captan to elute in 4–6 min. Maintain all parameters const thruout analysis. Typical values are: flow rate, 2.5 mL $CH_2Cl_2$/min, max.; pressure, ca 800 psi; chart speed 0.2"/min; mobile phase . . . immediately preceding and following sample injections must agree to within ± 2% of their mean. If not, repeat detn.

**Abbreviations** used throughout method are defined in "Definitions of Terms and Explanatory Notes."

***F. Calculation***

Measure peak hts to 3 significant figures, and calc. ratio for each injection. Average 4 std ratios, and the 2 sample ratios.

**Calculation symbols** are identified and show correct units.

$$\% \text{ Captan} = (R/R') \times (W'/W) \times P$$

where $R$ = av. sample ratio (captan peak ht/diethyl phthalate peak ht); $R'$ = av. std ratio (captan peak ht/diethyl phthalate peak ht); $W$ = mg sample; $W'$ = mg std, and $P$ = % purity of std.

Ref.: JAOAC **63**, 1231(1980).

**References** direct the user to the published collaborative study and any subsequent revisions in the method. Other informative references may be included.

CAS-133-06-2 (captan)

**Chemical Abstracts Service** Registry Number. A unique identifier that may be used to search a number of data-retrieval systems.

*Method shown is incomplete to allow space for description.

# Definition of Terms and Explanatory Notes

### *Official Methods*

(1) Official methods are designated first action or final action, and, in a few cases, procedures. A first action method has undergone collaborative study, has been recommended by the appropriate General Referee and Methods Committee, has been approved interim first action by the chairman of the Official Methods Board, and has been adopted official by the Association members at an annual meeting. A method may be adopted final action a minimum of 2 years after it has been adopted first action, and, again, after it has been recommended by the appropriate General Referee and Methods Committee and voted on by the Association members at an annual meeting.

A sampling or sample preparation procedure or other type of procedure for which an interlaboratory collaborative study is impractical may be adopted, as above, as a procedure.

All methods in this book—first action, final action, or procedure—are official methods of AOAC.

### *Reagents*

(2) Term "$H_2O$" means distilled water, except where otherwise specified, and except where the water does not mix with the determination, as in "$H_2O$ bath."

(3) Term "alcohol" means 95% ethanol by volume. Alcohol of strength $x$% may be prepared by diluting $x$ mL 95% alcohol to 95 mL with $H_2O$. Absolute alcohol is 99.5% by volume. Formulas of specially denatured alcohols (SDA) used as reagents are as follows:

| SDA No. | 100 | parts alcohol plus |
|---|---|---|
| 1 | 5 | wood alcohol |
| 2-B | 0.5 | benzene or rubber hydrocarbon solv. |
| 3-A | 5 | MeOH |
| 12-A | 5 | benzene |
| 13-A | 10 | ether |
| 23-A | 10 | acetone |
| 30 | 10 | MeOH |

"Reagent" alcohol is 95 parts SDA 3-A plus 5 parts isopropanol.

(4) Term "ether" means ethyl ether, peroxide-free by following test: To 420 mL ether in separator add 9.0 mL 1% $NH_4VO_3$ in $H_2SO_4$ (1 + 16). Shake 3 min and let separate. Drain lower layer into 25 mL glass-stoppered graduate, dilute to 10 mL with $H_2SO_4$ (1 + 16), and mix. Any orange color should not exceed that produced by 0.30 mg $H_2O_2$ (1 mL of solution prepared by diluting 1 mL 30% $H_2O_2$ to 100 mL with $H_2O$) and 9.0 mL 1% $NH_4VO_3$ in $H_2SO_4$ (1 + 16). Peroxides may be eliminated by passing ≤700 mL ether through 10 cm column of Woelm basic alumina in 22 mm id tube.

(5) Reagents listed below, unless otherwise specified, have approximate strength stated and conform in purity with Recommended Specifications for Analytical Reagent Chemicals of the American Chemical Society:

| | Assay |
|---|---|
| Sulfuric acid | 95.0–98.0% $H_2SO_4$ |
| Hydrochloric acid | 36.5–38.0% HCl |
| Nitric acid | 69.0–71.0% $HNO_3$ |
| Fuming nitric acid | ≥90% $HNO_3$ |
| Acetic acid | ≥99.7% $HC_2H_3O_2$ |
| Hydrobromic acid | 47.0–49.0% HBr |
| Ammonium hydroxide | 28–30% $NH_3$ |
| Phosphoric acid | ≥85% $H_3PO_4$ |

Where no indication of dilution is given, reagent concentration is the concentration given above.

(6) All other reagents and test solutions, unless otherwise described in the text, conform to requirements of the American Chemical Society. Where such specifications have not been prepared, use highest grade reagent. When anhydrous salt is intended, it is so stated; otherwise the crystallized product is meant.

(7) Unless otherwise specified, phenolphthalein (phthln) used as indicator is 1% alcohol solution; methyl orange is 0.1% aqueous solution; methyl red is 0.1% alcohol solution.

(8) Directions for standardizing reagents are given in the chapter on Standard Solutions and Certified Reference Materials.

(9) Unusual reagents not mentioned in reagent sections or cross referenced, other than common reagents normally found in laboratory, are italicized the first time they occur in a method.

(10) Commercially prepared reagent solutions must be checked for applicability to specific method. They may contain undeclared buffers, preservatives, chelating agents, etc.

(11) In expressions (1 + 2), (5 + 4), etc., used in connection with name of reagent, first numeral indicates volume reagent used, and second numeral indicates volume of $H_2O$. For example, HCl (1 + 2) means reagent prepared by mixing 1 volume of HCl with 2 volumes of $H_2O$. When one of the reagents is a solid, expression means parts by weight, first numeral representing solid reagent and second numeral $H_2O$. Solutions for which the solvent is not specified are aqueous solutions.

(12) In making up solutions of definite percentage, it is understood that $x$ g substance is dissolved in $H_2O$ and diluted to 100 mL. Although not theoretically correct, this convention will not result in any appreciable error in any methods given in this book.

(13) Chromic acid cleaning solution is prepared by (*1*) adding 1 L $H_2SO_4$ to approx. 35 mL saturated aqueous $Na_2Cr_2O_7$ solution; or (*2*) adding 2220 mL (9 lb) $H_2SO_4$ to approx. 25 mL saturated aqueous $CrO_3$ solution (170 g/100 mL). Reagents may be technical high grade. Use only after first cleaning by other means (e.g., detergent) and draining. Mixture is expensive and hazardous. Use repeatedly until it is diluted or has a greenish tinge. Discard carefully with copious amounts of $H_2O$.

(14) All calculations are based on table of international atomic weights.

### *Apparatus*

(15) Burets, volumetric flasks, and pipets conform to the following U.S. Federal specifications (available from General Services Administration, Specification Activity 3F1, Washington Navy Yard, Building 197, Washington, DC 20407):

| | | |
|---|---|---|
| Buret | NNN-B-00789a | May 19, 1965 |
| Flask, vol. | NNN-F-289d | Feb. 7, 1977 |
| Pipet, vol. | NNN-P-395d | Feb. 24, 1978 |
| Pipet, measuring | NNN-P-350c | July 16, 1973 |

See also Appendix V, "Testing of Glass Volumetric Apparatus," in NIST Specification Publication 260-54, "Certification and Use of Acidic Potassium Dichromate Solutions as an Ultraviolet Absorbance Standard SRM935" (available from NIST, Office of Standard Reference Materials, B316 Chemicals, Gaithersburg, MD 20899).

(16) Standard taper ($\$$) glass joints may be used instead of stoppers where the latter are specified or implied for connecting glass apparatus.

(17) Sieve designations, unless otherwise specified, are those described in U.S. Federal Specification RR-S-366e, November 9, 1973 (available from General Services Administration). Designation "100 mesh" (or other number) powder (material, etc.) means material ground to pass through standard sieve No. 100 (or other number). Corresponding international standard and U.S. standard sieves are given in Table 1.

(18) Term "paper" means filter paper, unless otherwise specified.

(19) Term "high-speed blender" designates mixer with 4 canted, sharp-edge, stainless steel blades rotating at the bottom of 4-lobe jar at 10,000–12,000 rpm, or with equivalent shearing action. Suspended solids are reduced to fine pulp by action of blades and by lobular container, which swirls suspended solids into blades. Waring Blender, or equivalent, meets these requirements.

(20) "Flat-end rod" is glass rod with one end flattened by heating to softening in flame and pressing vertically on flat surface to form circular disk with flat bottom at end.

(21) Designation and pore diameter range of fritted glassware are: extra coarse, 170–220 µm; coarse, 40–60; medium, 10–15; fine, 4–5.5; Jena designations and pore diameter are: 1, 110 µm; 2, 45; 3, 25; 4, 8.

(22) Unless otherwise indicated, temperatures are expressed as degrees Centigrade.

### *Standard Operations*

(23) Operations specified as "wash (rinse, extract, etc.) with two (three, four, etc.) 10 mL (or other volumes) portions of $H_2O$ (or other solvent)" mean that the operation is to be performed with indicated volume of solvent and repeated with same volume of solvent until number of portions required have been used.

(24) Definitions of terms used in methods involving spectrophotometry are those given in JAOAC **37**, 54(1954). Most important principles and definitions are:

**(a)** More accurate instrument may be substituted for less accurate instrument (e.g., spectrophotometer may replace colorimeter) where latter is specified in method. Wavelength specified in method is understood to be that of maximum absorbance ($A$), unless no peak is present.

**(b)** *Absorbance(s) (A).*—Negative logarithm to base 10 of ratio of transmittance ($T$) of sample to that of reference or standard material. Other names that have been used for quantity represented by this term are optical density, extinction, and absorbancy.

**(c)** *Absorptivity(ies) (a).*—Absorbance per unit concentration and cell length. $a = A/bc$, where $b$ is cm and $c$ is g/L, or $a = (A/bc) \times 1000$, if $c$ is mg/L. Other names that have been used for this or related quantities are extinction coefficient, specific absorption, absorbance index, and $E^{1\%}_{1cm}$.

**(d)** *Transmittance(s) (T).*—Ratio of radiant power transmitted by sample to radiant power incident on sample, when both are measured at same spectral position and with same slit width. Beam is understood to be parallel radiation and incident at right angles to plane parallel surface of sample. If sample is solution, solute transmittance is quantity usually desired and is detected directly as ratio of transmittance of solution in cell to transmittance of solvent in an equal cell. Other names that have been used for this quantity are transmittancy and transmission.

**(e)** *Standardization.*—Spectrophotomer may be checked for accuracy of wavelength scale by referring to Hg lines: 239.94, 248.3, 253.65, 265.3, 280.4, 302.25, 313.16, 334.15, 365.43, 404.66, 435.83, 546.07, 578.0, and 1014.0 nm. To check consistency of absorbance scale, prepare solution of 0.0400 g $K_2CrO_4$/L 0.05N KOH and determine absorbance at following wavelengths in 1 cm cell: 230 nm, 0.171; 275, 0.757; 313.2, 0.043; 375, 0.991; 400, 0.396. See NIST Spec. Pub. 378, "Accuracy in Spectrophotometry and Luminescence Measurements," 1973 (available from NIST, Office of Standard Reference Materials, B316, Chem., Gaithersburg, MD 20899).

**Table 1. Nominal Dimensions of Standard Test Sieves (USA Standard Series)**

| Sieve Designation | | Nominal | Nominal |
|---|---|---|---|
| International Standard[a] (ISO) | U.S.A. Standard | Sieve Opening, inches | Wire Diameter, mm |
| 12.5 mm[b] | $\frac{1}{2}$ in.[b] | 0.500 | 2.67 |
| 11.2 mm | $\frac{7}{16}$ in. | 0.438 | 2.45 |
| 9.5 mm | $\frac{3}{8}$ in. | 0.375 | 2.27 |
| 8.0 mm | $\frac{5}{16}$ in. | 0.312 | 2.07 |
| 6.7 mm | 0.265 in. | 0.265 | 1.87 |
| 6.3 mm[b] | $\frac{1}{4}$ in.[b] | 0.250 | 1.82 |
| 5.6 mm | No. $3\frac{1}{2}$ | 0.223 | 1.68 |
| 4.75 mm | No. 4 | 0.187 | 1.54 |
| 4.00 mm | No. 5 | 0.157 | 1.37 |
| 3.35 mm | No. 6 | 0.132 | 1.23 |
| 2.80 mm | No. 7 | 0.111 | 1.10 |
| 2.38 mm | No. 8 | 0.0937 | 1.00 |
| 2.00 mm | No. 10 | 0.0787 | 0.900 |
| 1.70 mm | No. 12 | 0.0661 | 0.810 |
| 1.40 mm | No. 14 | 0.0555 | 0.725 |
| 1.18 mm | No. 16 | 0.0469 | 0.650 |
| 1.00 mm | No. 18 | 0.0394 | 0.580 |
| 850 µm[c] | No. 20 | 0.0331 | 0.510 |
| 710 µm | No. 25 | 0.0278 | 0.450 |
| 600 µm | No. 30 | 0.0234 | 0.390 |
| 500 µm | No. 35 | 0.0197 | 0.340 |
| 425 µm | No. 40 | 0.0165 | 0.290 |
| 355 µm | No. 45 | 0.0139 | 0.247 |
| 300 µm | No. 50 | 0.0117 | 0.215 |
| 250 µm | No. 60 | 0.0098 | 0.180 |
| 212 µm | No. 70 | 0.0083 | 0.152 |
| 180 µm | No. 80 | 0.0070 | 0.131 |
| 150 µm | No. 100 | 0.0059 | 0.110 |
| 125 µm | No. 120 | 0.0049 | 0.091 |
| 106 µm | No. 140 | 0.0041 | 0.076 |
| 90 µm | No. 170 | 0.0035 | 0.064 |
| 75 µm | No. 200 | 0.0029 | 0.053 |
| 63 µm | No. 230 | 0.0025 | 0.044 |
| 53 µm | No. 270 | 0.0021 | 0.037 |

[a] These standard designations correspond to the values for test sieve apertures recommended by the International Organization for Standardization, Geneva, Switzerland.

[b] These sieves are not in the standard series but they have been included because they are in common usage.

[c] 1000 µm = 1 mm.

(25) *Least square treatment of data and calculation of regression lines.*—This technique finds the best fitting straight line for set of data such as standard curve. It calculates that straight line for which sum of squares of vertical deviations (usually $A$) of observations from the line is smaller than corresponding sum of squares of deviation from any other line. Equation of straight line is:

$$Y = a + bX$$

where $a$ is intercept at $Y$ axis ($X = 0$), and $b$ is slope of line.

Least square estimates of constants are:

$$b = \frac{\Sigma(X_iY_i) - [(\Sigma X_i \Sigma Y_i)/n]}{\Sigma X_i^2 - (\Sigma X_i)^2/n}$$

$$a = \bar{Y} - b\bar{X}$$

where $\Sigma$ = "sum of" the $n$ individual values of indicated operation, and $\bar{X}$ and $\bar{Y}$ are the averages of the $X$ and $Y$ points.

Example: To find "best" straight line relating $A$ $(Y)$ to concentration $(X)$:

| Observation No. ($i$) | Concn $X_i$ | Absorbance $Y_i$ | $X_1^2$ | $X_iY_i$ |
|---|---|---|---|---|
| 1 | 80 | 1.270 | 6400 | 101.6 |
| 2 | 60 | 1.000 | 3600 | 60.0 |
| 3 | 40 | 0.700 | 1600 | 28.0 |
| 4 | 30 | 0.550 | 900 | 16.5 |
| 5 | 20 | 0.250 | 400 | 5.0 |
| 6 | 10 | 0.100 | 100 | 1.0 |
| 7 | 0 | 0.050 | 0 | 0.0 |
| Totals: $n = 7$ | $\Sigma X_i = 240$ | $\Sigma Y_i = 3.92$ | $\Sigma X_1^2 = 13000$ | $\Sigma(X_iY_i) = 212.1$ |

$$\bar{X} = \Sigma X_i/n = 240/7 = 34.29$$

$$\bar{Y} = \Sigma Y_i/n = 3.92/7 = 0.56$$

$$b = \frac{212.1 - (240)(3.92)/7}{13000 - (240)^2/7} = \frac{77.7}{4771} = 0.0163$$

$$a = 0.56 - 0.0163(34.29) = 0.001$$

Best equation is then:

$$Y = 0.00 + 0.0163X$$

If for sample, $A = 0.82$, corresponding concentration ($X$) would be:

$$X = (Y - 0.00)/0.0163 = 0.82/0.0163 = 50.3$$

Many scientific and statistical calculators are preprogrammed to perform this calculation.

(26) *Recovery (R) of analyte from fortified sample by a method of analysis.*—Fraction of an analyte added to a sample (fortified sample) prior to analysis, which is measured (recovered) by the method. When the *same* analytical method is used to analyze both the unfortified and fortified samples, calculate $\%R$ as follows:

$$\%R = [(C_F - C_U)/C_A] \times 100$$

where $C_F$ = concentration of analyte measured in fortified sample;
$C_U$ = concentration of analyte measured in unfortified sample;
$C_A$ = concentration of analyte *added* in fortified sample.

(Note: $C_A$ is a calculated value, not a value measured by the method being used.)

Concentration of added analyte should be no less that concentration of analyte in unfortified sample. Sum of concentration of added analyte plus analyte present before fortification should be in same range as analyte concentration sought in actual samples. Addition of analyte must not cause measuring instrument to exceed linear dynamic range of standard curve. Both fortified and unfortified samples must be treated identically during analysis to minimize experimental bias.

(27) Common safety precautions are given in the safety chapter.

### Method Performance

(28) Efforts are being made to standardize the symbols and associated definitions for the statistical parameters that will accompany approved methods. Users of the method should consult the report of the collaborative study (reference given with the method) for complete details.

Beginning with methods published in "Changes in Official Methods of Analysis" (1989) JAOAC **72**, 188, the following statistical parameters are shown. Data from some studies may not be amenable to provide these measures of evaluation.

Within-laboratory precision:

$s_r$ repeatability standard deviation
$s_R$ reproducibility standard deviation

Among-laboratories precision:

$RSD_r$ repeatability relative standard deviation
$RSD_R$ reproducibility relative standard deviation

### Surplus Methods

(29) ★ This symbol indicates a method which has been declared surplus. Such methods are satisfactory methods, having been subjected to collaborative study and review. They are thought not to be in current use for various reasons: The purpose for which the method was developed no longer exists; the product for which the method was developed is no longer marketed; the method has been replaced by other methods; etc. These methods retain their official status but are carried only by reference. Any laboratory which uses a surplus method and wishes the text reprinted in the next edition must so notify AOAC.

### Editorial Conventions

(30) For sake of simplicity, abbreviations Cl and I instead of $Cl_2$ and $I_2$ are used for chlorine and iodine. Similar abbreviations have been used in other cases (O, N, H). The same abbreviation may also be used for the ion where no ambiguity will result.

(31) Reagents and apparatus referenced with only a letter, e.g., **(c)**, will be found in the reagent or apparatus section of that method.

(32) To conserve space, most of the articles and some prepositions have been eliminated.

### Manufacturers and Suppliers

(33) Names and addresses of manufacturers and suppliers, and trade names of frequently mentioned materials, are furnished below solely as a matter of identification and convenience, without implication of approval, endorsement, or certification. The same products available from other suppliers or other brands from other sources may serve equally well if proper tests indicate their use is satisfactory. These firms when mentioned in a method are given by name only (without addresses).

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*Aldrich Chemical Co., Inc.*, 940 W St. Paul Ave, Milwaukee, WI 53233
*Alltech Associates, Inc.*, 2051 Waukegon Rd, Deerfield, IL 60015

*American Cyanamid Co.*, Agricultural Div., 1 Cyanamid Plaza, Wayne, NJ 07470
*(ASBC) American Society of Brewing Chemists,* 3340 Pilot Knob Rd, St. Paul, MN 55121
*(ATCC) American Type Culture Collection,* 12301 Parklawn Dr, Rockville, MD 20852
*Analabs Inc.,* 140 Water St, Norwalk, CT 06854
*Analtech Inc.,* 75 Blue Hen Dr, PO Box 7558, Newark, DE 19714
*Applied Science,* 2051 Waukegan Rd, Deerfield, IL 60015
*J.T. Baker, Inc.,* 222 Red School Ln, Phillipsburg, NJ 08865
*BBL Microbiology Systems,* Div. of Becton, Dickinson & Co., PO Box 243, Cockeysville, MD 21030
*Beckman Instruments, Inc.,* 2500 Harbor Blvd, PO Box 3100, Fullerton, CA 92634
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*Brinkmann Instruments, Inc.,* Cantiague Rd, Westbury, NY 11590
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*Burrell Corp.,* 2223 Fifth Ave, Pittsburgh, PA 15219
*Calbiochem Corp.,* 10933 N Torrey Pines Rd, LaJolla, CA 92037
*Carborundum Co.,* PO Box 337, Niagara Falls, NY 14302
*(CGW) Corning Glass Works,* Laboratory Products Dept, Corning, NY 14830
*Curtin Matheson Scientific, Inc.,* 9999 Veterans Memorial Dr, PO Box 1546, Houston, TX 77038
*Difco Laboratories,* PO Box 331058, Detroit, MI 48232-7058
*Dohrmann,* Div. of Xertex Corp., 3240 Scott Blvd, Santa Clara, CA 95050
*Dow Chemical Co.,* Sample Coordinator, 9001 Bldg, PO Box 1706, Midland, MI 48647-1706
*Dow Corning Corp.,* PO Box 999, Midland, MI 48686-0997
*E.I. du Pont de Nemours & Co., Inc.,* Instrument Products Div., Concord Plaza, 1007 Market St, Wilmington, DE 19898
*Eastman Kodak Co.,* Eastman Organic Chemicals, 343 State St, Rochester, NY 14650
*Elanco Products Co.,* Div. of Eli Lilly & Co., Elanco Analytical Laboratories, Lilly Corporate Center, Indianapolis, IN 46285
*EM Sciences,* A Div. of EM Industries, 480 Democrat Rd, Gibbstown, NJ 08027
*Fischer & Porter Co.,* Lab Crest Scientific, E County Line Rd, Warminster, PA 18974
*Fisher Scientific Co.,* 1 Reagent Ln, Fair Lawn, NJ 07410
*Floridin Co.,* 3 Penn Center, Pittsburgh, PA 15235
*Foss Food Technology Corp.,* 10355 W 70th St, Eden Prairie, MN 55344
*Gelman Scientific Inc.,* 600 S Wagner Rd, Ann Arbor, MI 48106
*Gist-Brocades USA,* PO Box 241068, Charlotte, NC 28224-1068
*Hamilton Co.,* PO Box 17500, Reno, NV 89510
*Hess & Clark Laboratories,* Div. of Rhodia, Inc., 7th & Orange Sts, Ashland, OH 44805
*Hewlett-Packard Co.,* Avondale Div., Rte 41, PO Box 900, Avondale, PA 19311-0900
*Hewlett-Packard Co.,* Mail Stop 20B3, 3000 Hanover St, Palo Alto, CA 94304
*Hoffmann-La Roche, Inc.,* 340 Kingsland St, Nutley, NJ 07110
*ICI Americas, Inc.,* Western Research Center, 1200 S 47th St, PO Box 4023, Richmond, CA 94804-0023
*ICN Pharmaceuticals, Inc.,* Life Sciences Group, 26201 Miles Rd, Cleveland, OH 44128
*(IEC) International Equipment Co.,* Div. of Damon, 300 Second Ave, Needham Heights, MA 02194
*Kimble Glass Inc.,* Crystal Ave, Vineland, NJ 08360
*Kontes Glass Co.,* Spruce St, Vineland, NJ 08360
*Kopp Glass Co.,* 2108 Palmer St, Swissvale, PA 15218
*Labconco Corp.,* 8811 Prospect Ave, Kansas City, MO 64132
*Lurex Scientific,* 1298 North West Blvd, PO Box 2420, Vineland, NJ 08360
*Mallinckrodt Chemicals Works,* Science Products Div., 675 McDonnell Blvd, PO Box 5840, St. Louis, MO 63134
*Manville Filtration & Minerals,* PO Box 519, Lompoc, CA 93438
*Matheson Scientific, Inc.,* see *Curtin Matheson Scientific, Inc.*
*E. Merck,* Frankfurter Str 250, Postfach 4119, D6100 Darmstadt, West Germany
*Merck & Co., Inc.,* Chemical Div., PO Box 2000, Rahway, NJ 07065
*Mettler Instrument Corp.,* PO Box 71, Hightstown, NJ 08520
*Millipore Corp.,* Ashby Rd, Bedford MA 01730
*Mobay Corp.,* Agricultural Chemicals Div., Hawthorne Rd, PO Box 4913, Kansas City, MO 64120-0013
*Monsanto Chemical Co.,* 800 N Lindberg Blvd, St. Louis, MO 63167
*(NBS) National Bureau of Standards,* see NIST
*(NF) National Formulary,* see *USP*
*New York Laboratory Supply Co.,* 510 Hempstead Tnpk, West Hempstead, NY 11552
*(NIST) National Institute of Standards and Technology,* Gaithersburg, MD 20899
*Orion Research Inc.,* 529 Main St, Boston, MA 02149
*Perkin-Elmer Corp.,* 761 Main Ave, Mail Station 256, Norwalk, CT 06859-0256
*Pierce Chemical Co.,* PO Box 117, Rockford, IL 61105
*Rheodyne Inc.,* PO Box 996, Cotati, CA 94928
*Rohm & Haas Co.,* Independence Mall West, Philadelphia, PA 19105
*Salsbury Laboratories,* Charles City, IA 50616
*Sargent-Welch Scientific Co.,* 7300 N Linder Ave, PO Box 1026, Skokie, IL 60077
*(S&S) Sleicher & Schuell, Inc.,* 10 Optical Ave, Keene, NH 03431
*(SEPCO) Scientific Equipment Products Co.,* Div. of JAG Industries, Inc., 2201 Aisquith St, Baltimore, MD 21218
*Scientific Products Inc.,* Div. of Baxter Healthcare Corp., 1430 Waukegan Rd, McGaw Park, IL 60085-6787
*Searle Analytic, Inc.,* 2000 Nuclear Dr, Des Plaines, IL 60018
*Shell Chemical Co.,* 1 Shell Plaza, Houston, TX 77002
*Sigma Chemical Co.,* PO Box 14508, St. Louis, MO 63178
*G. Frederick Smith Chemical Co.,* PO Box 23214, Columbus, OH 43223
*Supelco, Inc.,* Supelco Park, Bellefonte, PA 16823-0048
*Technicon Instruments Corp.,* 511 Benedict Ave, Tarrytown, NY 10591
*Thomas Scientific,* 99 High Hill Rd, I-295, PO Box 99, Swedesboro, NJ 08085
*Tracor Instruments, Austin, Inc.,* 6500 Tracor Ln, Bldg 27-7, Austin, TX 78726-2100
*UVP, Inc.,* 5100 Walnut Grove Ave, PO Box 1501, San Gabriel, CA 91778-1501
*Union Carbide Corp.,* Old Ridgebury Rd, Danbury, CT 06817
*Uniroyal Chemical,* Elm St, Naugatuck, CT 06770
*The Upjohn Co.,* 7000 Portage Rd, Kalamazoo, MI 49001
*(USP) United States Pharmacopeial Convention, Inc.,* 12601 Twinbrook Pkwy, Rockville, MD 20852
*Varian Instrument Group,* 505 Julie Rivers Rd, Sugarland, TX 77478
*VWR Scientific,* PO Box 7900, San Francisco, CA 94120

*Waters Associates, Inc.*, 34 Maple St, Milford, MA 01757
*Whatman, Inc.*, 9 Bridewell Pl, Clifton, NJ 07014

### Abbreviations

(34) The following abbreviations, many of which conform with those of *Chemical Abstracts*, are used. In general, principle governing use of periods after abbreviations is that period is used where final letter of abbreviation is not the same as final letter of word it represents. Periods are not used with units, except inch(es) and gallon(s). Hour(s), second(s), inch(es), and foot(feet) appear as hr or h, sec or s, ″ or in., and ′ or ft, because of new abbreviations adopted in recent methods.

| *Abbreviation* | *Word* |
|---|---|
| *a* | absorptivity(ies) |
| *A* | absorbance(s) thruout (not restricted to formulas); not absorption. *A′* is used for std; $A_0$ for blank; 3 digit subscript numerals usually denote wavelengths in nm |
| AA | atomic absorption |
| Ac | $CH_3CO$- (acetyl, not acetate) |
| ACS | American Chemical Society |
| addn | addition |
| addnl | additional |
| alc. | alcoholic (not alcohol) |
| alk. | alkaline (not alkali) |
| alky | alkalinity |
| amp | ampere(s) |
| amt | amount |
| anal. | analytical(ly) |
| anhyd. | anhydrous |
| AOCS | American Oil Chemists' Society |
| APHA | American Public Health Association |
| app. | apparatus |
| approx. | approximate(ly) |
| aq. | aqueous |
| ASTM | American Society for Testing and Minerals |
| atm. | atmosphere, atmospheric |
| av. | average (except as verb) |
| Bé. | degree Baumé |
| bp | boiling point |
| Bu | butyl |
| C | degrees Celsius (Centigrade) |
| ca | about, approximately |
| calc. | calculate |
| calcd | calculated |
| calcg | calculating |
| calcn | calculation |
| Cat. No. | Catalog Number |
| centrf. | centrifuge |
| centrfd | centrifuged |
| centrfg | centrifuging |
| Chap. | Chapter |
| chem. | chemical(ly) |
| chromatgc | chromatographic |
| chromatgd | chromatographed |
| chromatgy | chromatography |
| Ci | curie(s) |
| CI | Colour Index |
| CIPAC | Collaborative International Pesticides Analytical Council |
| cm | centimeter(s) |
| compd | compound |
| com. | commercial(ly) |
| conc. | concentrate (as verb or noun) |
| concd | concentrated |
| concg | concentrating |

| *Abbreviation* | *Word* |
|---|---|
| concn | concentration |
| const | constant |
| contg | containing |
| cP | centipoise |
| cpm | counts per minute |
| cryst. | crystalline (not crystallize) |
| crystd | crystallized |
| crystg | crystallizing |
| crystn | crystallization |
| cu in. | cubic inch(es) |
| dc | direct current |
| det. | determine |
| detd | determined |
| detg | determining |
| detn | determination |
| diam. | diameter |
| diat. earth | diatomaceous earth |
| dil. | dilute |
| dild | diluted |
| dilg | diluting |
| diln | dilution |
| distd | distilled |
| distg | distilling |
| distn | distillation |
| DMF | *N,N*-dimethylformamide |
| DMSO | dimethyl sulfoxide |
| EDTA | ethylenedinitrilotetraacetic acid (or -tetraacetate) |
| *e.g.* | for example |
| elec. | electric(al) |
| equiv. | equivalent |
| est. | estimate |
| estd | estimated |
| estg | estimating |
| estn | estimation |
| Et | ethyl |
| EtOH | ethanol (the chemical entity $C_2H_5OH$) |
| evap. | evaporate |
| evapd | evaporated |
| evapg | evaporating |
| evapn | evaporation |
| ext | extract |
| extd | extracted |
| extg | extracting |
| extn | extraction |
| F | degrees Fahrenheit (°C = (5/9) × (°F − 32)) |
| FAO | Food and Agriculture Organization |
| Fig. | Figure (illustration) |
| fl oz | fluid ounce(s) (29.57 mL) |
| fp | freezing point |
| ft | foot (30.48 cm) |
| g | gram(s) |
| *g* | gravity (in centrfg) |
| gal. | gallon(s) (3.785 L) |
| GC | gas chromatography |
| gr. | grain(s) |
| g-s | glass-stoppered |
| HCHO | formaldehyde |
| HOAc | acetic acid (not HAc) |
| h or hr | hour(s) |
| ht | height |
| id | inner diameter (or dimension) |
| in. | inch(es) (2.54 cm) |
| inorg. | inorganic |
| insol. | insoluble |

| *Abbreviation* | *Word* |
|---|---|
| IR | infrared |
| ISO | International Organization for Standardization |
| JAOAC | Journal of the Association of Official Analytical Chemists (after 1965) |
| | Journal of the Association of Official Agricultural Chemists (before 1966) |
| kg | kilogram(s) |
| L | liter(s) |
| LC | liquid chromatography |
| lb | pound(s) (453.6 g) |
| liq. | liquid |
| m | meter(s); milli—as prefix |
| *m* | molal |
| *M* | molar (as applied to concn), not molal |
| ma | milliampere (cf amp) |
| mag. | magnetic(ally) |
| manuf. | manufacturer |
| max. | maximum |
| mech. | mechanical(ly) |
| Me | methyl |
| MeOH | methyl alcohol |
| mg | milligram(s) |
| min | minute(s) |
| min. | minimum |
| mixt. | mixture |
| mL | milliliter(s) |
| mm | millimeter(s) |
| mp | melting point |
| mμ | millimicron ($10^{-6}$ mm); use nanometer (nm) ($10^{-9}$ m) |
| mv | millivolt |
| MW | molecular weight |
| *N* | normal (as applied to concn); in equations, normality of titrating reagent |
| N | Newton ($10^5$ dynes) |
| *n* | refractive index |
| neg. | negative |
| neut. | neutral |
| neutze | neutralize |
| neutzd | neutralized |
| neutzg | neutralizing |
| neutzn | neutralization |
| NF | National Formulary |
| NFPA | National Food Processors Association |
| NIST | National Institute of Standards and Technology |
| ng | nanogram ($10^{-9}$ g) |
| nm | nanometer ($10^{-9}$ m); formerly mμ |
| No. | number |
| -OAc | acetate (cf Ac) |
| -OCN | cyanate |
| od | outer diameter (or dimension) |
| org. | organic |
| oxidn | oxidation |
| oz | ounce(s) (28.35 g) |
| p | pico ($10^{-12}$) as prefix |
| Pa | Pascal (1 Newton/m$^2$; $9.87 \times 10^{-6}$ atm.; $7.5 \times 10^{-3}$ mm Hg (torr); $1.45 \times 10^{-4}$ psi) |
| par. | paragraph(s) |
| pet ether | petroleum ether |
| phthln | phenolphthalein |
| pos. | positive |
| powd | powdered (as adjective) |
| ppb | parts per billion ($1/10^9$) |
| ppm | parts per million ($1/10^6$) |
| ppt | precipitate |

| *Abbreviation* | *Word* |
|---|---|
| pptd | precipitated |
| pptg | precipitating |
| pptn | precipitation |
| Pr | propyl |
| prep. | prepare |
| prepd | prepared |
| prepg | preparing |
| prepn | preparation |
| psi | pounds per square inch (absolute) |
| psig | pounds per square inch gage (atmospheric pressure = 0) |
| pt | pint(s) (473 mL) |
| QAC | quaternary ammonium compound |
| qt | quart(s) (946 mL) |
| qual. | qualitative(ly) |
| quant. | quantitative(ly) |
| ® | Trademark name—(Registered) |
| $R_f$ | distance spot moved/distance solv. moved (TLC) |
| r-b | round-bottom (flask) |
| ref. | reference |
| resp. | respectively |
| rpm | revolutions per minute |
| sat. | saturate |
| satd | saturated |
| satg | saturating |
| satn | saturation |
| -SCN | thiocyanate |
| SDF | special denatured formula (applied to alcohol) |
| s or sec | second(s) |
| sep. | separate(ly) |
| sepd | separated |
| sepg | separating |
| sepn | separation |
| sol. | soluble |
| soln | solution |
| solv. | solvent |
| sp gr | specific gravity (apparent density) |
| spectrophtr | spectrophotometer |
| spectrophtric | spectrophotometric(ally) |
| sq | square |
| SRM | Standard Reference Material of National Institute of Standards and Technology |
| std | standard |
| std dev. | standard deviation |
| stdzd | standardized |
| stdze | standardize |
| stdzg | standardizing |
| stdzn | standardization |
| *T* | transmittance |
| tech. | technical |
| temp. | temperature |
| titr. | titrate |
| titrd | titrated |
| titrg | titrating |
| titrn | titration |
| TLC | thin layer chromatography |
| U | unit |
| USDA | United States Department of Agriculture |
| USP | United States Pharmacopeia |
| UV | ultraviolet |
| v | volt(s) |
| v/v | both components measured by vol. |
| vac. | vacuum |
| vol. | volume; also volumetric when used with flask |

| *Abbreviation* | *Word* |
|---|---|
| w/w | both components measured by wt |
| WHO | World Health Organization |
| wt | weight |
| μ | micron (0.001 mm); use micrometer (μm) ($10^{-6}$ m) |
| μg | microgram(s) ($10^{-6}$ g) |
| μL | microliter(s) ($10^{-6}$ L) |
| μm | micrometer(s) ($10^{-6}$ m); formerly μ |
| Δ | difference (*e.g.*, $\Delta A = (A - A')$) |
| ′ | foot (feet) (1′ = 30.48 cm) |
| ″ | inch(es) (1″ = 2.54 cm) |
| / | per |
| % | percent (parts per 100); percentage |
| > | more than; greater than; above; exceeds (use with numbers only) |
| < | less than; under; below (use with numbers only) |
| ≤ | not more than; not greater than; equal to or less than |
| ≥ | not less than; equal to or greater than; equal to or more than; at least |
| Ŧ | standard taper |
| $ | standard spherical joint |

# Collaborative Study Procedures of the Association of Official Analytical Chemists

The Association of Official Analytical Chemists (AOAC) is a unique, nonprofit scientific organization whose primary purpose is to serve the needs of academia, government regulatory and research agencies, and industry for analytical methods for compliance, quality control, and research purposes. The goal of the Association is to provide methods which will perform with the necessary accuracy and precision under usual laboratory conditions (*1*). Since its formation in 1884, AOAC has provided a mechanism to select methods of analysis from published literature or develop new methods, collaboratively test them through interlaboratory studies, approve them, and publish the approved methods for a wide variety of materials relating to foods, drugs, cosmetics, pesticides, feeds, fertilizers, forensic science, and products affecting the public health and welfare. Its membership is composed of scientists from government, academia, and industry laboratories in many countries who work within AOAC's established procedures as researchers, methods collaborators, and committee members.

AOAC has more than 100 years of experience in utilizing the collaborative study as a means of determining the reliability of analytical methods for general purposes and, especially, for regulatory purposes. In fact, AOAC's major contribution to analytical science has been to bring the collaborative study technique for the validation of analytical methods to a high degree of perfection. In such a study, laboratories analyze identical sample sets which cover the range of applicability of a method previously selected as being useful and practical. The purpose of the study is to establish the characteristics of the method with respect to accuracy, precision, sensitivity, range, specificity, limit of detection, limit of reliable measurement, selectivity, practicality, and similar attributes, as required.

## ORGANIZATION AND PROCEDURES FOR AOAC COLLABORATIVE STUDIES

The collaborative study is organized and directed by an analyst designated as the Associate Referee for the specific subject under investigation. Currently, some 700 Associate Referees appointed by the Association are responsible for as many topics. An Associate Referee is selected for his or her knowledge, interest, and experience in the subject matter field. The Associate Referee operates under the scientific guidance and support of a General Referee, who is in turn responsible for a product area. The Associate Referee reviews the literature and selects one or two of the appropriate analytical methods available, modifying them as needed. Alternatively, he or she may develop or adapt a method used in his or her laboratory for the analyte and matrix under study, testing it thoroughly before designing a collaborative study. The General Referee is kept informed of such preliminary studies.

The samples analyzed in a collaborative study are normally prepared and distributed to the participants by the Associate Referee. The Association follows the "Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis," as accepted by IUPAC and adopted by AOAC (see p. 673) for the number of participating laboratories and number of materials.

Laboratories with at least some experience in the general subject matter are selected as collaborators. Because the objective of the study is to evaluate the method, as contrasted to evaluating the analyst (*2*), all analysts are instructed to follow the method exactly as written even though they may not concur with the Associate Referee's selection among possible alternatives. The content of the analyte in the samples is unknown to the participants.

All individual results obtained by the collaborators are reported to the Associate Referee, who compiles and evaluates them. Since statistical treatment of the data is considered essential in a rigorous evaluation of the method for accuracy, precision, sensitivity, and specificity, it is now required for all studies. The Association considers this of such importance that it provides statistical assistance in all cases where it is otherwise unavailable to the Associate Referee. A statistical manual (*3*) is also provided.

The Associate Referee makes the initial judgment on the performance of the method. If he or she recommends approval, it passes to the General Referee, the appropriate Methods Committee, and then to the chairman of the Official Methods Board. If all parties recommend approval, the method receives interim official first action approval. The method is then presented at the Association's annual business meeting for vote for adoption as official by the membership.

Approved methods and supporting data are published in the *Journal of the Association of Official Analytical Chemists*. They are subject to scrutiny and general testing by other analysts for 2 years before final adoption. They may be modified and restudied collaboratively as needed, should feedback from general use reveal flaws in the method or in its written set of directions. Approved methods are included in the Association's *Official Methods of Analysis*, the compendium of all adopted methods, which is updated every 5 years.

The preceding summary of AOAC's modus operandi recognizes the need for healthy skepticism toward results obtained by analytical methods which have not undergone such rigorous scrutiny and interlaboratory testing of their accuracy, precision, specificity, and practicality.

## SELECTION OF METHODS FOR STUDY

A certain degree of variability is associated with all measurements. Much of the research on analytical chemistry is an attempt to minimize that variability. But there are many different types of variability in analytical work. We often find that when we attempt to minimize one kind, we must necessarily permit expansion in another kind. In practical analytical chemistry, the problem often comes down to which variability is to be minimized.

Some examples of this point may be helpful. In atomic weight determination, everything—especially *practicality*—is sacrificed for *accuracy*. A high degree of accuracy and practicality is required in the assay of precious metals, but the fire assay used is generally *applicable* to little else besides metals and minerals. In clinical chemistry, within-laboratory *precision* (repeatability) is critical, and often is of greater interest to clinical laboratories than absolute accuracy or agreement with the values of other laboratories (reproducibility). In drug analysis, a high degree of accuracy is required in the therapeutic *range* because the analytical values determining the identity, strength, quality, and purity of pharmaceutical preparations, as laid down in pharmacopoeial specifications, are directly related to clinical value. With polynuclear hydrocarbons, *specificity* is important, since some of these compounds are carcinogenic while others are not. In applying the famous Delaney clause of the United States Federal Food, Drug, and Cosmetic Act, all attributes of the analytical methods are secondary to the detection of extremely small concentrations (*detectability*), or to exhibiting a high degree of response for small changes in concentration (*sensitivity*).

There is a very special case involving accuracy, where the "true value" is determined by the method of analysis. Many legal specifications and standards for food and agricultural products define ill-defined components such as moisture, fat, protein, and crude fiber in terms of reference methods. Therefore, the precision of these methods becomes the limiting factor for their performance. In fact, most analyses involved in commercial transactions require primarily that the buyer and seller agree on the same value (analytically and economically), regardless of where it stands on an absolute scale.

The point of these examples is that although methods of analysis are characterized by a number of attributes—accuracy, precision, specificity, sensitivity, detectability, dependability, and practicality—no method is so flawless that all these qualities can be maximized simultaneously. For any particular analysis, the analyst must determine, on the basis of the purpose of the analysis, which attributes are essential and which may be compromised.

Unfortunately, the literature is replete with examples indicating that an individual analyst, and especially the originator of a method of analysis, is not an unbiased judge of the relative merits of the methods of analysis which he or she develops and uses. In our experience, the collaborative study provides impartial data on the suitability of the method. The data, in many cases, speak for themselves.

The collaborative study, or ring test or round robin test, as it is called in other organizations, provides the basic information on the performance of analytical methods. The extent of the information will depend on the number of samples provided, the number of analyses performed, and the number of laboratories participating. The data should be unbiased because the composition of the samples is known only to the administrator of the study. Some of the requirements of the study and their relationship to the characteristics and attributes of the method are as follows:

(1) *Accuracy*. Samples must be of defined composition (by spiking, by formulation, or by analytical consensus).

(2) *Specificity*. Samples should contain related analytes.

(3) *Sensitivity*. Samples should differ from each other or from negative samples by a known amount.

(4) *Applicability*. Samples should include the concentration range and matrix components of interest.

(5) *Blanks*. Samples should include different matrices with "none" of the component of interest.

(6) *Precision*. Instructions should request replicate analyses by the same or different analysts in the same laboratory, preferably on different days. By far a better procedure is to include "blind" (unknown to the analyst) replicate samples in the series.

(7) *Practicality*. Instructions should request information as to the actual and elapsed time required for the analyses; the availability of reagents, equipment, and standards; and any necessary substitutions. When practice samples are included, the number of analyses required to achieve the stated recovery and repeatability should be reported.

## PROCEDURAL DETAILS OF COLLABORATIVE STUDY

As numerous beginners in this field have discovered, much preliminary work must be done before sending out samples:

(1) The method must be chosen and demonstrated to apply to the matrices and concentrations of interest.

(2) The critical variables in the method should have been determined and the need for their control must be emphasized [a ruggedness test (*4*) is useful for this purpose].

(3) The method should be written in detail by the Associate Referee and tested by an analyst not previously connected with its development.

(4) Unusual standards, reagents, and equipment must be available from usual commercial sources of supply, or sufficient quantities must be prepared or obtained to furnish to the participants.

(5) The samples must be identical and homogeneous so that the analytical sample error is only a negligible fraction of the expected analytical error.

(6) A sufficient number of samples must be prepared to cover typical matrices and the concentration range of interest (tolerance, maximum or minimum specifications, likely levels of occurrence, etc.).

(7) A minimum of 8 laboratories and sufficient samples must be included to provide a minimum of 40 data points. Additional laboratories and samples are recommended.

(8) Samples must be stable and capable of surviving the rigors of commercial transportation.

(9) Reserve samples should be prepared and preserved to replace lost samples and to permit reanalysis of samples considered as outliers to attempt to discover the cause of abnormal results.

(10) The instructions must be clear. They should be reviewed by someone not connected with the study to uncover potential misunderstandings and ambiguities.

(11) If the analyte is subject to change (e.g., bacterial levels, nitroglycerin tablets), provision must be made for all participants to begin the analysis at the same time.

(12) Practice samples of a known and declared composition should be furnished with instructions not to analyze the unknowns until a specified degree of recovery and repeatability (or other attribute) has been achieved.

(13) Provision should be made when necessary for submission of standard curves, tracings of recorder charts, or photographs of thinlayer plates in order to assist in determining possible causes of error.

## OTHER TYPES OF INTERLABORATORY STUDIES

This type of collaborative study, which is designed to determine the characteristics of a method, must be carefully distinguished from other types of interlaboratory studies which by design or through ignorance provide other kinds of information. The most important types of other studies are:

(1) Those studies which require the collaborators to investigate the variability of parts of methods or applicability to different types of samples. (An interlaboratory study is usually an inefficient way of obtaining this type of information.)

(2) Those studies which permit an analyst to use any method desired. Such studies invariably produce such a wide scatter of results that the data are of little value for evaluation of methods. They may be useful in selecting a method from a number of apparently equivalent methods, provided the purpose is emphasized beforehand and the participants provide a description of the method used in order to permit a correlation of the details of the methods with apparent biases and variabilities.

(3) Those studies which are used for quality control purposes, whose participants are not permitted sufficient time to gain familiarity with the method, or who permit deviations to enter into the performance of the analyses on the grounds that the deviation is obviously an improvement which could not possibly affect the results of the analysis, or who claim to have a superior method.

The following definitions were agreed on as part of the guidelines for collaboration between AOAC and the Collaborative International Pesticides Analytical Council Ltd. (CIPAC) (5).

*Collaborative study.* An analytical study involving a number of laboratories analyzing the same sample(s) by the same method(s) for the purpose of validating the performance of the method(s).

*Preliminary interlaboratory study.* An analytical study in which two or more laboratories evaluate a method to determine if it is ready for a collaborative study.

*Laboratory performance check.* The analysis of very carefully prepared and homogeneous samples, normally of known active ingredient content, to establish or verify the performance of a laboratory or analyst.

## SUMMARY

The collaborative study is an experiment designed to evaluate the performance of a method of analysis through the analysis of a number of identical samples by a number of different laboratories. With proper design, it provides an unbiased evaluation of the performance of a method in the hands of those analysts who will use it. A collaborative study must be distinguished from those studies designed to choose a method or to determine laboratory or analyst performance.

## REFERENCES

(*1*) Handbook for AOAC Members, AOAC, Arlington, VA (1989).

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(*3*) W. J. Youden and E. H. Steiner, "Statistical Manual of the AOAC: Statistical Techniques for Collaborative Tests. Planning and Analysis of Results of Collaborative Tests," AOAC, Arlington, VA (1975).

(*4*) W. J. Youden, "The Collaborative Test," *J. Assoc. Off. Agric. Chem.*, **46,** 55–62 (1963).

(*5*) "Guidelines for Collaboration Between the Association of Official Analytical Chemists (AOAC) and the Collaborative International Pesticides Analytical Council Ltd. (CIPAC)," *J. Assoc. Off. Anal. Chem.*, **57,** 447–449 (1974).

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# 1. Agricultural Liming Materials

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## 924.01 Sampling of Liming Materials

**Procedure**

(*Caution:* See safety note on calcium oxide.)

Take sample representative of lot or shipment. Avoid disproportionate amt of surface or any modified or damaged zone.

(**a**) *Burnt or lump lime, in bulk.*—Collect composite sample of $\geq$10 shovelfuls/car, with proportionate amts from smaller lots, taking each shovelful from different part of lot or shipment. Immediately crush to pass 5 cm (2″) diam. circular opening, mix thoroly and rapidly, reduce composite to ca 2 kg (5 lb) sample by riffling or quartering, and place in labeled, dry, air-tight container.

(**b**) *Hydrated lime and ground burnt lime, in bags.*—Select 10 bags from different parts of each lot or shipment of $\leq$20 tons and 1 addnl bag for each addnl 5 tons. Use sampling tube to withdraw top to bottom core from each bag selected. Combine cores, mix thoroly and rapidly, reduce composite to ca 1 kg (2 lb) by riffling or quartering, and place in dry, air-tight container.

(**c**) *Ground limestone and ground marl, in bags.*—Proceed as in (**b**).

(**d**) *Ground limestone, ground burnt lime, ground marl, and slag, in bulk.*—Use slotted sampling tube to withdraw samples to full sampler depth from 10 points in lot or shipment. Proceed as in (**b**), beginning "Combine cores, . . ."

Refs.: JAOAC **7,** 252(1924); **48,** 95(1965).

CAS-1317-65-3 (limestone)

## 924.02 Mechanical Analysis of Liming Materials

**Procedure**

(*Caution:* See safety note on calcium oxide.)

If entire sample is not to be dried, obtain lesser portions by riffling or quartering. Dry at 110° to const wt and cool to room temp.

Obtain 90–150 g dry sample by riffling or quartering. Break any agglomerates formed during drying by rolling dry sample with hard rubber roller on hard rubber mat, wet sieving, or by equally effective means that does not result in crushing the limestone.

*Wet sieving.*—Place 100 g sample on No. 200 sieve and wash with moderate stream of tap $H_2O$ at max. gage pressure of 0.28 kg/sq cm (4 lb/sq in.) until $H_2O$ passing sieve is clear, with care to avoid loss of sample by splashing. Dry material remaining on sieve at 105° and transfer to No. 100 sieve in series with No. 200 sieve of same diam. and depth. Shake 8 min in mech. shaker. (If wet sieving is used to break agglomerates, do wet sieving on sieve having smallest opening to be used in final testing. After drying, transfer to sieves to be used in final testing. If only 1 sieve is to be used, do not transfer.) Quant. transfer weighed sample to 8″ diam. std sieve or set of sieves (e.g., Nos. 10, 20, 40, 60, 80, and 100 or other appropriate combination).

Sieve by lateral and vertical motion accompanied by jarring action. Continue $\geq$5 min or until addnl 3 min of sieving time fails to change results of any sieve fraction by 0.5% of total sample wt. Do not overload any sieve when assaying closely sized materials.

Det. wt of each sieve fraction and report as % of total sample wt.

Refs.: JAOAC **7,** 252(1924); **55,** 539(1972); **48,** 95(1965); **52,** 322(1969).

## 924.03 Liming Materials

**Preparation of Sample**

**Procedure**

Reduce dried sample, **924.02**, to amt sufficient for analysis and grind $\geq$225 g (0.5 lb) reduced sample in mortar, ball mill, or other mech. app. to pass No. 60 sieve. Mix thoroly, and store in air-tight container.

Refs.: JAOAC **7,** 252(1924); **48,** 95(1965).

## 955.01 Neutralizing Value for Liming Materials

**Final Action**

(Uncorrected for sulfide content)

### *A. Reagents*

(**a**) *Sodium hydroxide std soln.*—0.25*N*. Prep. and stdze as in **936.16**.

(**b**) *Hydrochloric acid std soln.*—0.5*N*. Stdze against (**a**), using phthln.

### *B. Indicator Titrimetric Method*

Place 0.5 g burnt or hydrated lime (1 g ground limestone or ground marl), prepd as in **924.03**, in 250 mL erlenmeyer; add 50 mL HCl std soln and boil *gently* 5 min. Cool, and titr. excess acid with NaOH std soln, using phthln. For burnt and hydrated lime, report as % CaO; for limestone and marl, report as % $CaCO_3$ equivalence.

$$\% \ CaCO_3 \text{ equivalence of sample} = 2.5 \times (\text{mL HCl} - \text{mL NaOH}/2)$$

$$\% \ CaO \text{ equivalence} = 2.8 \times (\text{mL HCl} - \text{mL NaOH}/2)$$

### *C. Potentiometric Titration Method*

(Applicable to liming materials contg large amt of $Fe^{+2}$ or coloring matter, but not to silicate materials)

Proceed as in **955.01B** thru "Cool, . . ." Transfer to 250 mL beaker and insert glass and calomel electrodes of pH meter, buret contg 0.25*N* NaOH, and mech. stirrer. Stir at moderate speed to avoid splash. Deliver NaOH rapidly to pH 5, then dropwise until soln attains pH 7 and remains const 1 min while stirring. (If end point is passed, add, from 1 mL Mohr pipet, just enough 0.5*N* HCl to bring pH to <7, and back-titr. slowly to pH 7.) Add mL of excess acid, if used, to initial 50 mL in calcg. Report as % $CaCO_3$ or CaO equivalence as in **955.01B**.

Ref.: JAOAC **38,** 240(1955).

### D. Approximate Proportions of Calcium and Magnesium in Magnesic Limestone

Slightly acidify titrd soln, **955.01B** or **C**, transfer to 250 mL vol. flask, and dil. to vol. Det. Ca in 50 mL aliquot as in **927.02**, beginning ". . . dil. to ca 100 mL . . ." Subtract its $CaCO_3$ equivalence from total $CaCO_3$ equivalence, **955.01B** or **C**, and assign difference as $CaCO_3$ equivalence of the Mg content of the limestone.

CAS-7440-70-2 (calcium)
CAS-1317-65-3 (limestone)
CAS-7439-95-4 (magnesium)

## 928.01 Caustic Value for Liming Materials

**Titrimetric Method**

**Final Action 1965**

### A. Apparatus (Figure 928.01)

Use 500 mL Pyrex erlenmeyer, *A*, and fritted glass filter (Corning Glass Works No. 39535, 30F), *F*. Connect filter to siphon tube *B* with thick-wall rubber tubing. Use receiving flasks *M* and *N* calibrated *to deliver* 50 and 100 mL, resp. *S* is suction flask.

### B. Determination

Transfer portion of sample, **924.03**, to weighing bottle and det. wt bottle and contents in atm. of min. moisture and $CO_2$ content. With polished, narrow-point spatula calibrated to hold ca 1.5 g, withdraw sample to be used and det. exact wt by difference. Insert sample directly into dry flask, *A*, fitted with tight rubber stopper.

Prep. *sucrose soln immediately before use* by placing 25 g granulated sucrose in measuring flask calibrated *to deliver* 500 mL. Dissolve sucrose with cold *$CO_2$-free $H_2O$* and dil. to vol. Holding both erlenmeyer contg sample and flask contg sucrose soln in slightly inclined position, insert neck of sucrose soln flask short distance into erlenmeyer, and carefully transfer sucrose soln with synchronized rotary motion of both flasks to prevent granulation of lime. Stopper erlenmeyer securely, agitate, and add, if desired, some clean dry beads. Completely dissolve uncoated caustic lime by six 1 min agitations at 2 or 3 min intervals. Invert flask to trap any solid particles between stopper and neck and crush by carefully twisting stopper. Let stand 15 min and filter as follows:

Connect filter cone *F* with siphon *B* and close stopcock *D*. Connect receiving flasks, apply suction, and quickly connect erlenmeyer *A* contg lime soln with stopper *E*. Open stopcock *C* and filter 25–50 mL soln. Close *C* and open *D* to release suction. Remove *M* and replace with similar dry flask. Close *D*, open *C*, and continue filtration until both *M* and *N* are filled at least to marks. To disconnect system, close stopcock *C*, and gently press down outlet of flask *M* and then outlet of flask *N*, to remove any excess liq. above marks. Let intermediate connection empty, open stopcock *D*, and remove *M* and *N*. Titr. first 50 mL, or pilot aliquot, of filtered soln with 0.5*N* HCl, using phthln. To covered 200 mL beaker add twice vol. 0.5*N* acid required for this titrn, add second (100 mL) aliquot of filtered soln to this acid and phthln, and complete titrn.

Calc. caustic value of sample: $X = 7V/W$

where $X$ = % active CaO; $V$ = mL 0.5*N* acid used/100 mL lime soln; $W$ = g sample

Refs.: Ind. Eng. Chem. **20,** 312(1928). JAOAC **11,** 152(1928); **12,** 146(1929).

CAS-1305-78-8 (calcium oxide)

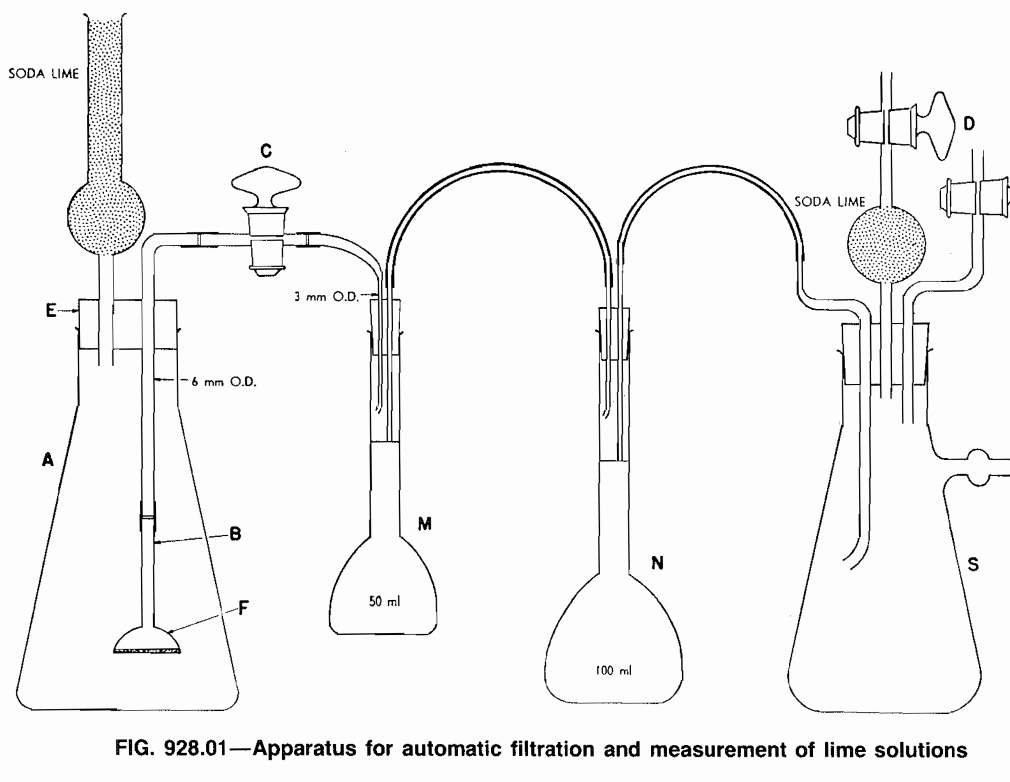


**FIG. 928.01—Apparatus for automatic filtration and measurement of lime solutions**

## 955.02 Carbon Dioxide in Liming Materials

### Knorr Alkalimeter Method

### Final Action 1965

#### A. Apparatus and Reagents

*Knorr alkalimeter with $CO_2$ absorption train.*—Fill guard tube of alkalimeter with Ascarite. Connect upper end of condenser to absorption train consisting of 5 or 6 U-shape, g-s drying tubes (or equiv.) joined in series. Fill first tube with $H_2SO_4$ and second with $Ag_2SO_4$-$H_2SO_4$ soln (10 g $Ag_2SO_4$ in 100 mL $H_2SO_4$) to remove acidic gases other than $CO_2$. Fill third tube with $Mg(ClO_4)_2$ to absorb $H_2O$. Fill inlet $^2/_3$ of fourth and succeeding tubes with Ascarite to absorb $CO_2$, and outlet $^1/_3$ of each tube with $Mg(ClO_4)_2$. Connect last tube in train with aspirating bottle or suction source.

Condition app. daily before use, and also when freshly filled tube is placed in train, by aspirating air at rate of 2–3 bubbles/sec thru dry alkalimeter assembly and absorption train until $CO_2$ absorption tubes attain const wt (usually 20–30 min). Tare against similarly packed tubes. Use std procedure for wiping tubes with dry, lint-free cloth before each weighing.

#### B. Determination

Transfer 3 g burnt or hydrated lime or 0.5–1.0 g limestone or marl, prepd as in **924.03**, to dry alkalimeter flask. Momentarily open stopcocks of first 2 $CO_2$ absorption tubes to air to equalize pressure, weigh tubes sep., and place in position in train. With assembled alkalimeter connected to absorption train, adjust rate of aspiration of air thru system to ca 2 bubbles/sec. Close funnel stopcock, remove alkalimeter guard tube, fill funnel with 50 mL HCl (1 + 4), and replace guard tube. Open funnel stopcock and let acid run slowly into flask, taking care that evolution of gas is so gradual as not to materially increase flow thru tubes. After all acid is added, agitate alkalimeter assembly to ensure complete dispersion of sample in acid soln. Continue aspiration, gradually heat contents of flask to bp, and boil 2–3 min after $H_2O$ begins to condense. Discontinue heating, and continue aspiration 15–20 min or until app. cools. Remove, equalize internal and external pressure, and reweigh absorption tubes.

Increase in wt = wt $CO_2$. (Material increase in wt of second tube usually indicates exhaustion of first tube, but may result from too rapid evolution of $CO_2$ in relation to aspiration rate.) Report % $CaCO_3$.

Ref.: JAOAC **38,** 413(1955).

CAS-124-38-9 (carbon dioxide)

## CALCIUM SILICATE SLAGS

## 944.01 Neutralizing Value for Calcium Silicate Slags

### Titrimetric Method

### Final Action 1965

(Uncorrected for sulfide content)

(**a**) *Blast furnace slag.*—Transfer 0.5 g sample, ground to pass No. 80 sieve, to 250 mL erlenmeyer. Wash down with small portions $H_2O$ and add 35 mL 0.5*N* HCl while swirling. Heat to gentle boil over burner, *agitating suspension continuously* until bulk of sample dissolves. Boil 5 min and cool to room temp.; then dil. with $CO_2$-free $H_2O$ to ca 150 mL and add 1 mL *30% $H_2O_2$* and 5 drops bromocresol green. (Dissolve 0.1 g tetrabromo-*m*-cresolsulfonphthalein in 1.5 mL 0.1 NaOH, and dil. to 100 mL with $H_2O$.) Back-titr. with *0.5N* NaOH, adding first 15 mL rapidly and titrg dropwise thereafter, vigorously agitating contents of stoppered flask after each addn, until indicator tint matches or slightly exceeds that of pH 5.2 phthalate buffer soln, **941.17C**, of like vol. and indicator concn, after 2–3 sec agitation.

(**b**) *Rock phosphate reduction furnace slag.*—Transfer 0.5 g sample to 250 mL beaker. Wash down with small portions $H_2O$ and add, stirring continuously, 50 mL HOAc (1 + 4). Heat to bp and boil 5 min, stirring frequently. Evap. to dryness on steam bath. Add 20 mL of the HOAc, dil. to 150 mL, and heat to bp; add $NH_4OH$ (1 + 1) to distinct yellow of Me red. Digest ca 10 min on hot plate. Filter by gravity thru 9 cm paper, catching filtrate in 100 × 50 mm lipped Pyrex crystg dish; wash beaker 3 times and paper 5 addnl times with *neut. 0.5N $NH_4OAc$*. Evap. filtrate on hot plate. Adjust heat so bubbles breaking thru viscous surface film are released gently to avoid spattering. (To expedite dehydration, repeat treatments with 25 mL hot $H_2O$ and evapn 2 or 3 times.) Continue heating residue on hot plate until no HOAc odor remains. Heat addnl 10 min at full heat of hot plate; then ignite 10 min at 550°. Cool, wet residue with 15 mL $H_2O$, place watch glass over dish, and add 25 mL *0.5N* HCl thru lip of dish. Heat 5 min over burner at gentle simmer. Rinse watch glass, filter suspended matter on 9 cm paper, catching filtrate in 250 mL erlenmeyer, and wash dish and filter 3 times with hot $H_2O$. Titr. excess acid with *0.5N* NaOH to distinct yellow of Me red.

Net acid used × 5 = neutzg value of slag in terms of % $CaCO_3$ equivalence.

Refs.: JAOAC **27,** 74, 532(1944); **28,** 310(1945); **31,** 71(1948).

CAS-471-34-1 (calcium carbonate)

## 948.01 Sulfide Sulfur in Calcium Silicate Slags

### Titrimetric Method

### Final Action 1965

(*Note:* $CdSO_4$ is toxic; see also safety note on toxic dusts.)

#### A. Reagents

(**a**) *Zinc dust.*—Low in Pb.

(**b**) *Absorbent soln.*—Dissolve 20 g $CdSO_4.2^2/_3H_2O$ in $H_2O$ and dil. to 1 L. Adjust to pH 5.6 potentiometrically or colorimetrically. If colorimetrically, match sep. 50 mL aliquot to buffer of same pH, **941.17C**.

(**c**) *Sodium hydroxide std soln.*—0.1*N*. Prep. and stdze as in **936.16**.

(**d**) *Std acid.*—0.1*N* HCl. Stdze against std alkali, (**c**), using Me red.

(**e**) *Methyl red indicator.*—Dissolve 0.2 g Me red in 100 mL alcohol.

#### B. Apparatus

Fit 250 mL erlenmeyer with 2-hole No. 5.5 stopper. Insert thru stopper 60 mL separator with stem drawn out to 2 mm and bent upward at tip, adjusting separator so stem is 6 mm from bottom of flask. Also insert thru stopper 6 mm glass outlet tube. Connect with amber rubber tubing to inlet of 25 × 150 mm tube half filled with $H_2O$ and heated to near bp before and during detn. Connect in series 2 addnl tubes of same size, each contg 25 mL absorbent soln and held in 600 mL beaker filled with cold $H_2O$.

#### C. Determination

Fill absorbent tubes with absorbent soln and heat $H_2O$ tube to gentle boiling. Weigh 1 g slag, ground to pass No. 80 sieve, into evolution flask, add 1 g Zn dust, and wash down sides with 5–10 mL $H_2O$; mix with flat-end rod and connect flask to app. Add 50 mL HCl (1 + 4) to separator and let acid flow into reaction flask while swirling contents. If necessary, apply pressure to transfer acid and close stopcock while a little of the acid is still above it. Heat to bp; then regulate to maintain active but not too vigorous boiling for 10 min. Swirl flask frequently after adding acid and for first 5 min of boiling. To disconnect, hold inlet in first absorbent tube firmly with one hand and quickly pull off rubber tubing with other hand without pinching.

Filter CdS suspension by gravity thru 9 cm paper into 250 mL erlenmeyer and wash with $H_2O$ to vol. of 100 mL. Add 4 drops Me red indicator and agitate vigorously while titrg slowly with 0.1*N* NaOH to exact tint of *ref. soln* (50 mL absorbent soln dild to 100 mL, with identical indicator concn, in 250 mL erlenmeyer). If end point is passed so that $Cd(OH)_2$ ppts, add 1–2 mL 0.1*N* HCl, let stand until ppt disappears, and complete titrn dropwise, agitating vigorously.

% $CaCO_3$ equivalence of sulfide *S* in sample = net mL 0.1*N* NaOH/2

$$\text{g Sulfide S/detn} = \text{mL } 0.1N \text{ NaOH} \times 0.0016$$

$$\% \text{ Sulfide S} = \text{g sulfide S} \times 100$$

Refs.: JAOAC **31,** 715(1948); **32,** 73(1949).

CAS-7704-34-9 (sulfur)

## GRAVIMETRIC ELEMENTAL ANALYSES

### 963.01 Elemental Analysis of Liming Materials

**Preparation of Sample Solution**

**First Action 1963**
**Final Action 1965**

(*Caution:* See safety notes on wet oxidation, nitric acid, and perchloric acid.)

Prep. samples as in **924.03**, preferably in agate mortar. Grind silicates to pass No. 100 sieve, and dry all samples at 105°.

Weigh 2 g limestone or 0.5 g silicate. If sample contains org. matter, transfer to Pt crucible and place in cold furnace. Raise temp. gradually to 1000° and hold 15 min. Transfer sample to 400 mL beaker and, if ignited, moisten cautiously with $H_2O$. Add 10 mL $HNO_3$ and evap. on hot plate at low heat until mixt. becomes pasty. Cool, and add 10 mL $H_2O$ and 20 mL 60% $HClO_4$. Boil to heavy fumes of $HClO_4$, cover, and fume slowly until soln is colorless or slightly yellow (5–10 min). Do not evap. to dryness. Cool to <100° and add 50 mL $H_2O$. Filter thru Whatman 41H or finer paper into 250 mL vol. flask. *Wash thoroly with hot $H_2O$ to remove all traces of $HClO_4$.* Reserve filtrate and washings for prepn of *Sample Solns X and Y,* **963.02**.

### 963.02 Silica in Liming Materials

**Gravimetric and Titrimetric Methods**

**First Action 1963**
**Final Action 1965**

(*See also* **965.07**.)

(*Caution:* See safety notes on hydrofluoric acid and perchloric acid.)

Transfer paper with $SiO_2$ to uncovered Pt crucible and heat gently with low flame until paper chars without flame. Partially cover crucible and cautiously burn C. Finally cover completely and heat with blast lamp or in furnace at 1150–1200°. Cool in desiccator and weigh. Repeat to const wt (*W*). Treat with ca 1 mL $H_2O$, 2 drops $H_2SO_4$ (1 + 1), and 10 mL HF. Cautiously evap. to dryness in hood. Heat 2 min at 1050–1100°, cool in desiccator, and weigh (*B*).

$$W - B = \text{g } SiO_2 \text{ in sample}$$

$$\text{g } SiO_2 \times 0.4674 = \text{g Si}$$

(**a**) *Sample Soln X.*—(0.008 g limestone or 0.002 g silicate/mL.) Fuse residue from Si detn with 0.5 g $Na_2CO_3$ by heating covered crucible 10 min over Meker burner. Cool, fill crucible 2/3 full with $H_2O$, and add 2 mL 60% $HClO_4$ dropwise, with stirring. Warm if necessary to dissolve melt. Add to filtrate and washings reserved for prepn of *Sample Soln X* in **963.01**. Dil. to 250 mL with $H_2O$.

(**b**) *Sample Soln Y.*—(0.00016 g limestone or 0.00004 g silicate/mL.) Dil. 10 mL *Sample Soln X* to 500 mL with $H_2O$.

Refs.: JAOAC **46,** 603(1963); **47,** 1019(1964).

CAS-7631-86-9 (silicon dioxide)

### 917.01 Aluminum, Iron, Phosphorus, and Titanium Oxides in Liming Materials

**Gravimetric Method**

**Final Action 1965**

(Alternatively, Al, Fe, Mn, P, and Ti may be detd colorimetrically as in **965.01**, **965.02**, **965.03**, **965.04**, **965.05**, and **965.06**.)

To 125 mL aliquot *Soln X* from **963.02(a)**, add 10 mL HCl and few drops Me red indicator; heat to gentle boil and add $NH_4OH$ (1+1) until ppt forms and indicator just changes to distinct yellow. Boil ≤2 min and filter rapidly. Wash ppt 6–8 times with hot *2% $NH_4NO_3$* soln. Return ppt and filter to original beaker, add 10 mL HCl, and macerate filter with policeman. Dil. with $H_2O$, heat to dissolve ppt, dil. to ca 200 mL, and reppt as above. Wash thoroly with the hot $NH_4NO_3$ soln until Cl-free. Combine first and second filtrates and save for Ca and Mg detns.

Place ppt in Pt crucible and dry. Ignite gently to oxidize C, heat to bright red ca 10 min, cool in desiccator, and weigh in covered crucible as $Fe_2O_3$ + $Al_2O_3$ + $P_2O_5$ + $TiO_2$.

Refs.: U.S. Geol. Survey Bull. **700,** p. 106. Ind. Eng. Chem. **9,** 1114(1917). JAOAC **48,** 95(1965).

CAS-1344-28-1 (aluminum oxide)
CAS-1309-37-1 (ferric oxide)
CAS-1314-56-3 (phosphorus pentoxide)
CAS-13463-67-7 (titanium dioxide)

### 917.02 Calcium in Liming Materials

**Gravimetric and Titrimetric Methods**

**Final Action 1965**

Conc. combined filtrates and washings from **917.01** to ca 50 mL; make slightly alk. with $NH_4OH$ (1+1); while still hot, add *satd $(NH_4)_2C_2O_4$ soln* dropwise as long as any ppt forms, and then enough excess to convert Mg salts also to oxalate. Heat to bp, let stand ≥3 hr, decant clear soln thru filter, pour 15–20 mL hot $H_2O$ on ppt, and again decant clear soln thru filter. Dissolve any ppt remaining on filter by washing with hot HCl (1+9) into original beaker, wash 6 times with hot $H_2O$, and then reppt at bp by adding $NH_4OH$ and a little satd

$(NH_4)_2C_2O_4$ soln. Let stand as before, filter thru same filter, and wash with hot $H_2O$ until Cl-free. Reserve filtrates and washings from both pptns for detn of Mg, **919.01B**.

Complete detn by one of following methods and report as % CaO:

(**a**) Ignite ppt in crucible, either over S-free blast lamp, or in elec. furnace at 950°, to const wt, cool in desiccator, and weigh as CaO.

(**b**) Incinerate filter over low flame, mix ignited ppt with finely pulverized and dried mixt. of equal parts of *$(NH_4)_2SO_4$ and $NH_4Cl$*, and drive off excess sulfate by carefully heating upper portion of crucible. Complete ignition, cool in desiccator, and weigh as $CaSO_4$.

(**c**) Perforate apex of cone; wash $CaC_2O_4$ ppt into beaker used for pptn; then wash filter with hot $H_2SO_4$ (1+4), and titr. at 85–90° with 0.1*N* $KMnO_4$.

Refs.: U.S. Geol. Survey Bull. **700**, p. 106. Ind. Eng. Chem. **9**, 1114(1917).

CAS-1305-78-8 (calcium oxide)

## 919.01 Magnesium in Liming Materials
### Gravimetric Method
### Final Action 1965

### *A. Reagent*

*Phosphate soln.*—Dissolve 100 g $(NH_4)_2HPO_4$ in hot $H_2O$, dil. to 1 L, and add 5 mL $CHCl_3$.

### *B. Determination*

To combined filtrates and washings, **917.02**, add 2 mL 1*M* citric acid, 100 mL $NH_4OH$, and 50 mL alcohol. Then add 25 mL of the phosphate soln, with const stirring, and let stand 12–24 hr. Filter, wash twice with $NH_4OH$ (1+9), and dissolve ppt in $HNO_3$ (1+4), washing soln into original beaker to vol. of 100–150 mL. Add 1/10 vol. $NH_4OH$ and 2 drops of the phosphate soln. Stir vigorously and let stand ≥3 hr. Filter thru gooch, wash with $NH_4OH$ (1+9), moisten filter with *satd soln of $NH_4NO_3$ made slightly ammoniacal*, ignite, and weigh as $Mg_2P_2O_7$. Report as % MgO. Correct wt $Mg_2P_2O_7$ for co-pptd $Mn_2P_2O_7$ by detg Mn as in **973.55A**.

Ref.: Washington, "Chemical Analysis of Rocks," 3rd Ed., 1919, p. 181.

CAS-1309-48-4 (magnesium oxide)

## CHELOMETRIC ELEMENTAL ANALYSES

## 962.01 Calcium and Magnesium in Liming Materials
### EDTA Titrimetric Methods
### First Action 1962
### Final Action 1965

(Not applicable to samples with high phosphate content or contg <2% Mg)

(*Caution:* See safety note on cyanides.)

### *A. Reagents*

(**a**) *Buffer soln.*—pH 10. Dissolve 67.5 g $NH_4Cl$ in 200 mL $H_2O$, add 570 mL $NH_4OH$, and dil. to 1 L.

(**b**) *Potassium hydroxide-potassium cyanide soln.*—Dissolve 280 g KOH and 66 g KCN in 1 L $H_2O$.

(**c**) *Potassium cyanide soln.*—2%. Dissolve 2 g KCN in 100 mL $H_2O$.

(**d**) *Eriochrome black T indicator soln.*—Dissolve 0.2 g indicator $(HOC_{10}H_6N{:}NC_{10}H_4(OH)(NO_2)SO_3Na)$ (Eastman Kodak P6361, or equiv.) in 50 mL MeOH contg 2 g $NH_2OH.HCl$. Store ≤1 month.

(**e**) *Magnesium std solns.*—0.25 and 1.00 mg/mL. Dissolve 0.25 and 1.00 g Mg turnings in HCl (1+10) and dil. each to 1 L with double distd $H_2O$.

(**f**) *Calcium std soln.*—1 mg/mL. Dissolve 2.4973 g $CaCO_3$, primary std grade, previously dried 2 hr at 285°, in HCl (1+10). Dil. to 1 L with double distd $H_2O$.

(**g**) *Calcein indicator.*—Grind together 1 g indicator (2′,7′-bis[[bis(carboxymethyl)amino]methyl]-fluorescein, sodium derivative, sodium salt), 10 g charcoal (Norite A is satisfactory), and 100 g KCl. (Indicator is described in Anal. Chem. **28**, 882 (1956), and is available from Eastman Kodak.)

(**h**) *Disodium dihydrogen EDTA std solns.*—*(1) 0.4%.*—Dissolve 4 g $Na_2H_2EDTA$ in 1 L $H_2O$. Stdze against std Ca and Mg solns. *(2) 0.1%.*—Prep. as in *(1)*, using 1 g $Na_2H_2EDTA$, and stdze against 0.25 mg/mL Mg std soln.

### *B. Standardization*

(**a**) *For calcium.*—Pipet 10 mL std Ca soln into 300 mL erlenmeyer and add 10 mL $H_2O$. Add 10 mL KOH-KCN soln and ca 35 mg calcein indicator. Using mag. stirrer and artificial light, titr. with 0.4% EDTA std soln to disappearance of all green. Titr. ≥3 aliquots and use av. to calc. titer Ca soln = 10/mL EDTA soln.

(**b**) *For magnesium.*—Pipet 10 mL 0.25 and 1.00 mg/mL Mg std solns into 300 mL erlenmeyers and add 100 mL $H_2O$. Add 5 mL pH 10 buffer, 2 mL 2% KCN soln, and 10 drops eriochrome black T indicator. Using mag. stirrer and artificial light, titr. with 0.1 and 0.4% EDTA std solns, resp., until color changes permanently from wine red to pure blue. Titr. ≥3 aliquots and use av. to calc. titer Mg soln = 2.5/mL EDTA soln, or 10/mL EDTA soln, resp.

### *C. Determination*

Dry sample at 110° to const wt and cool to room temp. Grind to pass No. 60 or 80 sieve and mix thoroly. Accurately weigh ca 0.5 g into 250 mL beaker, add 20 mL HCl (1+1), and evap. to dryness on hot plate. Dissolve residue in 5 mL HCl (1+10), dil. to ca 100 mL with $H_2O$, and digest over low flame 1 hr. Cool, transfer to 200 mL vol. flask, dil. to vol., mix, and let settle or filter.

(**a**) *For calcium.*—Pipet 10 mL aliquot into 300 mL erlenmeyer and titr. as in **962.01B(a)**, observing end point thru soln and away from light. % Ca = (Titer EDTA std soln for Ca) × mL EDTA std soln × 2/g sample.

(**b**) *For magnesium.*—(For agricultural limestones contg >4% Mg.) For Ca + Mg, pipet 10 mL aliquot into 300 mL erlenmeyer and titr. with 0.4% EDTA soln as in **962.01B(b)**.

% Mg = (Titer EDTA std soln for Mg) × [(mL EDTA std soln in Ca + Mg titrn) − (mL EDTA std soln in Ca titrn)] × 2/g sample.

(**c**) *For magnesium.*—(For agricultural limestones contg 2–4% Mg.) Pipet 10 mL aliquot (0.5–1.0 mg Mg) into 300 mL erlenmeyer and add exact vol. of 0.4% EDTA soln required for Ca detn. Titr. with 0.1% EDTA soln as in **962.01B(b)**.

% Mg = (Titer EDTA std soln for Mg) × mL EDTA std 0.1% soln × 2/g sample.

Refs.: JAOAC **45**, 1(1962); **46**, 611(1963); **48**, 95(1965); **50**, 190(1967).

CAS-7440-70-2 (calcium)
CAS-7439-95-4 (magnesium)

## COLORIMETRIC ELEMENTAL ANALYSES

### 965.01 Elemental Analysis of Liming Materials

**Preparation of Sample Solution**

**First Action 1965**
**Final Action 1975**

(Carry reagent blanks thru detn with stds and samples. Treat aliquots of blank soln (corresponding to aliquot sizes of sample solns taken for analysis) as in *Determination* for appropriate element and correct values for samples accordingly.)

Det. Al, Fe, Mn, P, and Ti in solns prepd by $HClO_4$ digestion, **963.01** and **963.02**, or NaOH fusion, **965.01**. Det. Si only in soln prepd by NaOH fusion.

(*Caution:* See safety notes on perchloric acid, NaOH, and KOH.)

Prep. samples as in **924.03**, preferably in agate mortar. Grind samples to pass No. 100 sieve and dry at 105°.

(**a**) *Sample Soln X.*—(0.005 g limestone or 0.002 g silicate/mL.) Place 0.5 g limestone or 0.2 g silicate in 75 mL Ni crucible. If sample contains org. matter, place uncovered crucible in cold furnace, raise temp. gradually to 900°, and hold 15 min. Remove crucible from furnace and let cool. Mix 0.3 g $KNO_3$ with sample and add 1.5 g NaOH pellets. Cover crucible with Ni cover and heat 5 min at dull redness over gas flame. (Do not fuse in furnace.) Remove from flame and swirl melt around sides. Cool, add ca 50 mL $H_2O$, and warm to disintegrate fused cake. Transfer to 150 mL beaker contg 15 mL 5*N* $HClO_4$ (1(60%)+1). Scrub crucible and lid with policeman, and wash any residue into beaker. Transfer to 100 mL vol. flask and dil. to vol. (*Sample Soln X*). (This soln is acidic and is normally clear and free of insol. matter. Occasionally particles of oxidized Ni from crucible appear. When this occurs, let particles settle before taking aliquots.)

(**b**) *Sample Soln Y.*—(0.00015 g limestone or 0.00004 g silicate/mL.) Dil. 15 mL limestone *Sample Soln X* or 10 mL silicate *Sample Soln X* to 500 mL with $H_2O$.

Ref.: JAOAC **47,** 1019(1964).

### 965.02 Aluminum in Liming Materials

**Colorimetric Method**

**First Action 1965**
**Final Action 1975**

#### A. Reagents

(**a**) *Aluminum std solns.*—(*1*) *Stock soln.*—100 μg Al/mL. To 0.1000 g pure Al metal in 30 mL beaker, add 6 mL HCl (1+1).

Cover with watch glass and warm gently until Al completely dissolves. Dil. to 1 L with $H_2O$. (*2*) *Working soln.*—4 μg Al/mL. Dil. 20 mL stock soln to 500 mL.

(**b**) *Aluminon soln.*—Dissolve sep. in $H_2O$: 0.5 g $NH_4$ aurintricarboxylate in 100 mL; 10 g acacia (gum arabic) in 200 mL; and 100 g $NH_4OAc$ in 400 mL. Filter acacia soln. Add 56 mL HCl to $NH_4OAc$ soln and adjust pH to 4.5 with HCl or $NH_4OH$. Combine 3 solns and dil. to 1 L with $H_2O$.

(**c**) *Antifoam soln.*—Disperse 0.03 g silicone defoamer (Dow Corning Corp. Antifoam A) in 100 mL $H_2O$.

(**d**) *Thioglycolic acid soln.*—Dil. 1 mL $HSCH_2COOH$ to 100 mL with $H_2O$.

#### B. Preparation of Standard Curve

Transfer aliquots of std soln contg 0, 4, 20, 40, 60, and 80 μg Al to 100 mL vol. flasks and proceed as in detn. Prep. std curve by plotting %*T* against μg Al on semilog paper.

#### C. Determination

Use *Sample Soln X* for limestones contg <0.2% or silicates contg <0.8% Al and adjust pH of aliquot to 4.5 with $NH_4OH$. For materials contg greater concns of Al, use *Sample Soln Y* and omit pH adjustment.

Transfer aliquot (≤20 mL contg <80 μg Al) of *Sample Soln X* or *Y* to 100 mL vol. flask. Dil. to 20 mL with $H_2O$. Add 2 mL thioglycolic acid soln, 0.5 mL antifoam soln, and 10 mL aluminon soln. Place flask in boiling $H_2O$ 20 min (250 mL beaker contg 125 mL $H_2O$ holds 100 mL vol. flask conveniently). Remove flask from $H_2O$ and let cool ca 30 min. Dil. to 100 mL with $H_2O$. Use 0 μg Al soln, **965.02B**, to set 100% *T* at 525 nm. Read %*T* for sample soln and det. μg Al from std curve. Calc. % Al in sample.

Ref.: JAOAC **47,** 1019(1964).

CAS-7429-90-5 (aluminum)

### 965.03 Iron in Liming Materials

**Colorimetric Method**

**First Action 1965**
**Final Action 1975**

#### A. Reagents

(**a**) *Iron std solns.*—(*1*) *Stock soln.*—100 μg Fe/mL. Dissolve 0.1000 g pure Fe metal in 5 mL 2*N* HCl and dil. to 1 L with $H_2O$. (*2*) *Working soln.*—5 μg Fe/mL. Dil. 25 mL stock soln to 500 mL.

(**b**) *2,4,6-Tripyridyl-s-triazine* (*TPTZ*) *soln.*—Dissolve 0.500 g TPTZ in few drops HCl and dil. to 1 L with $H_2O$.

(**c**) *Hydroxylamine hydrochloride soln.*—Dissolve 50 g $NH_2OH.HCl$ in $H_2O$. Add 10 mL TPTZ soln and 0.5 g $NaClO_4.H_2O$, and dil. to 500 mL with $H_2O$. Transfer to separator, add 25 mL *nitrobenzene,* and shake several min. Let phases sep. and discard lower nitrobenzene phase contg Fe. Repeat extn 2 or 3 times.

(**d**) *Acetate buffer soln.*—Dissolve 164 g anhyd. NaOAc in $H_2O$. Add 115 mL HOAc, 10 mL $NH_2OH.HCl$ soln, 0.05 g TPTZ, and 1 g $NaClO_4.H_2O$, and dil. to 1 L with $H_2O$. Transfer to separator, add 25 mL nitrobenzene, and shake several min. Let phases sep. and discard lower nitrobenzene phase. Repeat extn 3 or 4 times.

#### B. Preparation of Standard Curve

Treat aliquots of std soln contg 0, 5, 50, and 100 μg Fe as in detn. Prep. std curve by plotting %*T* against μg Fe on semilog paper.

#### C. Determination

Use *Sample Soln X* (≤5 mL) for limestones contg <0.05% or silicates contg <0.2% Fe and *Sample Soln Y* for materials contg greater concns of Fe.

Transfer aliquot (<100 μg Fe) of *Sample Soln X* or *Y* to 100 mL vol. flask. Add 3 mL $NH_2OH.HCl$ soln and 10 mL TPTZ soln. Add $NH_4OH$ dropwise until Fe derivative remains violet on mixing. Add 10 mL buffer soln and dil. to 100 mL. Use 0 μg Fe soln, **965.03B**, to set 100% *T* at 593 nm. Read %*T* for sample soln and det. μg Fe from std curve. Calc. % Fe in sample.

Ref.: JAOAC **47,** 1019(1964).

CAS-7439-89-6 (iron)

## 965.04 Manganese in Liming Materials

**Colorimetric Method**

**First Action 1965**
**Final Action 1975**

### A. Reagents

(**a**) *Manganese std soln.*—50 μg Mn/mL. Dissolve 0.0500 g pure Mn metal in 20 mL 0.5 *N* $H_2SO_4$ and dil. to 1 L with $H_2O$.

(**b**) *Acid mixture.*—Add 800 mL $HNO_3$ and 200 mL $H_3PO_4$ to $H_2O$ and dil. to 2 L.

### B. Preparation of Standard Curve

Treat aliquots of std soln contg 0, 50, 100, 300, and 500 μg Mn as in detn. Prep. std curve by plotting %*T* against μg Mn on semilog paper.

### C. Determination

Transfer aliquot (<500 μg Mn) of *Sample Soln X* to 150 mL beaker. Add 25 mL acid mixt. and 0.3 g $KIO_4$. Bring to bp and keep near boiling temp. 10 min after color develops. Let cool, transfer to 50 mL vol. flask, dil. to vol., and mix. Use 0 μg Mn soln, **965.04B**, to set 100% *T* at 525 nm. Read %*T* for sample soln and det. μg Mn from std curve. Calc. % Mn in sample.

Ref.: JAOAC **47,** 1019(1964).

CAS-7439-96-5 (manganese)

## 965.05 Phosphorus in Liming Materials

**Colorimetric Method**

**First Action 1965**
**Final Action 1975**

(Do not clean glassware with detergents contg P.)

### A. Reagents

(**a**) *Phosphorus std solns.*—(*1*) *Stock soln.*—100 μg P/mL. Dissolve 0.4393 g $KH_2PO_4$ in $H_2O$ and dil. to 1 L. (*2*) *Working soln.*—5 μg P/mL. Dil. 25 mL stock soln to 500 mL.

(**b**) *Ammonium molybdate soln.*—Dissolve 20 g $(NH_4)_6 \cdot Mo_7O_{24}.4H_2O$ in 500 mL $H_2O$. Add 285 mL $H_2SO_4$, cool, and dil. to 1 L with $H_2O$.

(**c**) *Hydrazine sulfate soln.*—Dissolve 2 g $N_2H_4.H_2SO_4$ in $H_2O$ and dil. to 1 L.

### B. Preparation of Standard Curve

Treat aliquots of std soln contg 0, 5, 50, and 75 μg P as in detn. Prep. std curve by plotting %*T* against μg P on semilog paper.

### C. Determination

Transfer aliquot (≤15 mL contg <75 μg P) of *Sample Soln X* to 100 mL vol. flask. Add 5 mL $NH_4$ molybdate soln and mix. Add 5 mL $N_2H_4.H_2SO_4$ soln, dil. to 70 mL with $H_2O$, and mix. Place flask in boiling $H_2O$ 9 min. Remove, *cool rapidly,* and dil. to vol. Use 0 μg P soln, **965.05B**, to set 100% *T* at 827 nm. Read %*T* for sample soln and det. μg P from std curve. Calc. % P in sample.

Ref.: JAOAC **47,** 1019(1964).

CAS-7723-14-0 (phosphorus)

## 965.06 Titanium in Liming Materials

**Colorimetric Method**

**First Action 1965**
**Final Action 1975**

### A. Reagents

(**a**) *Titanium std solns.*—(*1*) *Stock soln.*—100 μg Ti/mL. Place 0.1668 g $TiO_2$ and 2 g $K_2S_2O_7$ in Pt crucible. Heat covered crucible gently at first and then at dull red ca 15 min. Dissolve melt in 50 mL $H_2SO_4$ (1+1) and dil. to 1 L with $H_2O$. (*2*) *Working soln.*—5 μg Ti/mL. Dil. 25 mL stock soln to 500 mL.

(**b**) *Acetate buffer soln.*—pH 4.7. Dissolve 41 g anhyd. NaOAc in $H_2O$, add 30 mL HOAc, and dil. to 1 L.

(**c**) *Disodium-1,2-dihydroxybenzene-3,5-disulfonate* (*Tiron*) *soln.*—Dissolve 4 g Tiron in $H_2O$ and dil. to 100 mL.

### B. Preparation of Standard Curve

Treat aliquots of std soln contg 0, 5, 50, and 75 μg Ti as in detn, but do not add dithionite to stds. Prep. std curve by plotting %*T* against μg Ti on semilog paper.

### C. Determination

Transfer aliquot (<75 μg Ti) of *Sample Soln X* to 50 mL beaker. Dil. to ca 25 mL with $H_2O$. Add 5 mL Tiron soln and then $NH_4OH$ (1+9) dropwise until soln is neut. to Congo Red paper. (Tiron soln must be added before pH is adjusted.) Transfer to 50 mL vol. flask, add 5 mL buffer soln, dil. to vol. with $H_2O$, and mix thoroly. Add 25 mg *dithionite* ($Na_2S_2O_4$) and dissolve with min. agitation (to avoid reappearance of blue). Use 0 μg Ti soln, **965.06B**, to set 100% *T* at 410 nm. Read %*T* for sample soln within 15 min after adding dithionite. Det. μg Ti from std curve. Calc. % Ti in sample.

Ref.: JAOAC **47,** 1019(1964).

CAS-7440-32-6 (titanium)

## 965.07 Silicon in Liming Materials

**Colorimetric Method**

**First Action 1965**
**Final Action 1975**

(Clean all glassware with HCl (1+1).)

### A. Reagents

(**a**) *Silicon std soln.*—20 μg Si/mL. Place 0.0428 g pure $SiO_2$ in 75 mL Ni crucible and treat as in **965.01(a)**, but dil. with $H_2O$ to 1 L instead of 100 mL.

(**b**) *Tartaric acid soln.*—Dissolve 50 g tartaric acid in $H_2O$ and dil. to 500 mL. Store in plastic bottle.

(**c**) *Ammonium molybdate soln.*—Dissolve 7.5 g $(NH_4)_6 \cdot Mo_7O_{24}.4H_2O$ in 75 mL $H_2O$, add 10 mL $H_2SO_4$ (1+1), and dil. to 100 mL with $H_2O$. Store in plastic bottle.

(**d**) *Reducing soln.*—Dissolve 0.7 g $Na_2SO_3$ in 10 mL $H_2O$. Add 0.15 g 1-amino-2-naphthol-4-sulfonic acid and stir until dissolved. Dissolve 9 g $NaHSO_3$ in 90 mL $H_2O$, add to first soln, and mix. Store in plastic bottle.

### B. Preparation of Standard Curve

Treat aliquots of std soln contg 0, 20, 100, and 200 μg Si as in detn. Prep. std curve by plotting %*T* against μg Si on semilog paper.

***C. Determination***

Transfer 10 mL *Sample Soln Y* to 100 mL vol. flask (use *Sample Soln X* for limestones contg <0.2% Si) and add 1 mL $NH_4$ molybdate soln with swirling. Mix well, and let stand 10 min. Add 4 mL tartaric acid soln with swirling, and mix well. Add 1 mL reducing soln with swirling, dil. to vol., mix well, and let stand ≥30 min. Use 0 μg Si soln, **965.07B**, to set 100% *T* at 650 nm. Read %*T* for sample soln and det. μg Si from std curve. Calc. % Si in sample.

Ref.: JAOAC **47,** 1019(1964).

CAS-7440-21-3 (silicon)

# 2. Fertilizers


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### 929.01 Sampling of Solid Fertilizers

**Final Action 1974**

(**a**) *Bagged fertilizers*.—Use slotted single or double tube trier with solid cone tip, constructed of stainless steel or brass. (Do not use unplated brass for samples on which micronutrients are to be detd.) Trier length, exclusive of handle, should be approx. length of filled bag to be sampled, but >25″; length of slot >23″; width of slot ≥0.5″; and id ≥$^5/_8$″.

Take sample as follows: Lay bag horizontally and remove core diagonally from end to end. From lots of ≥10 bags, take core from each of 10 bags. When necessary to sample lots of <10 bags, take 10 cores but at least 1 core from each bag present. For small packages (≤10 lb), take 1 entire package as sample.

(**b**) *Bulk fertilizers, including railroad car-size lots*.—Use trier of design represented in Table **929.01**.

Draw 10 vertical cores distributed in std concentric sampling pattern (Fig. **929.01A**) of such design that each core represents approx. equal fractions of lot.

Bulk shipments may be sampled at time of loading or unloading by passing sampling cup, Fig. **929.01B** (mouth dimensions: width $^3/_4$″, length 16″ or as long as max. diam. of stream), thru entire stream of material as it drops from belt or chute. Make sampling such as to assure ≥10 equal-timed-spaced passes thruout transfer operation. Stream samples are not applicable unless uniform continuous flow of fertilizer is maintained for >3 min while lot is being sampled.

(**c**) *Preparation of sample*.—Place composite sample in airtight container and deliver entire sample to laboratory. Reduce composite sample in laboratory, using riffle.

Refs.: JAOAC **12,** 97(1929); **33,** 424(1950); **38,** 108,541 (1955); **50,** 190,382(1967); **51,** 859(1968); **55,** 709 (1972).

### 969.01 Sampling of Liquid Fertilizers

**Final Action**

(In absence of free ammonia)

(**a**) *Clear solns*.—(Mixed liqs and N solns.) Secure sample directly from mixing vat, storage tank, or delivery tank after thoro mixing. Take sample from surface or thru direct tap. Flush direct tap, or delivery line and faucet, and collect sample in glass or polyethylene container. Alternatively, lower sample container into well mixed material thru port in top of tank and let fill. Seal container tightly.

(**b**) *Fluid fertilizers with suspended material*.—(Salt suspensions and slurries.) Agitate material in storage until thoroly mixed (15 min usually adequate) before taking sample. Sample directly as in (**a**), or use 500 mL Missouri or Indiana sampling bottle, Fig. **969.01**. Lower sampling bottle from top opening to bottom of tank and raise slowly while filling. Transfer to sample bottle and seal tightly.

Alternatively, secure sample from tap on recirculation line after agitating *and* recirculating simultaneously until thoroly mixed. Draw sample while recirculating. If recirculation line is attached to manifold delivery line, allowing cross-contamination, pump ca 30 cm (1′) or 2000 L (500 gal.) into temporary storage tank, then sample from recirculation line as above or from delivery line. Transfer to sample bottle and seal tightly.

Ref.: JAOAC **52,** 592(1969).

### 959.01 Sampling of Ammoniacal Solutions

**First Action 1959**
**Final Action 1960**

#### *A. Apparatus*

(**a**) *Container*.—Polyethylene reagent-form bottle with buttress-type cap, 1 L (1 qt) capacity.

(**b**) *Sample flow control apparatus*.—Construct from following fittings: 1$^1/_2$ × $^1/_4$″ reducing bushing; $^1/_4$″ tee; $^1/_4$″ nipple 12–18″ long (length not critical); two $^1/_4$″ stainless steel, bluntnose needle valves with hose connections (Hoke No. 3712M4Y; Hoke Inc., 1 Tenakill Pk, Cresskill, NJ 07626). All fittings except valves can be either Al or stainless steel. (*See* Fig. **959.01**.)

Attach valves directly to tee, which is then attached to reducing bushing thru nipple. To both valves attach $^1/_4$″ id Tygon tubing (Hoke No. 62065 hose connection), 12″ length to sample valve and sufficient length to vent valve to reach disposal area or container. To free end of sample tubing attach 3″ length of $^1/_4$″ glass or stainless steel tubing inserted thru No. 4 rubber stopper. To exit end of metal tube attach addnl 6″ length of Tygon tubing. Make certain all connections are tight. App. can be attached directly to tank cars, but requires addnl coupling, which varies with installation, to attach to storage tanks. 1$^1/_2$″ "quick coupler" (Ever-Tite Coupling Co., 254 W 54th St, New York, NY 10019) suffices in most cases.

Ref.: JAOAC **42,** 500 (1959).

#### *B. Sampling*

Prep. sample bottle in laboratory by adding ca 500 mL $H_2O$, replacing cap, and weighing accurately (±0.1 g). Attach sampling app. to car or tank and, with sample valve closed, flush line thru vent valve. Partially collapse sample bottle, insert sample tube with stopper, and seal tightly. With sample tube dipping below surface of $H_2O$ in bottle, throttle vent valve to maintain small flow of soln and partially open sample valve, collecting ca 100 mL sample. (Bottle should not expand to full size during this time.) Close sample valve, remove sample tube, partially collapse bottle, and cap tightly. Reweigh (±0.1 g) and calc. wt sample. Cool to 20°, transfer to 1 or 2 L vol. flask, dil. to vol. with $H_2O$, mix thoroly, and take aliquots for analysis.

### 959.02 Sampling of Anhydrous Ammonia

**First Action 1959**
**Final Action 1960**

(*Caution:* Use extreme care in handling anhyd. $NH_3$. Suitable gas mask and rubber gloves are required. *See* safety note on ammonia. )

**Table 929.01 Trier Specifications**

| Trier | Length, in. | od, in. | id, in. | Compartments No. | Compartments Size, in. |
|---|---|---|---|---|---|
| Missouri | 59 | 1 1/8 | 7/8 | 8 | 3 |
| 552 Grain[a] | 63 | 1 3/8 | 1 1/8 | 11 | 3 1/2 |
| Missouri "D"[b] | 49 | 1 1/4 | 1 | 1 | 43 |

Triers available from:
[a] Seedburo Equipment Co., 1022 W Jackson Blvd, Chicago, IL 60607.
[b] Boyt Tool & Die Co., 917 Maple St, West Des Moines, IA 50265.

### *A. Sampling*

Use sample tube of thermal shock-resistant glass calibrated to contain 100 mL and graduated in 0.05 mL subdivisions up to 0.5 mL. (Dupont special oil centrf. tube or ASTM long-form oil tube is satisfactory.) Flush line and fill tube to 100 mL mark with sample in such manner that condensing moisture will not enter sample tube. (Skirt attached to end of sample line will drain moisture away.)

### *B. Water and Nitrogen*

Immediately close sample tube with tight-fitting rubber stopper into which is inserted tight-fitting piece of 6 mm id glass tubing 5–8 cm long, bent at its exit from outer end of stopper to let gases escape but to exclude entrance of moisture or moisture-laden air. Place in $H_2O$ bath at approx. air temp. and let $NH_3$ evap. When temp. of sample tube is ca that of bath, remove tube, wipe outer surface, and det. vol. of residue.

$$\%\ H_2O \text{ in sample} = \text{mL residue} \times C$$

where $C$ = 0.74, 0.70, or 0.66 for pressures in original containers of 100, 150, or 200 psi, resp.

$$\%\ N = (100 - \%\ H_2O) \times 0.8224$$

Ref.: JAOAC **42,** 500(1959).

CAS-7727-37-9 (nitrogen)

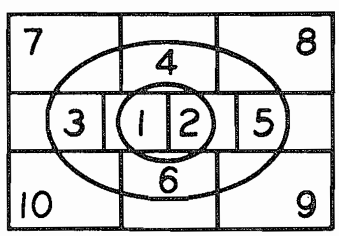


FIG. 929.01A—Sampling pattern

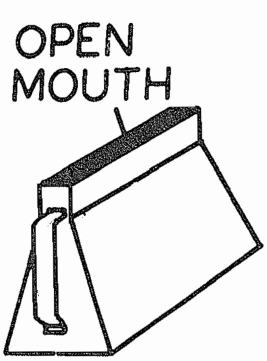


FIG. 929.01B—Sampling cup

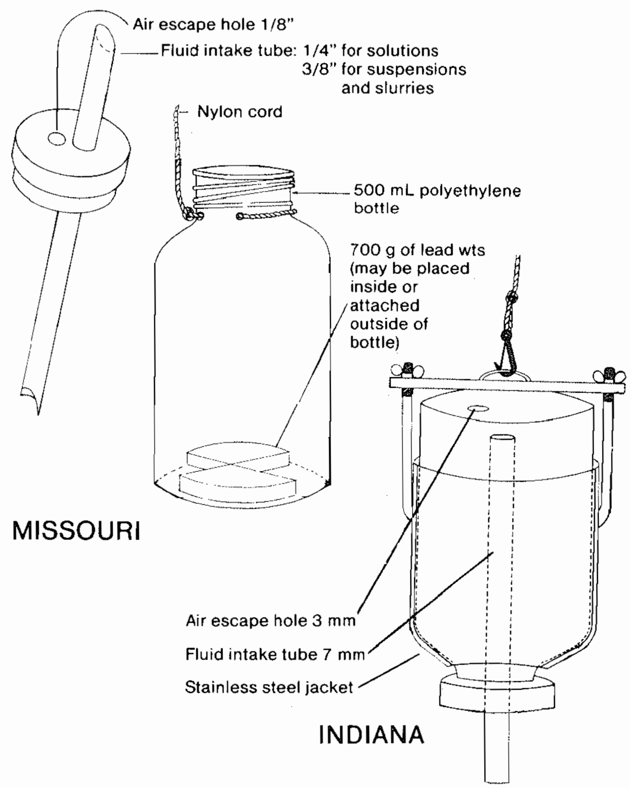


**FIG. 969.01—Missouri and Indiana weighted restricted-fill fluid fertilizer sampling bottles designed to fill while being lowered (and raised) in storage tanks**

## 929.02 Preparation of Fertilizer Sample

**Final Action**

Reduce gross sample to amt sufficient for analysis or grind ≥225 g (0.5 lb) of reduced sample without previous sieving.

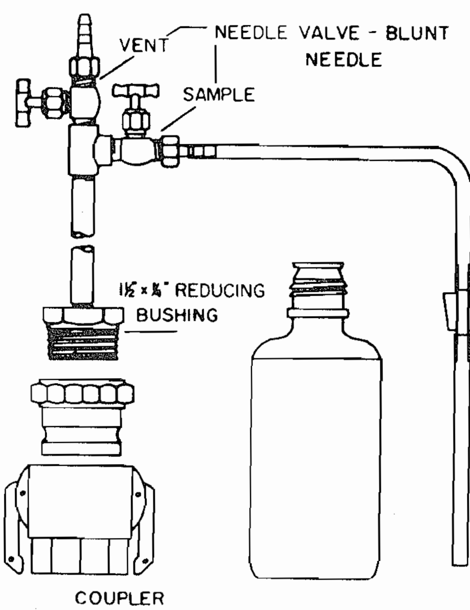


**FIG. 959.01—Sampling apparatus for ammoniacal solutions, including "quick coupler" for attaching to storage tanks**

For fertilizer materials and moist fertilizer mixts, grind to pass sieve with 1 mm circular openings, or No. 20 sieve; for dry mixts that tend to segregate, grind to pass No. 40 sieve. Grind as rapidly as possible to avoid loss or gain of moisture during operation. Mix thoroly and store in tightly stoppered bottles.

Refs.: JAOAC **12,** 98(1929); **24,** 253(1941).

## 917.03★ Bone, Tankage, and Basic Slag Fertilizers

**Mechanical Analysis**

**Final Action**
**Surplus 1970**

See **2.008**, 11th ed.

## 957.01 Phosphate Rock Fertilizers

**Mechanical Analysis**

**Final Action**

### *A. Apparatus*

(**a**) *Water pressure control.*—*See* Fig. **957.01**. Connect valve, *A*, std pressure gage, *B*, and aerator, *C*, with $^3/_8''$ diam. pipe.

(**b**) *Sieves.*—Nos. 100 and 200, bronze or stainless steel cloth, checked against certified sieves. Sieves 8″ diam. and 2″ in depth to sieve cloth are recommended for both wet and dry sieving, but other sizes may be used if detd to be suitable under conditions of method. (Other sieves in U.S. series may be used, with precaution to ensure complete sepn of sample into desired fractions.)

(**c**) *Sieve shaker.*—Ro-Tap (C-E Tyler, Inc., 3200 Bessemer City Rd, Hwy 274, PO Box 8900, Gastonia, NC 28053), Syntron (FMC Corp., Material Handling Equipment Div., Homer City, PA 15748), or other suitable machine.

### *B. Reagent*

*Dispersing agent.*—Dissolve 36 g Na hexametaphosphate and 8 g $Na_2CO_3$ in $H_2O$ and dil. to 1 L.

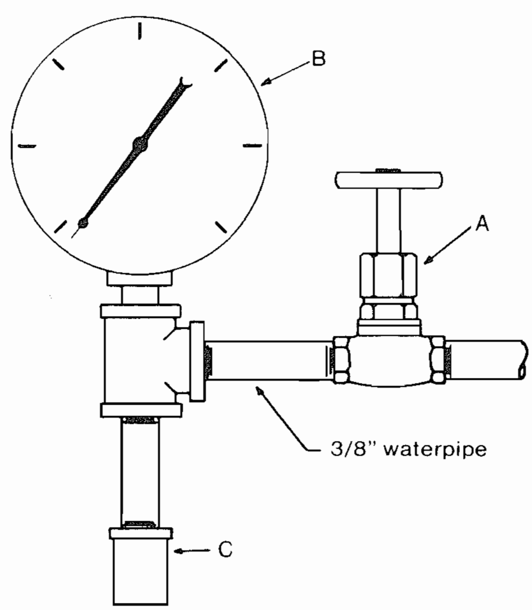


**FIG. 957.01—Apparatus for control of water pressure**

### *C. Determination*

(**a**) *Ground phosphate rock.*—Place 100 g sample on No. 200 sieve and wash with moderate stream of tap $H_2O$ at max. gage pressure of 0.28 kg/sq cm (4 lb/sq in.) until $H_2O$ passing sieve is clear, with care to avoid loss of sample by splashing. Dry material remaining on sieve at 105° and transfer to No. 100 sieve in series with No. 200 sieve of same diam. and depth. Shake 8 min in mech. shaker. Det. % sample passing No. 100 sieve by subtracting wt of material retained on that sieve from 100. Det. % sample passing No. 200 sieve by subtracting sum of wts of material retained on that sieve and on No. 100 sieve from 100.

(**b**) *Soft phosphate with colloidal clay.*—Add 100 g sample to rapidly stirred soln of 50 mL dispersing agent and 450 mL tap $H_2O$, with care to avoid contact of unwetted material with shaft of stirrer and side of beaker. Stir 5 min after addn of sample is completed. Transfer slurry to No. 200 sieve and proceed as in (**a**).

Ref.: JAOAC **40,** 711(1957).

## 955.03★ Ash (Acid-Insoluble) of Fertilizers

**Final Action**
**Surplus 1970**

See **2.015**, 11th ed.

# WATER

## 950.01 Water (Total) in Fertilizers

**Final Action**

(Not applicable to samples that yield volatile substances other than $H_2O$ at drying temp.)

Heat 2 g sample, **929.02**, 5 hr in oven at 100±1°. In case of $NaNO_3$, $(NH_4)_2SO_4$, and K salts, heat to const wt at 130±1°. Report % loss in wt as $H_2O$ at temp. used.

Ref.: JAOAC **33,** 260(1950).

## 965.08 Water (Free) in Fertilizers

**Vacuum-Desiccation Methods**

**First Action 1965**
**Final Action 1974**

(*Caution:* See safety note on magnesium perchlorate.)

### *A. Method I*

Place 2 g prepd sample, **929.02**, in tared weighing dish. (Weigh extremely hygroscopic or damp materials by difference in covered dishes.) Dry sample at 25–30° (precise results depend on as const a temp. as possible) in vac. desiccator over anhyd. $Mg(ClO_4)_2$, $P_2O_5$, or BaO, under ≥50 cm (20″) or ≤55 cm (22″) vac. (20–25 cm (8–10″) absolute pressure) 16–18 hr. Reweigh, and report % loss in wt as free $H_2O$.

### *B. Method II*

(Not applicable to samples which yield volatile substances other than $H_2O$)

Weigh 2 g prepd sample, **929.02**, into tared glass weighing dish. Dry sample 2 hr±10 min at 50±1.5° in oven under vac. of 48–53 cm (19–21″) (23–28 cm (9–11″) absolute pressure). (Temp. control within specified limits thruout oven chamber is essential.) Maintain vac. by passing desiccated air thru chamber. Cool dried sample in desiccator and reweigh. Report % loss in wt as free $H_2O$.

Refs.: JAOAC **46,** 582(1963); **47,** 32, 1040(1964); **48,** 98 (1965).

## 972.01 Water (Free) in Fertilizers

### Alternative Extraction Method

**First Action 1972**
**Final Action 1974**

#### *A. Principle*

Free $H_2O$ is extd with dioxane and detd by titrn with Karl Fischer reagent.

#### *B. Reagents*

(Keep exposure of org. reagents to air at min.)

(**a**) *Karl Fischer reagent.*—Stabilized single soln (Fisher Scientific Co., So-K-3, or equiv.) dild ca 1 + 1 with stabilized diluent (Fisher, So-K-5, or equiv.), or soln equiv. to 2.5 mg $H_2O$/mL. Stdze daily with ca 0.2 g Na tartrate.$2H_2O$. 1 mg Na tartrate.$2H_2O$ = 0.1566 mg $H_2O$.

(**b**) *Methanol.*—Low in $H_2O$.

#### *C. Determination*

Accurately weigh 2.5 g prepd sample, **929.02**, into 125 mL erlenmeyer, add 50.0 mL *1,4-dioxane,* stopper, mix by swirling, and let stand 15 min. Mix thoroly by swirling, and centrf. in closed tube. (*Caution:* See safety note on centrifuges.)

Transfer 10 mL aliquot to titrn vessel contg pretitrd MeOH and titr. with Karl Fischer reagent. (Discard contents of titrn vessel after 3 titrns, replace with enough MeOH to cover electrodes, and pretitr. before proceeding with next sample.) Det. blank on 10 mL dioxane as above and subtract from sample detns. Calc. and report as free $H_2O$.

Refs.: JAOAC **52,** 1127(1969); **55,** 699(1972).

# PHOSPHORUS

## 957.02 Phosphorus (Total) in Fertilizers

### Preparation of Sample Solution

**Final Action**

#### *A. Reagent*

*Magnesium nitrate soln.*—Dissolve 950 g P-free $Mg(NO_3)_2.6H_2O$ in $H_2O$ and dil. to 1 L.

#### *B. Preparation of Solution*

(*Caution:* See safety notes on wet oxidation, nitric acid, perchloric acid, sulfuric acid, and oxidizers.)

Treat 1 g sample by (**a**), (**b**), (**c**), (**d**), or (**e**), as indicated. Cool soln, transfer to 200 or 250 mL vol. flask, dil. to vol., mix, and filter thru dry filter.

(**a**) *Materials containing small quantities of organic matter.*—Dissolve in 30 mL $HNO_3$ and 3–5 mL HCl, and boil until org. matter is destroyed (30 min for liqs and suspensions).

★(**b**) *Fertilizers containing much Fe or Al phosphate, and basic slag.*★—*See* **2.017**, 10th ed.

(**c**) *Organic material like cottonseed meal alone or in mixtures.*—Evap. with 5 mL $Mg(NO_3)_2$ soln, **957.02A**, ignite, and dissolve in HCl.

★(**d**) *Materials or mixtures containing large amounts of organic matter.*★—*See* **2.017(d)**, 11th ed.

(**e**) *All fertilizers.*—Boil gently 30–45 min with 20–30 mL $HNO_3$ in suitable flask (preferably Kjeldahl for samples contg large amts of org. matter) to oxidize all easily oxidizable matter. Cool. Add 10–20 mL 70–72% $HClO_4$. Boil very gently until soln is colorless or nearly so and dense white fumes appear in flask. Do not boil to dryness at any time (Danger!). (With samples contg large amts of org. matter, raise temp. to fuming point, ca 170°, over period of ≥1 hr.) Cool slightly, add 50 mL $H_2O$, and boil few min.

Ref.: JAOAC **40,** 690(1957).

CAS-7723-14-0 (phosphorus)

## 958.01 Phosphorus (Total) in Fertilizers

### Spectrophotometric Molybdovanadophosphate Method

**Final Action**

(Not applicable to materials yielding colored solns or solns contg ions other than orthophosphate which form colored complexes with molybdovanadate. Not recommended for basic slag.)

#### *A. Apparatus*

*Photometer.*—Spectrophtr with stray light filter and matched 1 cm cells. Analyst must det. suitability for use and conditions for satisfactory performance. Means for dispelling heat from light source is desirable.

#### *B. Reagents*

(**a**) *Molybdovanadate reagent.*—Dissolve 40 g $NH_4$ molybdate.$4H_2O$ in 400 mL hot $H_2O$ and cool. Dissolve 2 g $NH_4$ metavanadate in 250 mL hot $H_2O$, cool, and add 450 mL 70% $HClO_4$. (*Caution:* See safety notes on perchloric acid.) Gradually add molybdate soln to vanadate soln with stirring, and dil. to 2 L.

(**b**) *Phosphate std soln.*—Dry pure $KH_2PO_4$ (52.15% $P_2O_5$) 2 hr at 105°. Prep. solns contg 0.4–1.0 mg $P_2O_5$ /mL in 0.1 mg increments by weighing 0.0767, 0.0959, 0.1151, 0.1342, 0.1534, 0.1726, and 0.1918 g $KH_2PO_4$ and dilg each to 100 mL with $H_2O$. Prep. fresh solns contg 0.4 and 0.7 mg $P_2O_5$/ mL weekly.

#### *C. Preparation of Standard Curve*

Pipet 5 mL aliquots of 7 std phosphate solns (2–5 mg $P_2O_5$ / aliquot) into 100 mL vol. flasks and add 45 mL $H_2O$. Then, within 5 min for entire series, add 20 mL molybdovanadate reagent by buret or pipet, dil. to vol. and mix. Let stand 10 min.

Select 2 absorption cells (std and sample cells) and fill both with 2 mg std. Set spectrophtr to 400 nm and adjust to zero *A* with std cell. Sample cell must check zero *A* within 0.001 unit; otherwise read *A* for sample cell and correct subsequent readings. (Choose cell showing pos. *A* against other as sample cell so that this pos. *A* is always subtracted.) Using sample cell, det. *A* of other stds with instrument adjusted to zero *A* for 2 mg std. After each detn empty and refill cell contg 2 mg std, and readjust zero to avoid error that might arise from temp. changes. Plot *A* against concn in mg $P_2O_5$ /mL std soln.

#### *D. Preparation of Solution*

Treat 1 g sample as in **957.02B**, preferably (**e**), when these acids are suitable solv. (Soln should be free of N oxides and NOCl.)

(**a**) For $P_2O_5$ content ≤5%, dil. to 250 mL.

(**b**) For $P_2O_5$ content >5%, dil. to such vol. that 5 or 10 mL aliquot contains 2–5 mg $P_2O_5$.

### *E. Determination*

Pipet, into 100 mL vol. flasks, 5 mL aliquots of std phosphate solns contg 2 and 3.5 mg $P_2O_5$/aliquot, resp., and develop color as in **958.01C**. Adjust instrument to zero *A* for 2 mg std, and det. *A* of 3.5 mg std. (It is essential that *A* of latter std be practically identical with corresponding value on std curve.)

(**a**) *Samples containing up to 5% $P_2O_5$.*—Pipet, into 100 mL vol. flask, 5 mL sample soln, **958.01D(a)**, and 5 mL std phosphate soln contg 2 mg $P_2O_5$. Develop color and det. *A* concurrently with and in same manner as for std phosphate solns in preceding par., with instrument adjusted to zero *A* for 2 mg std. Read $P_2O_5$ concn from std curve. With series of sample solns, empty and refill cell contg 2 mg std after each detn.

$$\% \ P_2O_5 \text{ in sample} = 100 \times [(\text{mg } P_2O_5 \text{ from std curve} - 2)/20]$$

(**b**) *Samples containing more than 5% $P_2O_5$.*—Pipet 5 or 10 mL sample soln, **958.01D(b)**, into 100 mL vol. flask. Without adding std phosphate soln, proceed as in (**a**).

$$\% \ P_2O_5 \text{ in sample} = 100 \times (\text{mg } P_2O_5 \text{ from std curve/mg sample in aliquot})$$

Refs.: JAOAC **41,** 517(1958); **42,** 503(1959); **44,** 133(1961).

CAS-1314-56-3 (phosphorus pentoxide)

## 962.02 Phosphorus (Total) in Fertilizers

### Gravimetric Quinolinium Molybdophosphate Method

**First Action 1962**
**Final Action 1965**

### *A. Reagents*

(Store solns in polyethylene bottles.)

(**a**) *Citric-molybdic acid reagent.*—Dissolve 54 g 100% molybdic anhydride ($MoO_3$) and 12 g NaOH with stirring in 400 mL hot $H_2O$, and cool. Dissolve 60 g citric acid in mixt. of 140 mL HCl and 200 mL $H_2O$, and cool. Gradually add molybdic soln to citric acid soln with stirring. Cool, filter, and dil. to 1 L. (Soln may be green or blue; color deepens on exposure to light.) If necessary, add 0.5% $KBrO_3$ soln dropwise until green color pales. Store in dark.

(**b**) *Quinoline soln.*—Dissolve 50 mL *synthetic* quinoline, with stirring, in mixt. of 60 mL HCl and 300 mL $H_2O$. Cool, dil. to 1 L, and filter.

(**c**) *Quimociac reagent.*—Dissolve 70 g Na molybdate.$2H_2O$ in 150 mL $H_2O$. Dissolve 60 g citric acid in mixt. of 85 mL $HNO_3$ and 150 mL $H_2O$, and cool. Gradually add molybdate soln to citric acid-$HNO_3$ mixt. with stirring. Dissolve 5 mL *synthetic* quinoline in mixt. of 35 mL $HNO_3$ and 100 mL $H_2O$. Gradually add this soln to molybdate-citric acid-$HNO_3$ soln, mix, and let stand 24 hr. Filter, add 280 mL acetone, dil. to 1 L with $H_2O$, and mix.

### *B. Preparation of Solution*

Treat 1 g sample as in **957.02B**, dilg to 200 mL.

### *C. Determination*

Pipet, into 500 mL erlenmeyer, aliquot contg ≤25 mg $P_2O_5$ and dil. to ca 100 mL with $H_2O$. Continue by one of the following methods:

(**a**) Add 30 mL citric-molybdic acid reagent and boil gently 3 min. (Soln must be ppt-free at this time.) Remove from heat and swirl carefully. Immediately add 10 mL quinoline soln from buret with continuous swirling. (Add first 3–4 mL dropwise and remainder in steady stream.) Or:

(**b**) Add 50 mL quimociac reagent, cover with watch glass, place on hot plate in well-ventilated hood, and boil 1 min.

After treatment by (**a**) or (**b**), cool to room temp., swirl carefully 3–4 times during cooling, filter into gooch with glass fiber filter paper previously dried at 250° and weighed, and wash with five 25 mL portions of $H_2O$. Dry crucible and contents 30 min at 250°, cool in desiccator to room temp., and weigh as $(C_9H_7N)_3H_3[PO_4.12MoO_3]$. Subtract wt reagent blank. Multiply by 0.03207 to obtain wt $P_2O_5$ (or by 0.01400 for P). Report as % $P_2O_5$ (or % P).

Refs.: Z. Anal. Chem. **189,** 243(1962). JAOAC **45,** 40, 999 (1962); **46,** 579(1963); **47,** 420(1964).

CAS-7723-14-0 (phosphorus)
CAS-1314-56-3 (phosphorus pentoxide)

## 969.02 Phosphorus (Total) in Fertilizers

### Alkalimetric Quinolinium Molybdophosphate Method

**First Action 1969**
**Final Action 1975**

### *A. Reagents*

(**a**) *Quimociac reagent.—See* **962.02A(c)**.

(**b**) *Sodium hydroxide std soln.*—(1 mL = 1 mg $P_2O_5$.) Dil. 366.32 mL 1*N* NaOH, **936.16**, to 1 L with $H_2O$.

(**c**) *Nitric acid std soln.*—Prep. $HNO_3$ soln equiv. to concn of (**b**) and stdze by titrg against (**b**), using phthln. (For greater precision, use $HNO_3$ soln corresponding to 1/5 concn of (**b**).)

(**d**) *Citric acid.*—10% (w/v).

(**e**) *Indicators.—(1) Thymol blue soln.*—0.1%. Add 2.2 mL 0.1*N* NaOH to 0.1 g thymol blue and dil. to 100 mL with 50% alcohol. *(2) Phenolphthalein.*—0.1%. Dissolve 0.1 g phthln in 100 mL 50% alcohol. *(3) Mixed indicator.*—Mix 3 vols (*1*) and 2 vols (*2*).

### *B. Preparation of Sample Solution*

Treat 1 g sample as in **957.02B**.

### *C. Determination*

(**a**) *Precipitation.*—Transfer aliquot contg ≤30 mg $P_2O_5$ and ≤5 mL concd acid to 500 mL erlenmeyer, add 20 mL citric acid soln, and adjust to ca 100 mL. Add 60 mL quimociac reagent, immediately cover with watch glass, and place on medium temp. hot plate. After soln comes to bp, move to cooler portion of hot plate and boil gently 1 min. Let cool until flask can be handled comfortably with bare hand.

(**b**) *Filtration and washing.*—Prep. pulped-paper pad ca 6 mm thick on perforated porcelain disk in funnel by adding ≥2 approx. equal increments of $H_2O$ suspension of pulped paper and sucking dry with vac. between addns. Swirl flask, pour contents onto filter, and wash flask with five ca 15 mL portions $H_2O$, adding washings to funnel. Immediately after funnel has emptied, wash down sides with ca 15 mL $H_2O$ to remove residual acetone, which causes excessively fast drying and later lump formation if allowed to evap. Wash with 3 addnl 15 mL portions $H_2O$, letting funnel empty between addns. Keep drying of ppt to min. Using only jet of $H_2O$, transfer ppt and pad to pptn flask and break up pad with jet of $H_2O$. Do not smear ppt against funnel or flask.

(**c**) *Titration.*—Titr. with std NaOH soln and add 3–5 mL excess. Add 1 mL mixed indicator and titr. with std $HNO_3$

soln to grey-blue end point. If overtitrd (greenish-yellow), add addnl excess std NaOH soln and titr. to grey-blue.

(**d**) *Blank*.—Det. blank on all reagents, adding known amt (1–2 mg) of $P_2O_5$. Use 1 + 9 dilns of std NaOH and $HNO_3$ for titrn and subtract theoretical titer equiv. to $P_2O_5$ added from experimental titer. Calc. difference equiv. to 0.3663*N* NaOH and subtract this blank from all sample detns.

Calc. and report as % $P_2O_5$.

Refs.: Z. Anal. Chem. **189,** 243(1962). JAOAC **45,** 40, 999 (1962); **49,** 1201(1966); **52,** 587(1969).

CAS-1314-56-3 (phosphorus pentoxide)

## 978.01 Phosphorus (Total) in Fertilizers

**Automated Method**

**First Action 1978**
**Final Action 1980**

### *A. Principle*

Samples are extd for direct available $P_2O_5$ or for total $P_2O_5$ detns. Destruction of coloring matter, hydrolysis of nonorthophosphates, and elimination of citrate effect are accomplished by digestion with 4*N* $HClO_4$ at 95°. Digested samples are reacted with molybdovanadate reagent, and *A* of resulting complex is read in flowcell at 420 nm in range 0.15–0.35 mg $P_2O_5$/mL.

### *B. Apparatus and Reagents*

(*Caution:* See safety notes on perchloric acid.)

(**a**) *Automatic analyzer*.—AutoAnalyzer with following modules (Technicon Instruments Corp., or equiv.): Sampler II or IV with 40/hr (4:1) cam; proportioning pump III; $P_2O_5$ anal. cartridge (with 2 heating baths, each contg 10.6 mL coil held at 95±1°; or AAI type heating bath contg one 40′ × 1.6 mm id coil and holding constant temp. of 95±1°); AAII single channel colorimeter with 15 × 1.5 or 2.0 mm id flowcell and 420 nm interference filters; voltage stabilizer; and recorder. Construct manifold as in flow diagram, Fig. **978.01**.

(**b**) *Molybdovanadate reagent*.—Dissolve 16.5 g $NH_4$ molybdate.$4H_2O$ in 400 mL hot $H_2O$, and cool. Dissolve 0.6 g $NH_4$ metavanadate in 250 mL hot $H_2O$, cool, and add 60 mL 70% $HClO_4$. Gradually add molybdate soln to vanadate soln with stirring. Add 2 mL wetting agent, (**e**), and dil. to 2 L.

(**c**) *Perchloric acid*.—4*N*. Add 342 mL 70% $HClO_4$ to 500 mL $H_2O$ in 1 L vol. flask. Add 1 mL wetting agent, and dil. to vol.

(**d**) *Sampler wash soln*.—Add 1 mL wetting agent to 1 L $H_2O$, and mix well.

(**e**) *Wetting agent*.—Ultrawet 60 L (Technicon No. T01-0214), or equiv.

(**f**) *Phosphorus std solns*.—*(1) Stock soln*.—10 mg $P_2O_5$/mL. Dissolve 9.5880 g dried (2 hr at 105°) $KH_2PO_4$ primary std (52.15% $P_2O_5$) in $H_2O$, and dil. to 500 mL with $H_2O$. *(2) Working solns*.—0.15, 0.19, 0.23, 0.27, 0.31, and 0.35 mg $P_2O_5$/mL. Using 25 mL buret, accurately measure 7.5, 9.5, 11.5, 13.5, 15.5, and 17.5 mL stock soln into six 500 mL vol. flasks. Dil. each to vol. with $H_2O$, and mix. *(3) Working soln for samples ≤7% $P_2O_5$*.—2 mg $P_2O_5$/mL. Pipet 100 mL stock soln into 500 mL vol. flask, dil. to vol. with $H_2O$, and mix.

### *C. Preparation of Samples*

Prep. samples for direct available $P_2O_5$ detn as in **960.03B(a)**. Prep. samples for total $P_2O_5$ detn as in **957.02B(a)** or (**e**), and dil. to 250 mL.

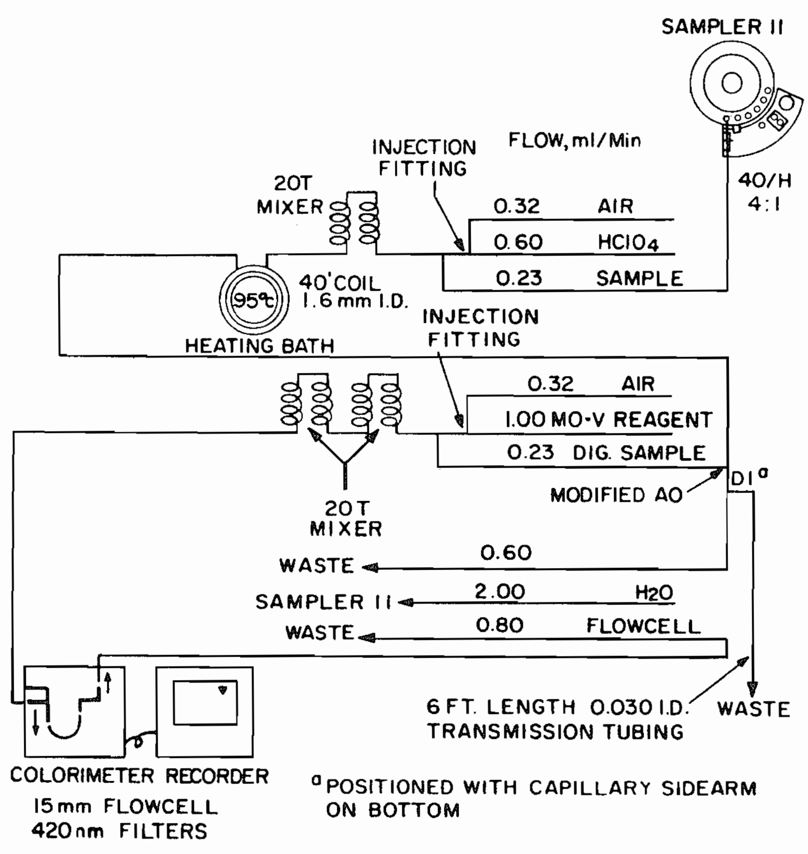


**FIG. 978.01—Flow diagram for automated analysis for phosphorus**

### D. Analytical System

(Technicon part numbers are given to aid in construction of manifold; equiv. coils, fittings, etc., are satisfactory.)

Sample, air, and 4$N$ $HClO_4$ are combined thru injection fitting (No. 116–0489) and mixed in 20T coil (No. 157–0248). Stream proceeds to heating bath(s) before resample thru modified AO fitting. Resample, air, and molybdovanadate reagent are combined thru injection fitting (No. 116–0489). Mixing and color development take place in two 20T coils (No. 157–0248) before measurement at 420 nm. If only total $P_2O_5$ samples are to be analyzed, heating bath can be removed and 4$N$ $HClO_4$ soln replaced by sampler wash soln, (**d**). Heating bath(s) and acid soln are necessary only when analyzing samples for direct available $P_2O_5$ or combination of direct available and total $P_2O_5$ detns.

If manifold is to be constructed following flow diagram, use clear std pump tubes for all air and soln flows. All fittings, coils, and glass transmission lines are AAII type and size. Use 1.6 mm glass transmission tubing for all connections after pump to colorimeter. Construct modified AO fitting, following heating bath, by using AO fitting, N13 stainless steel nipple connector, and 1.3 cm length of 0.035" id Tygon tubing. Insert N13 nipple approx. halfway into 0.035" Tygon tubing. Insert tubing into side arm of AO fitting far enough so resample line will not pump any air. Connect D1 fitting directly to waste side of AO fitting; position D1 fitting with capillary side arm on bottom. Attach 0.6 mL/min pump tube to top arm of D1 fitting, and attach 1.8 m (6') of 0.030" id transmission tubing to bottom arm. All air segments must pass thru 0.6 mL/min tube, leaving continuous column of liq. in 1.8 m length of tubing to provide for constant back pressure on heating bath coil. Length of resample pump tube should be ≤2.5 cm from shoulder at entrance end.

### E. Start-Up

Start automatic system, place all lines in resp. solns, and let equilibrate ≥30 min. Proceed as in **978.01G**.

### F. Shut-Down

Pump water thru reagent lines ≥30 min. Do not remove $HClO_4$ lines from reagent until 20 min after last sample is run.

### G. Check and Calibration

After equilibration, set colorimeter to damp 1 position and pump 0.15 mg $P_2O_5$/mL working std soln continuously thru system. Adjust colorimeter baseline to read 10% of full scale. Pump 0.35 mg $P_2O_5$/mL std and adjust std calibration to read 90% of full scale. Range of 0.15–0.35 mg $P_2O_5$/mL will expand to read 10–90% of full scale. Check of bubble flow pattern will give indication of performance of system. Perfect bubble pattern is required to obtain optimum peak shapes. Check for air bubble in flowcell if noisy conditions exist. To check system carryover, place three 0.35 mg/mL stds, followed by three 0.15 mg/mL stds, thru system. If first 0.15 mg/mL std following 0.35 mg/mL std is ≥1 chart division higher than other 2, carryover is indicated. If carryover occurs, check entire system for poor connections.

### H. Determination

Pipet aliquot of sample soln (*see* Table **978.01**) into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix by inversion 20 times. For sample contg ≤7% $P_2O_5$, pipet 10 mL working soln, (**f**)(*3*), into flask before diln. Place 0.15–0.35 mg $P_2O_5$/mL working std solns in tray in increasing order of concn, followed by group of samples. Analyze lowest concn std in duplicate, discarding first peak. Precede and follow each group of samples with std ref. curve to correct for possible drift. If drift between first and last set of stds is ≥2 chart divisions, repeat sample analysis. Prep. std curve by averaging peak hts of first and second set of stds. Plot av. peak ht of stds against mg $P_2O_5$/mL contained in each std. Read mg $P_2O_5$/mL for each sample from graph.

**Table 978.01 Standard Dilutions**

| % $P_2O_5$ Expected | Aliquot (mL) Direct Available | Total | Factor |
|---|---|---|---|
| 1–7 | 50 + "spike" | 25 + "spike" | 1 |
| 8–16 | no diln | 50 | 0.5 |
| 17–34 | 50 | 25 | 2 for direct available |
| ≥35 | 25 | 15 | 1.667 for total |

$$\% P_2O_5 = \text{mg } P_2O_5/\text{mL from graph } (-0.20, \text{ if spiked}) \times F \times 100$$

where $F$ = factor from Table **978.01**.

Refs.: JAOAC **61**, 533(1978).

CAS-1314-56-3 (phosphorus pentoxide)

## 977.01 Phosphorus (Water-Soluble) in Fertilizers

### Preparation of Solution

#### Final Action

Place 1 g sample on 9 cm filter and wash with small portions $H_2O$ until filtrate measures ca 250 mL. Add $H_2O$ in fine stream directed around entire periphery of filter paper in circular path, ensuring that $H_2O$ and solids are thoroly mixed with each addn. Let each portion pass thru filter before adding more and use suction if washing would not otherwise be complete within 1 hr. If filtrate is turbid, add 1–2 mL $HNO_3$, dil. to 250 mL, and mix.

Ref.: JAOAC **60**, 393,702(1977).

## 962.03 Phosphorus (Water-Soluble) in Fertilizers

### Gravimetric Quinolinium Molybdophosphate Method

#### Final Action

Pipet aliquot contg ≤25 mg $P_2O_5$ into 500 mL erlenmeyer. Dil., if necessary, to 50 mL, add 10 mL $HNO_3$ (1+1), and boil gently 10 min. Cool, dil. to 100 mL, and proceed as in **962.02C(b)**.

## 962.04 Phosphorus (Water-Soluble) in Fertilizers

### Alkalimetric Quinolinium Molybdophosphate Method

#### Final Action 1974

Pipet aliquot contg ≤30 mg $P_2O_5$ into 500 mL erlenmeyer. Dil., if necessary, to 50 mL, add 10 mL $HNO_3$ (1+1), boil gently 10 min, cool, and proceed as in **969.02C(a)**, beginning ". . . add 20 mL citric acid soln . . ."

## 970.01 Phosphorus (Water-Soluble) in Fertilizers

### Spectrophotometric Molybdovanadophosphate Method

**First Action 1970**
**Final Action 1974**

Adjust concn according to **958.01D(a)** or (**b**) and proceed as in **958.01E**.

## 963.03 Phosphorus (Citrate-Insoluble) in Fertilizers

**First Action 1963**
**Final Action 1964**

### A. Reagents

(**a**) *Ammonium citrate soln.*—Should have sp gr of 1.09 at 20° and pH of 7.0 as detd potentiometrically.

Dissolve 370 g cryst. citric acid in 1.5 L $H_2O$ and nearly neutze by adding 345 mL $NH_4OH$ (28–29% $NH_3$). If concn of $NH_3$ is <28%, add correspondingly larger vol. and dissolve citric acid in correspondingly smaller vol. $H_2O$. Cool, and check pH. Adjust with $NH_4OH$ (1+7) or citric acid soln to pH 7. Dil. soln, if necessary, to sp gr of 1.09 at 20°. (Vol. will be ca 2 L.) Keep in tightly stoppered bottles and check pH from time to time. If pH has changed from 7.0, readjust.

(**b**) *Other reagents and solns.—See* **957.02A**, **958.01B**, or **962.02A**.

### B. Preparation of Extract

(**a**) *Acidulated samples, mixed fertilizers, and materials containing water-soluble compounds.*—After removing $H_2O$-sol. $P_2O_5$, **977.01**, transfer filter and residue, within 1 hr, to 200 or 250 mL flask contg 100 mL $NH_4$ citrate soln previously heated to 65°. Close flask tightly with smooth rubber stopper, shake vigorously until paper is reduced to pulp, and relieve pressure by removing stopper momentarily. Continuously agitate stoppered flask in const temp. app. at exactly 65°. (Action of app. should be such that dispersion of sample in citrate soln is continually maintained and entire inner surface of flask and stopper is continually bathed with soln.)

Exactly 1 hr after adding filter and residue, remove flask from app. and immediately filter by suction as rapidly as possible thru Whatman No. 5 paper, or equiv., using buchner or ordinary funnel with Pt or other cone. Wash with $H_2O$ at 65° until vol. filtrate is ca 350 mL, allowing time for thoro draining before adding more $H_2O$. If material yields cloudy filtrate, wash with *5% $NH_4NO_3$ soln*. Prep. citrate-insol. residue for analysis by one of following methods:

(*1*) Dry paper and contents, transfer to crucible, ignite until all org. matter is destroyed, and digest with 10–15 mL HCl until all phosphate dissolves; or (2) treat wet filter and contents as in **957.02B(a)**, (**c**), (**d**), or (**e**). Dil. soln to 250 mL, or other suitable vol., mix well, and filter thru dry paper.

(**b**) *Nonacidulated samples.*—Place 1 g sample (ground to pass No. 40 sieve in case of Ca metaphosphate) on dry 9 cm paper. Without previous washing with $H_2O$, proceed as in (**a**). If sample contains much org. matter (bone, fish, etc.), dissolve residue insol. in $NH_4$ citrate as in **957.02B(c)**, (**d**), or (**e**).

### C. Determination —Final Action 1974

(**a**) *Gravimetric quinolinium molybdophosphate method.*—Treat 1 g sample as in **963.03B(a)** or (**b**). Transfer aliquot of citrate-insol. $P_2O_5$ contg ≤25 mg $P_2O_5$ and proceed as in **962.02C**.

(**b**) *Spectrophotometric molybdovanadophosphate method.*—Treat 1 g sample as in **963.03B(a)** or (**b**). Adjust concn of citrate-insol. $P_2O_5$ soln as in **958.01D(a)** or (**b**) and proceed as in **958.01E**.

(**c**) *Alkalimetric quinolinium molybdophosphate method.*—Treat 1 g sample by **963.03B(a)** or (**b**). Transfer aliquot of citrate-insol. $P_2O_5$ contg ≤5 mL concd acid to 500 mL erlenmeyer. Add 20 mL 10% citric acid soln and dil. to 100 mL with $H_2O$. Continue as in **969.02C(a)**, beginning "Add 60 mL quimociac reagent, . . ."

Refs.: JAOAC **5,** 443,460(1922); **6,** 384(1923); **14,** 182(1931); **19,** 269(1936); **22,** 254(1939); **42,** 503, 512(1959); **52,** 587(1969).
Z. Anal. Chem. **189,** 243(1962). JAOAC **45,** 40, 201, 999(1962); **46,** 579(1963); **47,** 420(1964).

CAS-1314-56-3 (phosphorus pentoxide)

## 960.01 Phosphorus (Citrate-Soluble) in Fertilizers

**Final Action 1960**

Subtract sum of $H_2O$-sol. and citrate-insol. $P_2O_5$ from total $P_2O_5$ to obtain citrate-sol. $P_2O_5$.

## 960.02 Phosphorus (Available) in Fertilizers

### Indirect Method

**Final Action 1960**

Subtract citrate-insol. $P_2O_5$ from total $P_2O_5$ to obtain available $P_2O_5$.

## 960.03 Phosphorus (Available) in Fertilizers

**Final Action**

### A. Reagents

(*Caution:* See safety notes on nitric acid, perchloric acid, and sulfuric acid.)

(**a**) *Nitric-perchloric acid mixture.*—Add 300 mL 70% $HClO_4$ to 700 mL $HNO_3$.

(**b**) *Ternary acid mixture.*—Add 20 mL $H_2SO_4$ to 100 mL $HNO_3$, mix, and add 40 mL 70% $HClO_4$.

(**c**) *Modified molybdovanadate reagent.*—Prep. as in **958.01B(a)** except use 250 mL 70% $HClO_4$ instead of 450 mL.

### B. Preparation of Solution

(**a**) *Acidulated samples, mixed fertilizers, and materials containing water-soluble compounds.—(1) Without filtration of citrate digest.*—Remove $H_2O$-sol. $P_2O_5$ as in **977.01**, collecting filtrate in 500 mL vol. flask, but do not add $HNO_3$ to filtrate. Treat $H_2O$-insol. residue with $NH_4$ citrate soln as in **963.03B(a)**. Exactly 1 hr after adding filter and residue, remove flask from app. and transfer contents to flask contg $H_2O$-sol. fraction. Cool to room temp. immediately, dil. to vol., mix thoroly, and let stand ≥2 hr before removing aliquot.

(*2*) *With filtration of citrate digest.*—If desired, wash by gravity into 500 mL Kohlrausch flask contg 5 mL $HNO_3$ (1+1), catching filtrate from insol. residue, **963.03B(a)**, in the Kohlrausch flask contg $H_2O$-sol. fraction, and wash residue until vol. soln in flask is ca 500 mL. Cool, dil. to 500 mL, and mix.

(**b**) *Nonacidulated samples*.—Place 1 g sample (ground to pass No. 40 sieve in case of Ca metaphosphate) on dry 9 cm paper. Without previous washing with $H_2O$, proceed as in (**a**)(*1*) or (*2*). If (*2*) is used, wash residue until vol. soln is ca 350 mL. Cool, dil. to 500 mL, and mix.

Refs.: JAOAC **43,** 478(1960); **44,** 133, 232(1961); **46,** 570 (1963); **60,** 702(1977).

### C. Alkalimetric Quinolinium Molybdophosphate Method —Final Action 1974

Treat 1 g sample by appropriate modification of **960.03B**. Transfer aliquot contg $\leq$30 mg $P_2O_5$ and $\leq$10 mL $NH_4$ citrate soln, **963.03A(a)**, to 500 mL erlenmeyer. Dil., if necessary, to 50 mL, add 10 mL $HNO_3$ (1+1), and boil gently 10 min. Cool, dil. to 100 mL, and continue as in **969.02C(a)**, beginning "Add 60 mL quimociac reagent, . . ."

Ref.: JAOAC **52,** 587(1969).

### D. Spectrophotometric Molybdovanadophosphate Method —Final Action 1961

(Not applicable to materials yielding colored solns or solns contg ions other than orthophosphate which form colored complexes with molybdovanadate. Not recommended for basic slag.)

Prep. std curve as in **958.01C**, using photometer, **958.01A**.
Pipet, into 100 mL vol. flasks, 5 mL aliquots std phosphate solns contg 2 and 3.5 mg $P_2O_5$/aliquot, **958.01B(b)**, resp., add 2 mL 70% $HClO_4$, and develop color as in **958.01C**. Adjust instrument to zero *A* for 2 mg std and det. *A* of 3.5 mg std. (*A* of latter must be practically identical with corresponding value on std curve.)

Prep. sample as in **960.03B**.

(**a**) *Samples containing up to 5% $P_2O_5$*.—Pipet 10 mL sample soln into 125 mL erlenmeyer, and treat by one of following methods (*Caution:* See safety notes on wet oxidation, nitric acid, and perchloric acid):

(*1*) Add 5 mL 20% $NaClO_3$ soln and 10 mL $HNO_3$-$HClO_4$ mixt., **960.03A(a)**. Boil gently until greenish-yellow color disappears (ca 20 min), cool, and add 2 mL HCl. After vigorous reaction subsides, evap. to fumes of $HClO_4$, and fume 2 min.

(*2*) Add 5 mL ternary acid mixt., **960.03A(b)**, swirl, boil gently 15 min, and digest at 150–200° until clear white salt or colorless soln remains. Evap. to white fumes and continue heating 5 min.

Cool, add 15 mL $H_2O$, and boil 5 min. Transfer to 100 mL vol. flask, dil. to 50 mL, swirl, and cool to room temp. Add 5 mL std phosphate soln contg 2 mg $P_2O_5$ and 20 mL modified molybdovanadate soln, **960.03A(c)**. Dil. to 100 mL, and continue as in **958.01E**.

(**b**) *Samples containing more than 5% $P_2O_5$*.—Dil. soln to such vol. that 5–10 mL aliquot contains 2–5 mg $P_2O_5$. Digest as in (**a**)(*1*) or (*2*). Without adding std phosphate soln, continue as in (**a**).

Ref.: JAOAC **44,** 233(1961).

### E. Gravimetric Quinolinium Molybdophosphate Method —First Action 1963 —Final Action 1964

(**a**) *Solns containing no organic phosphorus*.—Prep. sample as in **960.03B**. Pipet, into 500 mL erlenmeyer, aliquot contg $\leq$25 mg $P_2O_5$ and $\leq$10 mL original $NH_4$ citrate soln. Dil., if necessary, to ca 50 mL, add 10 mL $HNO_3$ (1+1), and boil gently 10 min. Cool, dil. to 150 mL, and proceed as in **962.02C(a)** or (**b**).

(**b**) *Solns containing organic phosphorus*.—(*Caution:* See safety notes on wet oxidation, nitric acid, and perchloric acid.) Select aliquot as in (**a**). Add 10 mL 20% $NaClO_3$ and 10 mL $HNO_3$-$HClO_4$ mixt., **960.03A(a)**. Boil vigorously until greenish-yellow color disappears (usually ca 30 min), cool, and add 2 mL HCl. After vigorous reaction subsides, evap. to white fumes, and continue heating 5 min. Cool, and proceed as in **962.02C(a)** or (**b**).

Refs.: JAOAC **46,** 570(1963); **47,** 420(1964).

# NITROGEN

## 920.01 Nitrates in Fertilizers
### Detection Method
### Final Action

Mix 5 g sample with 25 mL hot $H_2O$, and filter. To 1 vol. of this soln add 2 vols $H_2SO_4$, free from $HNO_3$ and oxides of N, and let cool. Add few drops *concd $FeSO_4$ soln* in such manner that fluids do not mix. If nitrates are present, junction at first shows purple, afterwards brown, or if only minute amt is present, reddish color. To another portion of soln add 1 mL *1% $NaNO_3$ soln* and test as before to det. whether enough $H_2SO_4$ was added in first test.

## 955.04 Nitrogen (Total) in Fertilizers
### Kjeldahl Method
### Final Action

(Provide adequate ventilation in laboratory and do not permit accumulation of exposed Hg.)

### A. Reagents

(**a**) *Sulfuric acid*.—93–98% $H_2SO_4$, N-free.

(**b**) *Mercuric oxide or metallic mercury*.—HgO or Hg, reagent grade, N-free.

(**c**) *Potassium sulfate* (*or anhydrous sodium sulfate*).—Reagent grade, N-free.

(**d**) *Salicylic acid*.—Reagent grade, N-free.

(**e**) *Sulfide or thiosulfate soln*.—Dissolve 40 g com. $K_2S$ in 1 L $H_2O$. (Soln of 40 g $Na_2S$ or 80 g $Na_2S_2O_3.5H_2O$ in 1 L may be used.)

(**f**) *Sodium hydroxide*.—(*Caution:* See safety notes on sodium and potassium hydroxide.) Pellets or soln, nitrate-free. For soln, dissolve ca 450 g solid NaOH in $H_2O$, cool, and dil. to 1 L. (Sp gr of soln should be $\geq$1.36.)

(**g**) *Zinc granules*.—Reagent grade.

(**h**) *Zinc dust*.—Impalpable powder.

(**i**) *Methyl red indicator*.—Dissolve 1 g Me red in 200 mL alcohol.

(**j**) *Hydrochloric or sulfuric acid std soln*.—0.5*N*, or 0.1*N* when amt of N is small. Prep. as in **936.15** or **890.01A**.

(**k**) *Sodium hydroxide std soln*.—0.1*N* (or other specified concn). Prep. as in **936.16**.

Stdze each std soln with primary std (*see* chapter on standard solutions) and check one against the other. Test reagents before use by blank detn with 2 g sugar, which ensures partial reduction of any nitrates present.

*Caution:* Use freshly opened $H_2SO_4$ or add dry $P_2O_5$ to avoid hydrolysis of nitriles and cyanates. Ratio of salt to acid (wt:vol.) should be ca 1:1 at end of digestion for proper temp. control. Digestion may be incomplete at lower ratio; N may be lost at higher ratio. Each g fat consumes 10 mL $H_2SO_4$, and each g carbohydrate 4 mL $H_2SO_4$ during digestion.

### B. Apparatus

(**a**) *For digestion.*—Use Kjeldahl flasks of hard, moderately thick, well-annealed glass with total capacity ca 500–800 mL. Conduct digestion over heating device adjusted to bring 250 mL $H_2O$ at 25° to rolling boil in ca 5 min or other time as specified in method. To test heaters, preheat 10 min if gas or 30 min if elec. Add 3–4 boiling chips to prevent superheating.

(**b**) *For distillation.*—Use 500–800 mL Kjeldahl or other suitable flask, fitted with rubber stopper thru which passes lower end of efficient scrubber bulb or trap to prevent mech. carryover of NaOH during distn. Connect upper end of bulb tube to condenser tube by rubber tubing. Trap outlet of condenser in such way as to ensure complete absorption of $NH_3$ distd over into acid in receiver.

### C. Improved Kjeldahl Method for Nitrate-Free Samples

(*Caution:* See safety notes on sulfuric acid, sodium hydroxides, and mercury.)

Place weighed sample (0.7–2.2 g) in digestion flask. Add 0.7 g HgO or 0.65 g metallic Hg, 15 g powd $K_2SO_4$ or anhyd. $Na_2SO_4$, and 25 mL $H_2SO_4$. If sample >2.2 g is used, increase $H_2SO_4$ by 10 mL for each g sample. Place flask in inclined position and heat gently until frothing ceases (if necessary, add small amt of paraffin to reduce frothing); boil briskly until soln clears and then ≥30 min longer (2 hr for samples contg org. material).

Cool, add ca 200 mL $H_2O$, cool <25°, add 25 mL of the sulfide or thiosulfate soln, and mix to ppt Hg. Add few Zn granules to prevent bumping, tilt flask, and add layer of NaOH without agitation. (For each 10 mL $H_2SO_4$ used, or its equiv. in dild $H_2SO_4$, add 15 g solid NaOH or enough soln to make contents strongly alk.) (Thiosulfate or sulfide soln may be mixed with the NaOH soln before addn to flask.) Immediately connect flask to distg bulb on condenser, and, with tip of condenser immersed in std acid and 5–7 drops indicator in receiver, rotate flask to mix contents thoroly; then heat until all $NH_3$ has distd (≥150 mL distillate). Remove receiver, wash tip of condenser, and titr. excess std acid in distillate with std NaOH soln. Correct for blank detn on reagents.

$$\% \text{ N} = [(\text{mL std acid} \times \text{normality acid}) - (\text{mL std NaOH} \times \text{normality NaOH})] \times 1.4007/\text{g sample}$$

Ref.: JAOAC **38,** 56(1955).

### D. Improved Kjeldahl Method for Nitrate-Containing Samples

(Not applicable to liqs or to materials with high $Cl:NO_3$ ratio. *Caution:* See safety notes on sulfuric acid and mercury.)

Place weighed sample (0.7–2.2 g) in digestion flask. Add 40 mL $H_2SO_4$ contg 2 g salicylic acid. Shake until thoroly mixed and let stand, with occasional shaking, ≥30 min; then add (*1*) 5 g $Na_2S_2O_3.5H_2O$ or (*2*) 2 g Zn dust (as impalpable powder, not granulated Zn or filings). Shake and let stand 5 min; then heat over low flame until frothing ceases. Turn off heat, add 0.7 g HgO (or 0.65 g metallic Hg) and 15 g powd $K_2SO_4$ (or anhyd. $Na_2SO_4$), and boil briskly until soln clears, then ≥30 min longer (2 hr for samples contg org. material).

Proceed as in second par. of **955.04C**.

Ref.: JAOAC **51,** 446(1968).

CAS-7727-37-9 (nitrogen)

## 970.02 Nitrogen (Total) in Fertilizers

**Comprehensive Nitrogen Method**
**First Action 1970**
**Final Action 1975**

(Applicable to all fertilizer samples.)

(*Caution:* See safety notes on sulfuric acid and mercury salts.)

### A. Reagents

(**a**) *Chromium metal.*—100 mesh, low N (Fisher Scientific Co. No. C-318 or Sargent-Welch Scientific Co. No. C11432 is satisfactory).

(**b**) *Alundum.*—Boiling stones. 8–14 mesh (Thomas Scientific No. 1590–D18, or equiv.).

(**c**) *Dilute sulfuric acid.*—Slowly add 625 mL $H_2SO_4$ to 300 mL $H_2O$. Dil. to ca 1 L and mix. After cooling, dil. to 1 L with $H_2O$ and mix. Avoid absorption of $NH_3$ from air during prepn, particularly if stream of air is used for mixing.

(**d**) *Sodium thiosulfate or potassium sulfide soln.*—160 g $Na_2S_2O_3.5H_2O$/L or 80 g $K_2S$/L.

For other reagents, *see* **920.02A**.

### B. Determination

Place 0.2–2.0 g sample contg ≤60 mg nitrate N in 500–800 mL Kjeldahl flask and add 1.2 g Cr powder. Add 35 mL $H_2O$ or, with liqs, amt to make total vol. 35 mL. Let stand 10 min with occasional gentle swirling to dissolve all nitrate salts. Add 7 mL HCl and let stand ≥30 sec but ≤10 min.

Place flask on preheated burner with heat input set at 7.0–7.5 min boil test, **920.02B(a)**. After heating 3.5 min, remove from heat and let cool.

Add 22 g $K_2SO_4$, 1.0 g HgO, and few granules Alundum. Add 40 mL dil. $H_2SO_4$, (**c**). (If adequate ventilation is available, 25 mL $H_2SO_4$ may be added instead of dil. $H_2SO_4$. If org. matter which consumes large amt of acid exceeds 1.0 g, add addnl 1.0 mL $H_2SO_4$ for each 0.1 g org. matter in excess of 1.0 g.)

Place flask on burners set at 5 min boil test. (Pre-heated burners reduce foaming with most samples. Reduce heat input if foam fills ≥$^2/_3$ of bulb of flask. Use variable heat input until this phase is past.) Heat at 5 min boil test until dense white fumes of $H_2SO_4$ clear bulb of flask. Digestion is now complete for samples contg ammoniacal, nitrate, and urea N. For other samples, swirl flask gently and continue digestion 60 min more.

Proceed as in **955.04C**, second par., substituting **970.02A(d)** for **920.02A(e)**.

Refs.: JAOAC **53,** 450(1970); **57,** 10(1974); **68,** 441(1978).

CAS-7727-37-9 (nitrogen)

## 978.02 Nitrogen (Total) in Fertilizers

**Modified Comprehensive Nitrogen Method**
**First Action 1978**
**Final Action 1984**

(Applicable to all fertilizer samples)

### A. Reagents

*See* **920.02A(a)**, (**c**), (**f**), (**i**), (**j**), (**k**), **970.02A(a)**, (**b**), and in addn:

*Copper sulfate pentahydrate (or anhydrous copper sulfate).*—Reagent grade, N-free.

### B. Determination

(*Caution:* See safety notes on wet oxidation, sulfuric acid, and sodium hydroxide.)

Proceed as in **970.02B**, par. 1 and 2, using 0.2–1.6 g sample. For samples contg orgs other than urea or urea-form, use ≥0.5 g sample.

Add 15 g $K_2SO_4$ or 12 g anhyd. $Na_2SO_4$, 0.4 g anhyd. $CuSO_4$ or 0.6 g $CuSO_4.5H_2O$, and ca 0.8 g Alundum granules. Add 37 mL $H_2SO_4$ (1+1). (If adequate ventilation is available, 20 mL $H_2SO_4$ may be added instead of $H_2SO_4$ (1+1). If org. matter other than urea exceeds 1.0 g, add addnl 1.0 mL $H_2SO_4$ for each 0.1 g fat or 0.2 g other org. matter in excess of 1.0 g.)

Proceed as in **970.02B**, par. 4, substituting 75 min for 60 min in last sentence.

Cool flask until it can be handled without gloves, and add ca 250 mL $H_2O$. Swirl to dissolve contents, and cool <25°. Add ca 0.8 g Alundum granules to minimize bumping, tilt flask, and add layer of NaOH without agitation. (For each 10 ml $H_2SO_4$ used, or its equiv. in $H_2SO_4$ (1+1), add 15 g solid NaOH or enough soln to make contents strongly alk.) Proceed as in **955.04C**, par. 2, beginning "Immediately connect flask to distg bulb . . ."

Ref.: JAOAC **61**, 299(1978).

CAS-7727-37-9 (nitrogen)

## 970.03 Nitrogen (Total) in Fertilizers

**Raney Powder Method**

**First Action 1970**
**Final Action 1975**

(Applicable to all fertilizer samples except "nitric phosphates" contg nonsulfate S. *Caution:* See safety notes on sulfuric acid and mercury salts.)

### A. Reagents

(**a**) *Raney catalyst powder No. 2813.*—50% Ni, 50% Al (W. R. Grace & Co., Davison Chemical Division, 10 E Baltimore St, PO Box 2117, Baltimore, MD 21203-2117). *Caution:* Raney catalyst powders react slowly in $H_2O$ or moist air to form alumina; avoid prolonged contact with air or moisture during storage or use.

(**b**) *Sulfuric acid-potassium sulfate soln.*—Slowly add 200 mL $H_2SO_4$ to 625 mL $H_2O$ and mix. Without cooling, add 106.7 g $K_2SO_4$ and continue stirring until all salt dissolves. Dil. to ca 1 L and mix. Cool, dil. to 1 L with $H_2O$, and mix. Avoid absorption of $NH_3$ from air during prepn particularly if stream of air is used for mixing.

For other reagents, see **920.02A**.

### B. Determination

Place 0.2–2.0 g sample contg ≤42 mg nitrate N in 500–800 mL Kjeldahl flask (800 mL flask is preferred with samples which foam considerably, especially orgs). Add 1.7 g Raney catalyst powder, 3 drops *tributyl citrate*, and 150 mL $H_2SO_4$-$K_2SO_4$ soln. If org. matter exceeds 0.6 g, add addnl 2.5 mL of this soln for each 0.1 g org. matter in excess of 0.6 g.

Swirl to mix sample with acid and place flask on cold burner. If burner has been in use, turn off completely ≥10 min before placing flask on burner. After flask is on burner, set heat input to 5 min boil test. When sample starts boiling, reduce heat to pass 10 min boil test. After 10 min, raise flask to vertical position and add 0.7 g HgO and 15 g $K_2SO_4$. (Contents of Kel-Pak No. 5 (Curtin Matheson Scientific, Inc.) without plastic container may be used.) Replace flask in inclined position and increase heat to 4–5 min boil test. (Reduce heat input if foam fills ≥$^2/_3$ of bulb of flask. Use variable heat input until this phase is past.) Heat at 4–5 min boil test until dense white fumes of $H_2SO_4$ clear bulb of flask. Digestion is now complete for samples contg only ammoniacal, nitrate, and urea N. For other samples, swirl flask gently and continue digestion addnl 30 min.

Proceed as in **955.04C**, second par. If 800 mL Kjeldahl flasks have been used, add 300 instead of 200 mL $H_2O$.

Refs.: JAOAC **53**, 450(1970); **57**, 10(1974).

CAS-7727-37-9 (nitrogen)

## 920.03 Nitrogen (Ammoniacal) in Fertilizers

**Magnesium Oxide Method**

**Final Action**

(Not applicable in presence of urea)

Place 0.7–3.5 g, according to $NH_3$ content of sample, in distn flask with ca 200 mL $H_2O$ and ≥2 g *carbonate-free MgO*. Connect flask to condenser by Kjeldahl connecting bulb, distil 100 mL liq. into measured amt std acid, **920.02A(j)**, and titr. with std NaOH soln, **920.02A(k)**, using Me red, **920.02A(i)**.

## 920.04★ Nitrogen (Ammoniacal) in Fertilizers

**Formaldehyde Titration Method**

**Surplus 1970**

(Applicable to $NH_4NO_3$ and $(NH_4)_2SO_4$)

*See* **2.058**, 11th ed.

## 920.05★ Nitrogen (Ammoniacal and Nitrate) in Fertilizers

**Ferrous Sulfate-Zinc-Soda Method**

**Final Action 1965**
**Surplus 1970**

(Not applicable in presence of org. matter, Ca cyanamide, and urea)

*See* **2.059**, 11th ed.

## 892.01 Nitrogen (Ammoniacal and Nitrate) in Fertilizers

**Devarda Method**

**Final Action**

(Not applicable in presence of org. matter, Ca cyanamide, and urea)

Place 0.35 or 0.5 g sample in 600–700 mL flask and add 300 mL $H_2O$, 3 g *Devarda alloy* (Cu 50, Al 45, Zn 5), and

5 mL NaOH soln (42% by wt), pouring latter down side of flask so that it does not mix at once with contents. By means of Davisson (J. Ind. Eng. Chem. **11**, 465(1919)) or other suitable scrubbing bulb that will prevent passing over of any spray, connect with condenser, tip of which always extends beneath surface of std acid in receiving flask. Mix contents of distg flask by rotating. Heat slowly at first and then at rate to yield 250 mL distillate in 1 hr. Collect distillate in measured amt std acid, **920.02A(j)**, and titr. with std NaOH soln, **920.02A(k)**, using Me red, **920.02A(i)**.

Refs.: Chem. Ztg. **16,** 1952(1892). JAOAC **6,** 391(1923); **15,** 267(1932).

## 930.01 Nitrogen (Nitrate) in Fertilizers

### Robertson Method

### Final Action

(Applicable in presence of Ca cyanamide and urea. *Caution:* See safety notes on sulfuric acid and mercury.)

(**a**) Det. total N as in **955.04D**, **970.02B**, or **970.03B**.

(**b**) Det. $H_2O$-insol. N as in **945.01**, but use 2.5 g sample. Dil. filtrate to 250 mL.

(**c**) Place 50 mL portion filtrate in 500 mL Kjeldahl flask and add 2 g $FeSO_4.H_2O$ and 20 mL $H_2SO_4$. (If total N is >5%, use 5 g $FeSO_4.7H_2O$.) Digest over hot flame until all $H_2O$ is evapd and white fumes appear, and continue digestion at least 10 min to drive off nitrate N. If severe bumping occurs, add 10–15 glass beads. Add 0.65 g Hg, or 0.7 g HgO, and digest until all org. matter is oxidized. Cool, dil., add the $K_2S$ soln, and complete detn as in **955.04C**. Before distn, add pinch of mixt. of Zn dust and granular "20-mesh" Zn to each flask to prevent bumping.

Total N (**a**) − $H_2O$-insol. N (**b**) = $H_2O$-sol. N.
$H_2O$-sol. N − N obtained in (**c**) = nitrate N.

Refs.: JAOAC **13,** 208(1930); **15,** 267(1932); **56,** 392(1973).

## 930.02 Nitrogen (Nitrate) in Fertilizers

### Jones Modification of Robertson Method

### Final Action

(Applicable when $H_2O$-sol. N need not be detd.)

(*Caution:* See safety notes on sulfuric acid and mercury.)

Weigh 0.5 g sample into Kjeldahl flask, add 50 mL $H_2O$, and rotate gently. Add 2 g $FeSO_4.7H_2O$ and rotate. Add 20 mL $H_2SO_4$. Digest over hot flame. When $H_2O$ evaps and white fumes appear, add 0.65 g Hg and proceed as in **955.04C**.

Total N − N thus found = nitrate N

Refs.: JAOAC **13,** 208(1930); **15,** 267(1932).

## 935.01★ Nitrogen (Water-Insoluble) in Cyanamide

### Final Action
### Surplus 1970

See **2.063**, 11th ed.

## 945.01 Nitrogen (Water-Insoluble) in Fertilizers

### Method I

### Final Action

(*See* **955.05B(a)** and (**b**) for urea-formaldehyde or mixts contg such compds.)

Place 1 or 1.4 g sample in 50 mL beaker, wet with alcohol, add 20 mL $H_2O$, and let stand 15 min, stirring occasionally. Transfer supernate to 11 cm Whatman No. 2 paper in 60° long-stem funnel 60 mm diam., and wash residue 4 or 5 times by decanting with $H_2O$ at room temp. (20–25°). Finally transfer all residue to filter and complete washing until filtrate measures 250 mL. Det. N in residue as in **955.04C**.

## 970.04 Nitrogen (Water-Insoluble) in Fertilizers

### Method II

### First Action 1974

#### A. Apparatus

*Extraction tube.*—Glass, 250 × 10 mm id, 12 mm od, constricted to 3–4 mm at one end.

#### B. Determination

Weigh 3.0 g unground mixed sample and place in extn tube contg small glass wool plug. Place addnl glass wool pad on top of sample. Connect 250 or 500 mL separator to column with 75 mm piece of rubber tubing. Close stopcock of separator and add 250 mL deionized $H_2O$. Open stopcock and let quick rush of $H_2O$ pass thru column. After initial rush of $H_2O$, close stopcock. Adjust flow thru stopcock to ca 2 mL/min. Squeeze rubber connection to bring level of $H_2O$ ca 25 mm above column bed. System then operates as constant-head feeder.

After $H_2O$ wash is complete, disconnect column from rubber tubing. Invert column over Kjeldahl flask and force contents into flask with aid of pressure bulb. Wash traces of sample from tube into Kjeldahl flask and wash sample from walls of digestion flask with min. $H_2O$. Det. N in residue as in **970.02** or **970.03**.

Refs.: JAOAC **53,** 808(1970); **56,** 853(1973).

## 930.03★ Nitrogen Activity in Fertilizers

### Removal of Water-Soluble Nitrogen

### Final Action
### Surplus 1967

(**a**) *Mixed fertilizers.—See* **2.058**, 10th ed.
(**b**) *Raw materials.—See* **2.058**, 10th ed.

## 920.06★ Nitrogen Activity in Fertilizers

### Water-Insoluble Organic Nitrogen Soluble in Neutral Permanganate

### Final Action
### Surplus 1987

*See* **2.059**, 10th ed.

## 920.07★ Nitrogen Activity in Fertilizers

### Water-Insoluble Organic Nitrogen Distilled from Alkaline Permanganate

**Final Action**
**Surplus 1987**

*See* **2.060–2.061**, 10th ed.

## 955.05 Nitrogen Activity Index (AI) of Urea-Formaldehyde Fertilizers

**Final Action 1965**

(Applicable to urea-formaldehyde compds and mixts contg such compds)

### A. Reagent

*Phosphate buffer soln.*—pH 7.5. Dissolve 14.3 g $KH_2PO_4$ and 91.0 g $K_2HPO_4$ in $H_2O$ and dil. to 1 L. Dil. 100 mL of this soln to 1 L.

### B. Determination

(**a**) Crush sample (do not grind) to pass No. 20 sieve.

(**b**) Det. cold $H_2O$-insol. N (*WIN*) as in **945.01**, keeping temp. at 25±2°. Stir at 5 min intervals during 15 min standing.

(**c**) Det. hot $H_2O$-insol. N (*HWIN*) in phosphate buffer soln as follows: Place accurately weighed sample contg 0.1200 g *WIN* in 200 mL tall-form beaker. Add ca 0.5 g $CaCO_3$ to mixed fertilizers contg urea-HCHO compds. From supply of boiling buffer soln, add 100 mL from graduate to sample, stir, cover, and immerse *promptly* in boiling $H_2O$ bath so that liq. in beaker is below $H_2O$ level in bath. Maintain bath at 98–100°, checked with thermometer, and stir at 10 min intervals. After exactly 30 min, remove beaker from bath and filter promptly thru 15 cm Whatman No. 12 fluted paper. If filtration takes >4 min, discard detn. Repeat detn, stirring in 1 g Celite filter-aid just before removing beaker from bath, and filter.

Wash insol. residue completely onto paper with boiling $H_2O$ and continue washing until total vol. used is 100 mL. Complete washing before filtrate becomes cloudy or its temp. drops to <60°. Det. total N (*HWIN*) in wet paper and residue as in **955.04C**, using 35 mL $H_2SO_4$ when $CaCO_3$ has been added.

$$\text{Activity index (AI)} = (\%\text{WIN} - \%\text{HWIN}) \times 100/\%\text{WIN}$$

Refs: JAOAC **38,** 436(1955); **44,** 245(1961).

## 959.03 Urea in Fertilizers

### Urease Method

**First Action 1959**
**Final Action 1960**

### A. Reagent

*Neutral urease soln.*—Use fresh com. 1% urease soln, or dissolve 1 g urease powder in 100 mL $H_2O$, or shake 1 g jack bean meal with 100 mL $H_2O$ 5 min. Transfer 10 mL soln to 250 mL erlenmeyer, dil. with 50 mL $H_2O$, and add 4 drops Me purple (available from Fisher Scientific Co.; No. So-I-9). Titr. with 0.1*N* HCl to reddish purple; then back-titr. to green with 0.1*N* NaOH. From difference in mL, calc. vol. 0.1*N* HCl required to neutze remainder of soln (usually ca 2.5 mL/100 mL), add this amount of acid, and shake well.

Verify enzyme activity of urease source periodically. Discard any source which does not produce soln capable of hydrolyzing 0.1 g urea/20 mL soln.

### B. Determination

Weigh 1–10±0.01 g sample (≤1.0 g urea) and transfer to 15 cm Whatman No. 12 fluted filter paper. Leach with ca 300 mL $H_2O$ into 500 mL vol. flask. Add 75–100 mL satd $Ba(OH)_2$ soln to ppt phosphates. Let settle and test for complete pptn with few drops satd $Ba(OH)_2$ soln. Add 20 mL 10% $Na_2CO_3$ soln to ppt excess Ba and any sol. Ca salts. Let settle and test for complete pptn. Dil. to vol., mix, and filter thru 15 cm Whatman No. 12 fluted paper. Transfer 50 mL aliquot to 200 or 250 mL erlenmeyer and add 1–2 drops of Me purple. Acidify with 2*N* HCl and add 2–3 drops excess. Neutze soln with 0.1*N* NaOH to first change in color of indicator. Add 20 mL neutral urease soln, close flask with rubber stopper, and let stand 1 hr at 20–25°. Cool flask in ice-$H_2O$ slurry and titr. at once with 0.1*N* HCl to full purple; then add ca 5 mL excess. Record total vol. added. Back-titr. excess HCl with 0.1*N* NaOH to neut. end point.

$$\%\ \text{Urea} = [(\text{mL } 0.1N\ \text{HCl} - \text{mL } 0.1N\ \text{NaOH}) \times 0.3003]/\text{g sample}$$

Refs.: Ind. Eng. Chem. Anal. Ed. **7,** 259(1935). JAOAC **41,** 637(1958); **42,** 494(1959); **43,** 123(1960).

CAS-57-13-6 (urea)

## 983.01 Urea and Methyleneureas (Water-Soluble) in Fertilizers

### Liquid Chromatographic Method

**First Action 1983**
**Final Action 1984**

### A. Principle

Sample is ground to pass 40 mesh sieve, extd with $H_2O$, and filtered. Urea, methylenediurea (MDU), and dimethylenetriurea (DMTU) are detd by liq. chromatgy using external stds and refractive index detection.

### B. Apparatus

(**a**) *Liquid chromatograph.*—With refractive index detector and pump capable of delivering mobile phase at 2 mL/min at pressures up to 2000 psig. Operating conditions: flow rate 1.0 mL/min (1500 psi); attenuator 8×; ambient temp.; injection vol. 10 μL. Sample injector with fixed sample loop preferred.

(**b**) *Chromatographic column.*—Partisil 5 ODS-3, 4.6 mm id × 25 cm (Whatman, Inc.; other manufacturers' small particle reverse phase columns may be substituted with adjustments in operating conditions).

(**c**) *Strip chart recorder.*—Range to match output of detector.

### C. Reagents

(**a**) *Mobile phase.*—LC grade $H_2O$.

(**b**) *Purified methylenediurea (MDU) and dimethylenetriurea (DMTU).*—Ext 50 g N-only ureaformaldehyde (UF) fertilizer with acetone 8 h on soxhlet extractor. Select UF fertilizer with high MDU/DMTU-to-urea ratio. Remove thimble from extractor, let air-dry, and collect residue. Mix 30 g acetone-washed residue in 300 mL $H_2O$ and filter or centrf. Inject 100 mL supernate onto Waters Associates PrepPak 500 C-18 cartridge (5.7×30 cm) in preparative liq. chromatograph (Waters Associates Inc. Prep-500, or equiv.) at ambient temp. and with $H_2O$ mobile phase at 150 mL/min. Collect top third of MDU and DMTU peaks. Evap. collected fractions to dryness in hood, using heat lamps. Dry using vac. over $P_2O_5$. Confirm identity using anal. liq. chromatogy and elemental analysis: mp of purified material, detd in Pyrex, should be 205–207°d for MDU and 231–232°d for DMTU.

**(c)** *External std solns.*—(*A*) Accurately weigh ca 1.0 g each of urea (Baker Analyzed Reagent) and purified MDU, transfer both weighed compds to same 100 mL vol. flask, and dil. to vol. with $H_2O$. (*B*) Accurately weigh 0.0125, 0.025, 0.050, and 0.10 g purified DMTU into sep. 50 mL vol. flasks. (*C*) Pipet 2, 5, 10, and 15 mL of mixed urea/MDU stds (*A*) into the vol. flasks from (*B*), resp. Dil. to ca 40 mL with $H_2O$ and warm as necessary to dissolve DMTU. Cool to room temp. and dil. to vol. Approx. std contents = (*1*) 0.25 mg DMTU + 0.4 mg urea/MDU per mL; (*2*) 0.50 mg DMTU + 1.0 mg urea/MDU per mL; (*3*) 1.00 mg DMTU + 2.0 mg urea/MDU per mL; (*4*) 2.00 mg DMTU + 3.0 mg urea/MDU per mL.

### D. Preparation of Sample

Grind sample to pass 40 mesh sieve. Accurately weigh 2.000 g well mixed ground sample into 200 mL vol. flask. Add 150 mL distd or deionized $H_2O$, place on wrist-action shaker 20 min, and dil. to vol. with $H_2O$. Using glass fiber paper, filter portion into 4 mL vial. Filter again thru 0.45 μm filter before injection.

### E. Determination and Calculations

Inject 10 μL of each mixed std until peak hts agree ±2%. Inject 10 μL sample. Repeat stds after all samples have been injected. Std peak hts should agree within 3% of initial std peak hts. Average peak hts for each component and plot mg/mL vs peak hts.

$$\% \text{ Urea N} = \text{mg/mL (from graph)} \times 9.33/\text{g sample}$$
$$\% \text{ MDU N} = \text{mg/mL (from graph)} \times 8.484/\text{g sample}$$
$$\% \text{ DMTU N} = \text{mg/mL (from graph)} \times 8.236/\text{g sample}$$

Ref.: JAOAC **66,** 769(1983).

CAS-57-13-6 (urea)
CAS-13547-17-6 (methylenediurea)

## 988.01 Triamino-*s*-Triazine in Fertilizer Mixes

**Liquid Chromatographic Method**
**First Action 1988**

### A. Principle

Ground sample is extd with $H_2O$ and filtered. Triamino-*s*-triazine is detd by liq. chromatgy using external std and UV detection at 254 nm.

### B. Apparatus

**(a)** *Liquid chromatograph.*—With UV detection at 254 nm. Operating conditions: flow rate 1.0 mL/min (1200 psi); column temp. ambient; chart speed 0.5 cm/min; injection vol. 20 μL; sample injector with fixed sample loop preferred. Pump LC mobile phase thru column until system is equilibrated. Allow 10 min run time for each injection. Retention time for triamino-*s*-triazine is 4–5 min. Re-equilibrate baseline before each injection.

**(b)** *LC column.*—LiChrosorb RP-18, 25 cm × 4.5 mm. (Use this type column; chemistry of triamino-*s*-triazine requires use of polar solv. system.)

**(c)** *Strip chart recorder.*—To match output of detector.

**(d)** *pH meter.*—Sensitivity 0.01. Stdze with pH 4 buffer soln.

**(e)** *Filters.*—2.4 cm glass fiber (Whatman 934-H or equiv.).

### C. Reagents

**(a)** *Sodium phosphate.*—Anhyd., dibasic. $Na_2HPO_4$, reagent grade or equiv.

**(b)** *Diethylamine.*—Reagent grade or equiv.

**(c)** *Phosphoric acid.*—Reagent grade or equiv.

**(d)** *Water.*—Deionized or distd.

**(e)** *Buffer soln.*—pH 4.0.

**(f)** *Mobile phase.*—Deionized $H_2O$ contg 1% (w/v) anhyd. $Na_2HPO_4$ and 1 mL diethylamine/L. Adjust to pH 4 with $H_3PO_4$.

**(g)** *Triamino-s-triazine std solns.*—(*1*) *Stock std soln.*—500 mg/L (ppm). Accurately weigh 50.0 mg triamino-*s*-triazine ref. std (Melamine Chemicals, Inc., PO Box 748, Donaldsonville, LA 70346) into 100 mL vol. flask. Dissolve in and dil. to vol. with deionized $H_2O$. (*2*) *Working std solns.*—50, 125, and 250 mg/L. Pipet 10, 25, and 50 mL stock std soln into sep. 100 mL vol. flasks and dil. to vol. with deionized $H_2O$. Use as calibration stds.

### D. Preparation of Sample

Grind ≥225 g sample (triamino-*s*-triazine granules or dry-mix blends with other fertilizers) to pass No. 40 sieve, mix thoroly, and store in tightly stoppered bottle.

Accurately weigh 5–8 g well mixed, ground sample and transfer to 2 L vol. flask. Dil. to vol. with deionized $H_2O$ and stir 2 h using stir bar and mag. stirrer. Filter portion for analysis thru 1 μm glass fiber filter. Pipet 1 mL filtrate into 100 mL vol. flask and dil. to vol. with deionized $H_2O$.

### E. Determination

Equilibrate column with mobile phase for 30–60 min. Inject 20 μL std soln until peak hts agree ±2%. Inject 20 μL sample with attenuation set to give largest possible on-scale peaks. Reinject std after every 10th sample to verify calibration and ensure accurate quantitation.

### F. Calculations

Calc. amt triamino-*s*-triazine as follows:

$$\text{Triamino-}s\text{-triazine, } \% = (PH/PH') \times [C/(5 \times W)] \times 100$$

where $PH$ and $PH'$ = peak hts for sample and std, resp.; $C$ = concn of std, ppm; and $W$ = sample wt, g.

Ref.: JAOAC **71,** 611(1988).

CAS-108-78-1 (1,3,5-triazine-2,4,6-triamine; melamine)

## 960.04 Biuret in Fertilizers

**Spectrophotometric Method**
**First Action 1960**
**Final Action 1980**

(Applicable to urea only. Do not use for mixed fertilizers.)

### A. Reagents

**(a)** *Alkaline tartrate soln.*—Dissolve 40 g NaOH in 500 mL $H_2O$, cool, add 50 g $NaKC_4H_4O_6.4H_2O$, and dil. to 1 L. Let stand 1 day before use.

**(b)** *Copper sulfate soln.*—Dissolve 15 g $CuSO_4.5H_2O$ in $CO_2$-free $H_2O$ and dil. to 1 L.

**(c)** *Biuret.*—To recrystallize, weigh ca 10 g reagent grade biuret, transfer to 2 L beaker, add 1 L absolute alcohol, and dissolve. Conc. by gentle heating to ca 250 mL. Cool at 5° and filter thru fritted glass funnel. Repeat crystn and dry final product 1 hr at 105–110° in oven. Remove from oven, place in desiccator, and cool to room temp.

**(d)** *Biuret std soln.*—1 mg/mL. Dissolve 1.0000 g recrystd biuret in $CO_2$-free $H_2O$ and dil. to 1 L.

### B. Preparation of Standard Curve

Transfer series of aliquots, 2–50 mL, of std biuret soln to 100 mL vol. flasks. Adjust vol. to ca 50 mL with $CO_2$-free $H_2O$, add 1 drop Me red, and neutze with 0.1*N* $H_2SO_4$ to pink color. Add, with swirling, 20 mL alk. tartrate soln and then 20 mL $CuSO_4$ soln. Dil. to vol., shake 10 sec, and place in $H_2O$ bath 15 min at 30±5°. Also prep. reagent blank. Det. *A* of each soln against blank at 555 nm (instrument with 500–570 nm filter is also satisfactory) with 2–4 cm cell. Plot std curve.

### C. Determination

Continuously stir ≤10 g sample contg 30–125 mg biuret in 150 mL ca 50° $H_2O$ 30 min. Filter and wash into 250 mL vol. flask, and dil. to vol. Transfer 50 mL aliquot to 100 mL vol. flask and proceed as in **960.04B**.

Refs.: JAOAC **43,** 499(1960); **57,** 1360(1974); **59,** 22(1976); **60,** 323(1977); **62,** 153, 330(1979); **63,** 222(1980).

CAS-108-19-0 (biuret)

## 976.01 Biuret in Fertilizers

**Atomic Absorption Spectrophotometric Method**

**First Action 1976**
**Final Action 1980**

### A. Apparatus and Reagents

(**a**) *Atomic absorption spectrophotometer.*—IL Model 353 (Instrumentation Laboratory, Inc., 113 Hartwell Ave, Lexington, MA 02173), or equiv., with Cu hollow cathode lamp.

(**b**) *Copper sulfate soln.*—Dissolve 15 g $CuSO_4.5H_2O$ in $H_2O$ and dil. to 1 L.

(**c**) *Buffer soln.*—pH 13.4. Dissolve 24.6 g KOH and 30 g KCl in $H_2O$ and dil. to 1 L.

(**d**) *Starch soln.*—Treat 1 g sol. starch with 10 mL cold $H_2O$, triturate to thin paste, and pour gradually into 150 mL boiling $H_2O$ contg 1 g oxalic acid. Boil until soln clears, cool, and dil. to 200 mL. Prep. fresh weekly.

(**e**) *Bromocresol purple indicator.*—Dissolve 0.1 g bromocresol purple in 19 mL 0.1*N* NaOH and dil. to 250 mL with $H_2O$.

(**f**) *Biuret.*—*See* **960.04A(c)**.

(**g**) *Biuret std soln.*—0.4 mg/mL. Dissolve 0.4000 g recrystd biuret in warm $H_2O$, cool, transfer to 1 L flask, and dil. to vol.

(**h**) *Copper std solns.*—Dil. aliquots of Cu stock soln, **965.09B(b)**, with $H_2O$ to obtain ≥4 std solns within range of detn, 1–4 μg Cu/mL final soln.

### B. Preparation of Standard Curve

Transfer aliquots of biuret std soln contg 0, 2, 4, 6, 8, 10, and 12 mg biuret to sep. 100 mL vol. flasks, dil. to ca 30 mL with $H_2O$, and add 25 mL alcohol to each. While stirring with mag. stirrer, add 2 mL starch soln, 10 mL $CuSO_4$ soln, and 20 mL buffer soln. Remove stirring bar, rinse, dil. to vol., mix thoroly, and let stand 10 min. With vac., filter ca 50 mL thru dry 150 mL medium porosity fritted glass funnel into dry flask. Transfer 25 mL aliquots of each filtrate to 250 mL vol. flasks, acidify with 5 mL 1*N* HCl, and dil. to vol. with $H_2O$. Proceed as in **965.09**, using std solns, **976.01A(h)**, to det. complexed Cu in soln by AA spectrophotometry after adding equiv. amts of alcohol, KOH soln, buffer soln, and 1*N* HCl. Take ≥3 readings of each soln. From mean value of Cu concn, prepare std curve relating mg Cu found to mg biuret added. Redet. daily.

### C. Determination

(**a**) *In urea.*—Accurately weigh sample contg <10 mg biuret, dissolve in $H_2O$, transfer to 100 mL vol. flask, add 25 mL alcohol, and proceed as in **976.01B**, beginning "While stirring with mag. stirrer, . . ." From Cu found, calc. biuret concn, using std curve.

(**b**) *In mixed fertilizers.*—Transfer accurately weighed sample contg <40 mg biuret to 250 mL beaker and add 1 mL $H_2O$ for each g of sample (5 g max.). Warm, add 65 mL alcohol and 7 drops bromocresol purple, and adjust pH to first blue color (pH 6–7) with 20% KOH. Place on hot plate, heat to bp, cool, and, if pH has changed, make final adjustment to first blue. Vac.-filter thru alcohol-washed paper pulp pad into 100 mL vol. flask. (If filtrate is not clear, improper pH adjustment has been made. Add HCl and readjust to pH 6–7.) Wash pad and ppt with alcohol and dil. to vol. with alcohol. Transfer 25 mL aliquot to 100 mL vol. flask, and proceed as in **976.01B**, beginning "While stirring with mag stirrer, . . ." From Cu found, calc. biuret concn, using std curve and appropriate diln factors. (Final aliquot can be varied to give Cu concn between 1 and 4 μg/mL.)

Refs.: JAOAC **59,** 22(1976); **62,** 153(1979); **63,** 222(1980).

CAS-108-19-0 (biuret)

# POTASSIUM

## 935.02★ Potassium in Fertilizers

**Lindo-Gladding Method**

**Final Action**
**Surplus 1970**

*See* **2.076–2.078**, 11th ed.

## 949.01★ Potassium in Fertilizers

**Wet-Digestion Method**

**Final Action**
**Surplus 1970**

*See* **2.079–2.080**, 11th ed.

## 945.02★ Recovery of Platinum

**Procedure**

**Final Action**
**Surplus 1970**

*See* **2.081–2.083**, 11th ed.

## 983.02 Potassium in Fertilizers

**Flame Photometric Method**
**(Manual or Automated)**

**First Action 1983**
**Final Action 1985**

(*Caution:* See safety notes on flame photometer.)

### A. Method Parameters

Any flame photometer, manual or automated, capable of detecting K, using Li as internal std, and meeting method performance characteristics described below, is satisfactory. Samples are extd with ammonium oxalate soln or ammonium citrate soln. Appropriate dilns of ext are mixed with $LiNO_3$ internal std soln and aspirated or pumped into flame photometer. $La_2O_3$ is added to $LiNO_3$ soln to eliminate the phosphate effect. Final soln to be introduced to flame should have the following composition: (a) concn of $K_2O$ in range such that std curve response is linear over that range, (b) const amt of Li in range 5 to 40 ppm, (c) selected concn of La <1400 ppm, and (d) 0.2N $HNO_3$. Exact concn of $LiNO_3$ and $La_2O_3$ are optimized for particular instrumentation as described in performance specifications below. Ratio of K intensity at 768 nm to Li intensity at 671 nm is detd, and compared with similar ratios from std set of ≥6 stds, prepd from NBS or primary std $KH_2PO_4$. Stds are arranged in ascending order and evenly distributed thru chosen range.

### B. Preparation of Sample

(**a**) *Ammonium oxalate extraction.*—Weigh 1 g sample into 500 mL vol. flask, add 50 mL 4% $(NH_4)_2C_2O_4$ and 125 mL $H_2O$, boil 30 min, and cool. Dil. to vol. with $H_2O$, mix, and filter or let stand until clear.

(**b**) *Ammonium citrate extraction from direct available phosphorus extract.*—Prep. as in **960.03B**. (If solns must be held overnight, add 3–4 drops of $CHCl_3$.)

### C. Performance Specifications

*System performance criteria.*—Detailed example of specific instrumental system capable of meeting specified performance criteria follows this performance section. It is necessary to verify that this or any other particular system meets all of the following performance criteria before samples are analyzed. Levels specified are to be considered min. acceptable levels. Various criteria are written for automated instrument, but should also apply to manual instrument systems.

(**a**) *$LiNO_3$ concentration level.*—Amt of $LiNO_3$ in final soln aspirated into flame is adjusted partly for convenience of instrument parameters, but should be such that Li and K channels give roughly equal responses. This can be detd either by displaying each channel's output sep., or by displaying ratio of K to Li response and then interchanging Li and K filters and displaying ratio again.

Using either procedure, sample midrange $K^+$ stds under analysis conditions while varying concn of $Li^+$ until acceptable concn of $Li^+$ is found.

(**b**) *Noise.*—Adjust detector output to 90% full scale with high std sampled continuously. Noise must be <2% full scale peak to peak. Note that some instruments, flow injection analysis systems, for example, are not designed to pump samples continuously. In this case, substitute repeated sampling for continuous sampling, and consider noise to be difference between adjacent peak maxima. (Optimum performance on example instrument system described in this method is ca $^1/_2$% peak to peak. To reduce noise on example system, stabilize flame, stabilize pumping rate, stabilize back-pressure, change pump tubes, clean manifold, and/or rework manifold to ensure adequate mixing. To det. min. noise limit of instrument, collect system waste soln, connect short length of tubing directly to photometer aspirator, and aspirate waste soln directly into flame.)

(**c**) *Carryover.*—Adjust detector output to give ca 10% and 90% full scale response for low and high stds, resp. Sample 3 high stds followed by 5 low stds on system under analysis conditions. Carryover, defined as difference between first low std and mean of other low stds, may not be >1% full scale. (Optimum performance on example system is negligible carryover. To reduce carryover on example system, clean manifold and aspirator, check manifold connections for dead space, redesign manifold shortening hydraulic system wherever possible, decrease sampling rate, and/or reduce std range.)

(**d**) *Drift.*—Adjust instrument to give detector response ca 50% full scale with middle std sampled continuously. Sample middle std continuously for the time it would take to analyze 30 samples. For instruments not designed to sample continuously, draw smooth line thru 30 middle std peaks. Drift may not exceed 1% full scale per any 10 sample segment. (Optimum performance on example system is zero drift. To reduce drift on example system, stabilize room and soln temps, adjust manifold to maintain const back-pressure, and/or stabilize flame.) As long as drift does not exceed 1% per 10 peak level, routine data may be further improved by inserting a middle std periodically between groups of samples. This allows mathematical peak ht correction, assuming linear drift.

(**e**) *Precision.*—With instrument calibrated for 10% and 90% full scale for low and high stds, resp., sample 30 middle stds under analysis conditions. Range of instrument response may not vary >2% full scale. (Optimum performance on example system is 0.7% full scale. To improve precision on example system, reduce noise, check sampler timing, and/or decrease sampling rate.)

(**f**) *Std curve.*—Std curve consists of ≥6 different stds, evenly distributed thru std concn range. Prep. solns from NIST or primary std $KH_2PO_4$, dried 2 h at 105°. Include factor for actual purity of std material in calcns of std concn.

With instrument calibrated for ca 10% and 90% response for low and high stds, resp., run stds in order of ascending concn under analysis conditions. Response should be linear. Mathematically perform first degree least squares fit to std curve data. Alternatively, use calculator capable of least squares fits. First order least squares fit may be performed as follows: Assume that points to be fitted are $(X_1, Y_1, (X_2, Y_2), \ldots (X_n, Y_n)$. Calc. means by:

$$\bar{X} = \frac{1}{n}\Sigma X_j \qquad \bar{Y} = \frac{1}{n}\Sigma Y_i$$

Slope of least square fitted line is given by:

$$b_1 = \frac{\Sigma(X_j - \bar{X})(Y_i - \bar{Y})}{\Sigma(X_j - \bar{X})^2} = \frac{(\Sigma X_j Y_i) - n\overline{XY}}{(\Sigma X_j^2) - 1/n(\Sigma X_j)^2}$$

Intercept for line is given by:

$$b_0 = \bar{Y} - b_1\bar{X}$$

Equation of resulting line is:

$$Y = b_0 + b_1 X$$

Using derived equation and individual std responses, calc. concn for each std. Compare calcd and known concns for each std. Calcd value may not differ from known value by >±2% in any one instance. Also, av. of absolute values of those % differences may not be >1%. (Optimum performance on example system is 0.75% and 0.37%, resp. To improve std curve fit, optimize parameters (**b**) thru (**e**) above and/or reduce std range.)

(**g**) *Phosphate effect.*—For example system, amt of $La_2O_3$ in $LiNO_3$ reagent is sufficient to eliminate phosphate effect (depression of instrument response to K by phosphate ion). If other than example automated system is used, elimination of phosphate effect must be verified. Using $KNO_3$, prep. 200 mL soln of $K_2O$ with concn equal to twice that of highest std. Pipet 50 mL of that soln into each of two 100 mL vol. flasks. Dil. one to vol. and mix. Add sufficient $NH_4H_2PO_4$ soln to the other flask such that concn of $P_2O_5$ will be as high as highest concn of $P_2O_5$ anticipated in any sample ext. Dil.to vol. and mix. Sample 10 portions of each soln, alternating, under analysis conditions. Average 10 responses for each soln. Av. responses of the 2 solns must not differ from each other by >1%. Select min. amt of $La_2O_3$ which will eliminate phosphate effect. (Optimum performance of example system is <0.5%. To improve performance, adjust amount of $La_2O_3$.)

(**h**) *Overall performance of system.*—Performance characteristics mentioned above are worst case examples. A system functioning marginally in many categories would probably fail the following overall performance check.

Verify overall performance as follows: Ext and analyze once each 20 different Magruder samples, or other similar performance check samples previously detd by interlaboratory study. Also ext and analyze 5 independent 1 g portions of NIST or primary std $KH_2PO_4$. Randomize Magruder and $KH_2PO_4$ sample order. Calc. % $K_2O$. Av. bias of Magruder results, $\Sigma$ (Magruder grand av. − calcd % $K_2O$)/20, must be $<\pm 0.1$. Av. of absolute value of differences must be <0.4. (Optimum values on example system are ca $\pm 0.02$ and $-0.2$, resp.)

For 5 analyses of $KH_2PO_4$, difference between mean of calcd % $K_2O$ and known % $K_2O$ must not be $>\pm 0.2$, and std deviation must not be >0.25. (Optimum values for example system are $\pm 0.1$ and 0.15, resp.)

(**i**) *Ongoing performance checks.*—(*1*) Conduct daily performance check by analyzing same performance check sample at least once in every 60 regular samples, and at least once in each run. (*2*) Repeat (**h**) above at least twice per year, and whenever system has not been used for prolonged periods.

### *Example Automated Instrument System*

### *D. Apparatus*

*Automatic analyzer.*—AutoAnalyzer with following modules (available from Technicon Instruments Corp.): sampler II or IV, pump III, flame photometer IV, and recorder. Computer or calculator capable of least square fits is desirable.

### *E. Reagents*

(**a**) *Ammonium oxalate soln.*—Dissolve 40 g $(NH_4)_2C_2O_4$ in 1 L $H_2O$.

(**b**) *Ammonium citrate soln.*—Should have sp. gr. of 1.09 at 20° and pH of 7.0 as detd potentiometrically.

Dissolve 370 g cryst. citric acid in 1.5 L $H_2O$ and nearly neutze by adding 345 mL $NH_4OH$ (28–29% $NH_3$). If concn of $NH_3$ is <28%, add correspondingly larger vol. and dissolve citric acid in correspondingly smaller vol. $H_2O$. Cool, and check pH. Adjust with $NH_4OH$ (1+7) or citric acid soln to pH 7. Dil. soln, if necessary, to sp. gr. of 1.09 at 20°. (Vol. will be ca 2 L.) Keep in tightly stoppered bottles and check pH from time to time. If pH has changed from 7.0, readjust.

(**c**) *Lithium nitrate soln.*—Dissolve 1.642 g $La_2O_3$ in 30 mL $HNO_3$, add 0.9935 g dried (2 h at 105°) $LiNO_3$ and 1 mL Flaminox 1% soln (Fisher Scientific Co.), and dil. to 1 L with $H_2O$.

(**d**) *Sampler wash and dilution water soln.*—Dil. 1 mL Flaminox 1% soln to 1 L with $H_2O$.

(**e**) *Potassium std solns.*—(*1*) *Stock std soln.*—1 mg $K_2O$/mL. Dissolve 2.889 g dried (2 h at 105°) $KH_2PO_4$ (NIST SRM 200) in $H_2O$, and dil. to 1 L. (*2*) *Working std solns.*—10, 20, 30, 40, 50, and 55 μg $K_2O$/mL. Accurately measure by buret 10, 20, and 30 mL stock std soln into 1 L vol. flasks, and 20, 25, and 27.5 mL into 500 mL vol. flasks. Add 0.2 g $(NH_4)_2C_2O_4$ per 500 mL final vol. if samples are prepd by ammonium oxalate extn, or add 12 mL ammonium citrate soln per 500 mL final vol. if samples are prepd by ammonium citrate extn. Dil. to vol. with $H_2O$ and mix. (Add 3 mL $CHCl_3$ to preserve citrate std solns for long periods.)

### *F. Analytical System*

Assemble manifold as in Fig. **983.02**. Use 1.6–2.0 mm id glass transmission tubing for all reagent flow upstream from D1 fitting. Use clear std pump tubes for air and soln stream flow.

Air and $H_2O$ are combined thru injection fitting (116-0492-01). Hard thin-wall polyethylene tubing (ca 0.30 in. id) connects air bar tubing to injection fitting. Sample is introduced immediately downstream thru second injection fitting (194-G012-01), designed to eliminate double peaks in recorder output. Mixing of sample and $H_2O$ occurs in double 10-turn coil with insert (157-B089). $LiNO_3$ reagent is introduced thru insert. Another 10-turn coil (157-0251) further mixes solns.

Portion of soln is aspirated to flame photometer thru A4 fitting (116-0200-04). Hard, thin-wall polyethylene tubing (ca 0.045 in. id) connected to photometer is inserted and glued to tee arm of A4 fitting. Remaining unaspirated soln is drawn thru double 10-turn mixing coil (157-9248-01) and thru D1 fitting (116-0203-01). Large diam. branch of D1 fitting leads to pump and waste. Small diam. branch of D1 fitting is con-

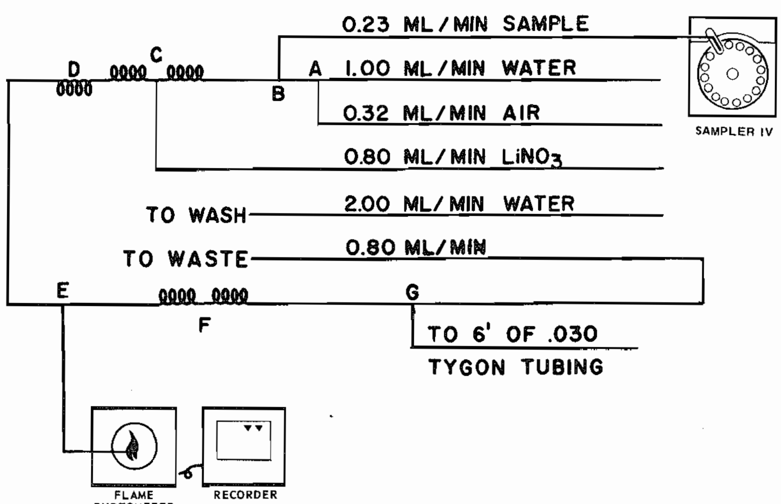


**FIG. 983.02—Manifold for $K_2O$ in fertilizers. A, injection fitting 116-0492-01; B, injection fitting 194-G012-01; C, double 10-turn coil with insert 157-B089; D, 10-turn coil 157-0251; E, A4 fitting 116-0200-04; F, double 10-turn coil 157-0248-01; G, D1 fitting 116-0203-01**

nected to 6 ft (1.83 m) of Tygon tubing (0.030 in. id) to waste. D1 fitting is oriented with small diam. branch low, so that only soln, and no air, enters 0.030 in. tubing. This establishes const back-pressure and therefore stable aspiration conditions at flame photometer.

### G. Startup and Shutdown Procedures

Start system and place reagent lines in proper solns. Let equilibrate 30 min before beginning calibration. Adjust flame photometer as follows: (*1*) damping control to damp 3 position; (*2*) flame ht of main cone ca 4 cm; (*3*) atomizer adjust control set to give atomization rate of ca 1.3–1.4 mL/min. Rate of atomization is detd by subtracting rate of flow to waste from rate of flow upstream from (disconnected) A4 fitting. Use 0.3 and 0.6 neut. density filters for Li and K detectors, resp.

Initially it may be necessary to manually fill system downstream from A4 fitting with $H_2O$, making certain that 6 ft of 0.030 tubing is filled. System is shut down after pumping $H_2O$ thru reagent lines ≥15 min.

### H. Checkout and Calibration

After equilibration, pump 10 μg $K_2O$/mL std thru system and adjust baseline control of photometer to read 10% full scale. Pump 55 μg $K_2O$/mL std thru system and adjust std calibration control to read 90% full scale. If noisy conditions exist, check for aspiration of air at A4 fitting, or check for air entering lower arm of D1 fitting. If drift exists, check room and solns for temp. stability. Std curves should be virtually linear.

### I. Determination

Pipet aliquots of sample solns, Table **983.02**, into 250 mL vol. flask, dil. to vol. with $H_2O$, and mix 15 times. For 10 mL aliquots of citrate extns, add 4 mL ammonium citrate soln to aliquots before dilg to vol. Run samples in groups of 10. Place 10 thru 55 μg $K_2O$/mL stds in order in sampler, preceded by extra 10 μg/mL std. Place 30 μg/mL std after every 10th sample, to be used for drift correction. End series with two 30 μg/mL stds. Sample at rate of 40/h, 2:1 sample-to-wash ratio.

### J. Calculations

Correct sample peak hts for drift. Correct peak hts of first 10 samples as follows:

$$H_c = H - [(D_1 - D_0)/14][L + 3]$$

where $H_c$ = corrected peak ht; $H$ = uncorrected peak ht; $D_1$ = ht of first drift correction std; $D_0$ = ht of 30 μg std in initial std sequence; and $L$ = position No. of sample peak to be corrected. Correct subsequent sample peak hts as follows:

$$H_c = H - [D_x - D_0] - [(D_y - D_x)/11][P]$$

where $D_x$ = ht of drift std preceding sample to be corrected; $D_y$ = ht of drift std following sample to be corrected; and $P$ = position No. of sample within group of 10.

Calc. least squares fitted curve of emission against $K_2O$ concn. Calc. μg $K_2O$/mL of corrected peak hts from equation:

$$\% K_2O = (\mu g\ K_2O/mL \times 12.5)/(aliquot \times g\ sample)$$

Ref.: JAOAC **66**, 1242(1983).

**Table 983.02 $K_2O$ Aliquots**

| $K_2O$ Expected, % | Aliquot, mL |
|---|---|
| <2 | 250 (no diln) |
| 2–6.49 | 100 |
| 6.50–19.99 | 30 |
| ≥20 | 10 |

CAS-7440-09-7 (potassium)
CAS-12136-45-7 (potassium oxide)

## 955.06★ Potassium in Fertilizers
**Flame Photometric Method**
**Final Action**
**Surplus 1986**

See **2.108–2.113**, 14th ed.

## 971.01★ Potassium in Fertilizers
**Automated Flame Photometric Method**
**First Action 1971**
**Final Action 1973**
**Surplus 1986**

See **2.114–2.118**, 14th ed.

## 958.02 Potassium in Fertilizers
**Volumetric Sodium Tetraphenylboron Method I**
**First Action 1958**
**Final Action 1960**

(*Caution:* See safety notes on formaldehyde.)

### A. Reagents

(**a**) *Formaldehyde soln.*—37%.

(**b**) *Sodium hydroxide soln.*—20%. Dissolve 20 g NaOH in 100 mL $H_2O$.

(**c**) *Sodium tetraphenylboron (STPB) soln.*—Approx. 1.2%. Dissolve 12 g $NaB(C_6H_5)_4$ in ca 800 mL $H_2O$. Add 20–25 g $Al(OH)_3$, stir 5 min, and filter (Whatman No. 42 paper, or equiv.) into 1 L vol. flask. Rinse beaker sparingly with $H_2O$ and add to filter. Collect entire filtrate, add 2 mL 20% NaOH, dil. to vol. with $H_2O$, and mix. Let stand 48 hr and stdze. Adjust so that 1 mL STPB = 1% $K_2O$. Store at room temp.

(**d**) *Benzalkonium chloride (BAC) soln.*—Approx. 0.625%. Dil. 38 mL 17% Zephiran chloride (Winthrop Laboratories; also available at local pharmacies as benzalkonium chloride) to 1 L with $H_2O$, mix, and stdze. Cetyltrimethylammonium bromide may be substituted for Zephiran chloride. If other concn is used, adjust vol.

(**e**) *Clayton Yellow (Titan Yellow; Colour Index No. 19540).*—0.04%. Dissolve 40 mg in 100 mL $H_2O$.

### B. Standardization of Solutions

(**a**) *BAC soln.*—To 1.00 mL STPB soln in 125 mL erlenmeyer, add 20–25 mL $H_2O$, 1 mL 20% NaOH, 2.5 mL HCHO, 1.5 mL *4%* $(NH_4)_2C_2O_4$, and 6–8 drops indicator, (**e**). Titr. to pink end point with BAC soln, using 10 mL semimicro buret. Adjust BAC soln so that 2.00 mL = 1.00 mL STPB soln.

(**b**) *Sodium tetraphenylboron soln.*—Dissolve 2.500 g $KH_2PO_4$ in $H_2O$ in 250 mL vol. flask, add 50 mL 4% $(NH_4)_2C_2O_4$ soln, dil. to vol. with $H_2O$, and mix. (It is not necessary to bring to boil.) Transfer 15 mL aliquot (51.92 mg $K_2O$, 43.10 mg K) to 100 mL vol. flask; add 2 mL 20% NaOH, 5 mL HCHO, and 43 mL STPB reagent. Dil. to vol. with $H_2O$, mix *thoroly,* let stand 5–10 min, and pass thru dry filter. Transfer 50 mL aliquot of filtrate to 125 mL erlenmeyer, add 6–8 drops indicator, (**e**), and titr. excess reagent with BAC soln. Calc. titer as follows:

$F$ = 34.61/(43 mL − mL BAC) = % $K_2O$/mL STPB reagent

Factor $F$ applies to all fertilizers if 2.5 g sample is dild to 250 mL and 15 mL aliquot is taken for analysis. If results are to be expressed as K rather than as $K_2O$, substitute 28.73 for 34.61 in calcg $F$.

### C. Determination

Place 2.5 g sample (1.25 g if $K_2O$ >50%) in 250 mL vol. flask, add 50 mL 4% $(NH_4)_2C_2O_4$ and 125 mL $H_2O$, and boil 30 min. (If org. matter is present, add 2 g *K-free C* before boiling.) Cool, dil. to vol. with $H_2O$, mix, and pass thru dry filter or let stand until clear. Transfer 15 mL aliquot sample soln to 100 mL vol. flask and add 2 mL 20% NaOH and 5 mL HCHO. Add 1 mL std STPB soln for each 1% $K_2O$ expected in sample plus addnl 8 mL excess to ensure complete pptn. Dil. to vol. with $H_2O$, mix *thoroly*, let stand 5–10 min, and filter thru dry paper (Whatman No. 12 or equiv.). Transfer 50 mL filtrate to 125 mL erlenmeyer, add 6–8 drops indicator, (**e**), and titr. excess reagent with std BAC soln.

% $K_2O$ in sample = (mL STPB added − mL BAC) × $F$

where $F$ = % $K_2O$/mL STPB reagent. (Multiply by 2 if 1.25 g sample was used.)

Refs: Anal. Chem. **29,** 1044(1957); **30,** 1882(1958). JAOAC **41,** 533(1958); **43,** 472(1960).

CAS-7440-09-7 (potassium)
CAS-13547-17-6 (potassium oxide)

## 969.04 Potassium in Fertilizers

**Volumetric Sodium Tetraphenylboron Method II**

**First Action 1969**
**Final Action 1970**

(For use with sample prepd for available P detn)

(*Caution:* See safety notes on formaldehyde.)

### A. Reagents

*See* **958.02A(a)**, (**b**), (**c**), (**d**), and (**e**).

### B. Standardization of Solutions

(**a**) *BAC soln.*—In 125 mL erlenmeyer, add 2.5 mL neut. $NH_4$ citrate soln, **963.03A(a)**, 15–20 mL $H_2O$, 4 mL HCHO, and 2.5 mL 20% NaOH soln. Swirl; then add 4.00 mL STPB soln and 6–8 drops indicator, **958.02A(e)**. Titr. to pink end point with BAC soln, using 10 mL semimicro buret. Adjust BAC soln so that 2.00 mL = 1.00 mL STPB soln.

(**b**) *Sodium tetraphenylboron soln.*—Dissolve 1.4447 g primary std $KH_2PO_4$ in $H_2O$ in 500 mL vol. flask, add 100 mL neut. $NH_4$ citrate soln, **963.03A(a)**, dil. to vol. with $H_2O$, and mix. Transfer 25 mL aliquot (25.00 mg $K_2O$, 20.75 mg K) to 100 mL vol. flask; add 8 mL HCHO and 5 mL 20% NaOH, swirl, and add 25 mL STPB reagent. Dil. to vol. with $H_2O$, mix *thoroly*, let stand 5–10 min, and pass thru dry filter. Transfer 50 mL aliquot of filtrate to 125 mL erlenmeyer, add 6–8 drops indicator, **958.02A(e)**, and titr. excess reagent with BAC soln. Calc. titer as follows:

$F$ = 25 mg $K_2O$/(25 mL STPB − mL BAC)
= mg $K_2O$/mL STPB reagent

If results are to be expressed as K rather than $K_2O$, substitute 20.75 for 25 in calcg $F$.

### C. Preparation of Sample

Prep. as in **960.03B**.

### D. Determination

Transfer 25 mL aliquot of sample soln to 100 mL vol. flask. (If org. matter is present, treat 100 mL portion with 1 g K-free C and filter before transferring aliquot.) Add 8 mL HCHO *first* and then 5 mL 20% NaOH soln, and wash down sides of flask with $H_2O$. Swirl and add 1 mL STPB for each 1.5 mg $K_2O$ expected in sample aliquot plus addnl 8 mL excess to ensure complete pptn. Dil. to vol. with $H_2O$, mix *thoroly*, let stand 5–10 min, and pass thru dry filter (Whatman No. 12, or equiv.). Transfer 50 mL aliquot filtrate to 125 mL erlenmeyer, add 6–8 drops indicator, **958.02A(e)**, and titr. excess reagent with std BAC soln.

% $K_2O$ in sample = (mL STPB added − mL BAC) × $F$ × 2

Calcn applies to all fertilizers if 1 g sample is dild to 500 mL and 25 mL aliquot is taken for analysis.

Ref: JAOAC **52,** 566(1969).

CAS-7440-09-7 (potassium)
CAS-13547-17-6 (potassium oxide)

# OTHER ELEMENTS

## 965.09 Nutrients (Minor) in Fertilizers

**Atomic Absorption Spectrophotometric Method**

**First Action 1965**
**Final Action 1969**

(*Caution:* See safety notes on atomic absorption spectrophotometer.)

### A. Apparatus and Reagent

(**a**) *Atomic absorption spectrophotometer.*—Several com. models are available. Since each design is somewhat different, with varying requirements of light source, burner flow rate, and detector sensitivity, only general outline of operating parameters is given in Table **965.09**. Operator must become familiar with settings and procedures adapted to his own app. and use table only as guide to concn ranges and flame conditions.

(**b**) *Disodium EDTA soln.*—2.5%. Dissolve 25 g $Na_2H_2$ EDTA in 1 L $H_2O$ and adjust to pH 7.0 with 5$N$ NaOH, using pH meter.

### B. Standard Solutions

(Do not use <2 mL pipets or <25 mL vol. flasks. Automatic diln app. may be used. Prep. std solns in 0–20 μg range fresh daily.)

(**a**) *Calcium solns.*—*(1) Stock soln.*—25 μg Ca/mL. Dissolve 1.249 g $CaCO_3$ in min. amt 3$N$ HCl. Dil. to 1 L. Dil. 50 mL to 1 L. *(2) Working std solns.*—0, 5, 10, 15, and 20 μg Ca/mL contg 1% La. To 25 mL vol. flasks add 0, 5, 10, 15, and 20 mL Ca stock soln. Add 5 mL La stock soln and dil. to 25 mL.

(**b**) *Copper stock soln.*—1000 μg Cu/mL. Dissolve 1.000 g pure Cu metal in min. amt $HNO_3$ and add 5 mL HCl. Evap. almost to dryness and dil. to 1 L with 0.1$N$ HCl.

(**c**) *Iron stock soln.*—1000 μg Fe/mL. Dissolve 1.000 g pure Fe wire in ca 30 mL 6$N$ HCl with boiling. Dil. to 1 L.

(**d**) *Lanthanum stock soln.*—50 g La/L. Dissolve 58.65 g $La_2O_3$ in 250 mL HCl, adding acid slowly. Dil. to 1 L.

(**e**) *Magnesium stock soln.*—1000 μg Mg/mL. Place 1.000 g pure Mg metal in 50 mL $H_2O$ and slowly add 10 mL HCl. Dil. to 1 L.

(**f**) *Manganese stock soln.*—1000 μg Mn/mL. Dissolve 1.582 g $MnO_2$ in ca 30 mL 6*N* HCl. Boil to remove Cl and dil. to 1 L.

(**g**) *Zinc stock soln.*—1000 μg Zn/mL. Dissolve 1.000 g pure Zn metal in ca 10 mL 6*N* HCl. Dil. to 1 L.

(**h**) *Other std solns.*—Dil. aliquots of solns (**b**), (**c**), (**e**), (**f**), and (**g**) with 0.5*N* HCl to make ≥4 std solns of each element within range of detn.

### C. Preparation of Sample Solutions

(*Caution:* See safety notes on wet oxidation, hydrofluoric acid, and perchloric acid.)

(**a**) *Inorganic materials and mixed fertilizers.*—Dissolve 1.00 g well ground sample in 10 mL HCl in 150 mL beaker. Boil and evap. soln nearly to dryness on hot plate. *Do not bake residue.* Redissolve residue in 20 mL 2*N* HCl, boiling gently if necessary. Filter thru fast paper into 100 mL vol. flask, washing paper and residue thoroly with $H_2O$. Measure absorption of soln directly, or dil. with 0.5*N* HCl to obtain solns within ranges of instrument. If Ca is to be detd, add enough La stock soln to make final diln 1% La (i.e., 5 mL La to 25 mL flask, 20 mL to 100 mL flask, etc.).

(**b**) *Fertilizers containing organic matter (tankage, corncobs, cottonseed hulls, etc.).*—Place 1.00 g sample in 150 mL beaker (Pyrex, or equiv.). Char on hot plate and ignite 1 hr at 500° with muffle door propped open to allow free access of air. Break up cake with stirring rod and dissolve in 10 mL HCl as in (**a**).

(**c**) *Fertilizers containing fritted trace elements.*— Dissolve ≤1.00 g well ground sample in 5 mL $HClO_4$ and 5 mL HF. Boil and evap. to dense $HClO_4$ fumes. Dil. carefully with $H_2O$, filter, and proceed as in (**a**). Alternatively, dissolve sample in 10 mL HCl, 5 mL HF, and 10 mL MeOH. Evap. to dryness. Add 5 mL HCl and evap. Repeat HCl addn and evapn. Dissolve residue as in (**a**). (Normally Pt ware should be used; Pyrex or other glassware may be used if Na, K, Ca, and Fe are not to be detd.)

(**d**) *For manganese.—(1) Acid-soluble, for both* $Mn^{+2}$ *and* $Mn^{+4}$.—**See** (**a**), (**b**), and (**c**), and **972.02**(**b**). *(2) Acid-soluble, for* $Mn^{+2}$ *only.—See* **972.02**(**a**), **940.02**, and **941.02**★. *(3) Water-soluble, for* $Mn^{+2}$ *only.—See* **972.03**.

(**e**) *For iron and zinc.—(1) Aqueous extraction.*—Place 1.00 g sample in 250 mL beaker, add 75 mL $H_2O$, and boil 30 min. Filter into 100 mL vol. flask, washing paper with $H_2O$. Dil. to vol. and redil. if necessary. (*2*) *Chelation extraction.*—Place 1.00 g sample in 250 mL beaker, and add 5 cm (2″) mag. stirrer bar and 100 mL 2.5% EDTA soln. Stir exactly 5 min, and filter thru Whatman No. 41 paper, or equiv. If filtrate is cloudy, refilter immediately thru fine paper (Whatman No. 5, or equiv.). Redil., if necessary, with 0.5*N* HCl.

### D. Determination

(P interferes in Ca and may interfere in Mg detn with air-$C_2H_2$ burners. Eliminate interference by adding La stock soln to std and sample solns so that final dilns contain 1% La. P does not interfere with Ca detn when $N_2O$-$C_2H_2$ flame is used.)

Set up instrument as in Table **965.09**, or previously established optimum settings for app. to be used. Less sensitive secondary lines (Gatehouse, and Willis, Spectrochim. Acta **17**, 710(1961)) may be used to reduce necessary diln, if desired. Read ≥4 std solns within anal. range before and after each group of 6–12 samples. Flush burner with $H_2O$ between samples, and re-establish 0 absorption point each time. Prep. calibration curve from av. of each std before and after sample group. Read concn of samples from plot of absorption against μg/mL.

**Table 965.09 Operating Parameters**

| Element | Wave-length, Å | Flame | Range, μg/mL | Remarks |
|---|---|---|---|---|
| Ca | 4227 | Rich Air-$C_2H_2$ | 2–20 | 1% La, 1% HCl |
| | 4227 | Rich $N_2O$-$C_2H_2$ | 2–20 | Requires special burner |
| Cu | 3247 | Air-$C_2H_2$ | 2–20 | |
| Fe | 2483 | Rich Air-$C_2H_2$ | 2–20 | |
| Mg | 2852 | Rich Air-$C_2H_2$ | 0.2–2 | May need La |
| Mn | 2795 | Air-$C_2H_2$ | 2–20 | |
| Zn | 2138 | Air-$C_2H_2$ | 0.5–5 | |

### E. Calculations

$$\% \text{ Element} = (\mu g/mL) \times (F/\text{sample wt}) \times 10^{-4}$$

$F$ = mL original diln × mL final diln/mL aliquot, if original 100 mL vol. is dild.

Refs.: JAOAC **48,** 406, 1100(1965); **50,** 401(1967); **51,** 847(1968); **58,** 928(1975).

CAS-7440-70-2 (calcium)
CAS-7440-50-8 (copper)
CAS-7439-89-6 (iron)
CAS-7439-95-4 (magnesium)
CAS-7439-96-5 (manganese)
CAS-7440-66-6 (zinc)

## 949.02 Boron (Acid-Soluble) in Fertilizers

**Titrimetric Method**

**Final Action**

### A. Apparatus

Use high sensitivity glass electrode pH meter for titrn. Use assembly with burets, electrodes, and mech. stirrer, arranged for convenient use with 250 mL beaker. Use ordinary 50 mL burets for the 0.025*N* NaOH and 0.02*N* HCl.

### B. Reagents

(**a**) *Boric acid std soln.*—Dissolve 1 g $H_3BO_3$ in $H_2O$ and dil. to 1 L. 1 mL = 0.1748 mg B.

(**b**) *Sodium hydroxide std soln.*—$CO_2$-free, ca 0.025*N*. Stdze as follows: Pipet 25 mL std $H_3BO_3$ soln into 250 mL beaker, add 3.0 g NaCl, acidify to Me red, dil. to 150 mL, boil to expel $CO_2$, cool, and titr. potentiometrically as in **949.02C**. Det. blank by repeating titrn, substituting 25 mL $H_2O$ for $H_3BO_3$ soln. Calc. B equivalence as follows:

$$\text{mg B/mL} = 4.369/[(\text{mL NaOH soln}) - (\text{mL blank})]$$

Protect from atm. $CO_2$ by soda-lime tubes or other suitable means.

(**c**) *Methyl red indicator.*—Dissolve 0.1 g Me red in 50 mL alcohol, dil. to 100 mL with $H_2O$, and filter if necessary.

### C. Determination

Weigh sample within 1 mg (1.0 g for up to 0.45% B, smaller samples for above that content) and place in 250 mL beaker. Add ca 50 mL $H_2O$ and 3 mL HCl. Heat to bp and keep hot until carbonates are decomposed. Keep soln hot but do not boil during following phosphate removal:

Add *10%* $Pb(NO_3)_2$ *soln,* usually 10 mL, or 1 mL for each 1.2% $P_2O_5$ if $P_2O_5$ content is known to be >12%. Add $NaHCO_3$, little at time, until soln approaches neutrality (often observed by formation of white ppt in addn to insol. matter already present). Add few drops Me red and continue adding $NaHCO_3$ gradually until *just* alk. to Me red (yellow or very slightly orange). Keep mixt. hot but not boiling ($H_2O$ bath or steam bath is best) 30 min, adding addnl small amts of $NaHCO_3$ if needed to keep same indicator color. (If indicator is bleached by nitrate present, add more; if color is obscured by org. matter, use external spot tests to follow neutzn.) After neutzn and heating, 40–50 mL soln should remain.

Filter hot soln into 250 mL beaker and wash solids thoroly with hot $H_2O$. Acidify filtrate with few drops HCl and boil briefly to expel most of $CO_2$. Neutze hot soln with 0.5*N* NaOH, and reacidify with 0.5*N* HCl, using 0.3–0.5 mL excess. Dil. to ca 150 mL and boil gently few min to expel remaining $CO_2$. Cool to room temp. in running $H_2O$. Roughly neutze mixt., using $CO_2$-free 0.5*N* NaOH, and place beaker in titrn assembly with electrodes and stirrer immersed. Start stirrer and adjust pH to exactly 6.30 by adding 0.025*N* NaOH or 0.02*N* HCl as required. (When properly adjusted, pH should be steady; drifting usually is due to incomplete removal of $CO_2$.) When reading of pH 6.30 is steady, read 0.025*N* NaOH buret, add 20 g *mannitol* or cryst. D-*sorbitol*, and titr. with 0.025*N* NaOH to pH 6.30. (Conveniently done with slidewire type instrument by opening pH meter circuit when mannitol is added, leaving scale setting at 6.30, closing circuit again when indicator color shows that end point is being approached, and carefully adding std NaOH soln until galvanometer needle returns to zero. Somewhat slow approach to equilibrium, characteristic of glass electrode, can be anticipated with practice so as not to overrun end point.) When end point is reached, read buret again. Obtain reagent blank by repeating detn with all reagents but without sample.

% B = (mL NaOH soln in detn − mL blank) × (mg B/mL NaOH soln)/(10 × g sample)

Refs.: JAOAC **32,** 422(1949); **33,** 132(1950); **36,** 623(1953); **38,** 407(1955).

CAS-7440-42-8 (boron)

## 949.03 Boron (Water-Soluble) in Fertilizers

### Titrimetric Method

### Final Action

(Not applicable in presence of >5% urea or urea-formaldehyde resins)

Weigh 2.5 g sample into 250 mL beaker. Add 125 mL $H_2O$, boil gently ca 10 min, and filter hot thru Whatman No. 40 paper, or equiv., into 400 mL beaker. Wash solids well with 6 portions hot $H_2O$ and dil. to ≥200 mL with $H_2O$. Heat filtrate just to bp. Add 15 mL *10% $BaCl_2$ soln* to ppt sulfates and phosphates, and add *powd $Ba(OH)_2$*, cautiously with stirring, until just alk. to phthln, avoiding large excess. Boil in open beaker ≥60 min to expel $NH_3$. (Samples colored by org. matter should be boiled longer.) If necessary, add $H_2O$ to keep vol. to ≥150 mL. Add and stir 1–2 teaspoonfuls Filter-Cel or other inert filtering aid, and filter with suction thru packed paper pads into 500 mL Pyrex erlenmeyer. Wash ppt 6 times with hot boiled $H_2O$. (Avoid too large wash vols which increase vol. in flask to point of dangerous bumping in next step.)

Make filtrate just colorless to phthln with HCl (1+5), add Me red, and make just pink with the acid. Add 5 or 6 boiling stones and stirring rod, cover with watch glass, and boil 5 min to remove $CO_2$. Cool in cold $H_2O$ while covered. Wash cover glass, stirrer, and sides of flask. Titr. to yellow of Me red with *std 0.05N NaOH*, **936.16**. Add 20 g D-*mannitol* and 1 mL or more phthln, shake, and wash down sides of flask. Titr. to pink end point. Det. blank in exactly same manner as sample.

1 mL 0.05*N* NaOH = 0.000540 g B or 0.00477 g $Na_2B_4O_7.10H_2O$

Or, (Titer − blank) × factor = lb $Na_2B_4O_7.10H_2O$/ton(factor = 3.807 for 0.05*N* NaOH).

Refs.: JAOAC **32,** 422(1949); **33,** 132(1950); **36,** 623(1953); **38,** 407(1955).

CAS-7440-42-8 (boron)

## 982.01 Boron (Acid- and Water-Soluble) in Fertilizers

### Spectrophotometric Method

### First Action 1982

### Final Action 1985

#### *A. Apparatus and Reagents*

(**a**) *Spectrophotometer*.—Beckman Model 24/25 (replacement model DV-64), or equiv.

(**b**) *Precision pipet*.—100 µL Sherwood Lancer (Monoject Scientific, 200 Express St, Plainview, NY 11803), or equiv.

(**c**) *Dispenser pipet*.—Automatic (Repipet, Labindustries, Inc., 620 Hearst Ave, Berkeley, CA 94710), or equiv., 5 mL capacity.

(**d**) *Boron std solns*.—(*1*) *Stock soln*.—100 µg/mL. Dissolve 0.5716 g boric acid in $H_2O$ and dil. to 1 L with $H_2O$. Mix well and transfer to plastic bottle. (*2*) *Working solns*.—0, 5, 10, 15, 20, 25, 30, and 45 µg/mL. Pipet 0, 5, 10, 15, 20, 25, 30, and 45 mL stock soln into sep. 100 mL vol. flasks, dil. to vol. with 1% HCl, mix well, and transfer to plastic bottles. Solns are stable.

(**e**) *Azomethine H color reagent*.—Dissolve 0.9 g azomethine H (Pierce Chemical Co.) and 2.0 g ascorbic acid in 100 mL $H_2O$. Store in refrigerator and discard after 14 days.

(**f**) *Buffer-masking soln*.—Dissolve 140 g ammonium acetate, 10 g potassium acetate, 4 g nitrilotriacetic acid, disodium salt 99+% (Aldrich Chemical Co., Inc., No. 10629-1), 10 g (ethylenedinitrilo) tetraacetic acid, and 350 mL 10% acetic acid (v/v) in $H_2O$ and dil. to 1 L with $H_2O$. Soln is stable.

(**g**) *Color developing reagent*.—Place 35 mL azomethine H color reagent and 75 mL buffer-masking soln into 250 mL vol. flask and dil. to vol. with $H_2O$. Prep. fresh daily.

#### *B. Preparation of Sample Solutions*

(**a**) *Acid-soluble boron*.—Weigh 2.00 g sample into 100 mL vol. flask, add 30 mL $H_2O$ and 10 mL HCl, stopper, and shake 15 min. Dil. to vol. with $H_2O$, mix well, and filter immediately into plastic bottle. Dil. as necessary, so final soln for color measurement falls within std curve.

(**b**) *Water-soluble boron*.—Weigh 2.00 g sample into 250 mL beaker, add 50 mL $H_2O$, and boil ca 10 min. Filter hot thru Whatman No. 40 paper, or equiv., into 100 mL vol. flask. Wash ppt 6 times with hot, boiled $H_2O$ until vol. in flask is ca 95 mL. Cool, add 1.0 mL HCl, dil. to vol. with $H_2O$, and mix. Transfer to plastic bottle immediately; dil. as necessary so final soln for color measurement falls within std curve.

#### *C. Determination*

Pipet 100 µL aliquots of 0, 5, 10, 15, 20, 25, 30, and 45 µg/mL std and 100 µL aliquots of sample solns into sep. 10 mL erlenmeyers. Add 5.0 mL color developing reagent by automatic pipet dispenser (5 mL pipet is suitable but slower) and let stand 1 h at room temp. Transfer to 1 cm cell and read *A* at 420 nm against $H_2O$. Correct for reagent blank (0 mg/mL std). Construct std curve by plotting *A* against µg/mL stds and read concns (µg/mL) of sample solns from std curve.

#### D. Calculation

Boron, % = (μg/mL from std curve) × diln factor × (100/g sample) × $10^{-6}$

Ref.: JAOAC **65**, 234(1982).

CAS-7440-42-8 (boron)

### 945.03 Calcium (Acid-Soluble) in Fertilizers

**Titrimetric Method I**

**Final Action**

(Presence of other analytes pptd by oxalate, such as Ba and Sr, will cause pos. bias in results.)

Weigh 2.5 g sample into 250 mL vol. flask, add 30 mL $HNO_3$ and 10 mL HCl, and boil 30 min. Cool, dil. to vol., mix, and filter if necessary. Transfer 25 mL aliquot to beaker and dil. to 100 mL. Add 2 drops bromophenol blue (grind 0.1 g bromophenol blue powder with 1.5 mL 0.1N NaOH and dil. to 25 mL with $H_2O$). Add $NH_4OH$ (1+4) until indicator changes from yellow to green (not blue). If overrun, bring back with HCl (1+4). (This gives pH of 3.5–4.0.) Dil. to 150 mL, bring to bp, and add 30 mL satd hot $(NH_4)_2C_2O_4$ soln slowly, stirring constantly. If color changes from green to blue or yellow again, adjust to green with HCl (1+4). If yellow, adjust with $NH_4OH$ to green. Digest on steam bath 1 hr, or let stand overnight, and cool to room temp. Filter supernate thru quant. paper, gooch, or fritted glass filter, and wash ppt thoroly with $NH_4OH$ (1+50). Place paper or crucible with ppt in original beaker and add mixt. of 125 mL $H_2O$ and 5 mL $H_2SO_4$. Heat to ≥70° and titr. with 0.1*N* $KMnO_4$ until first slight pink appears. Correct for blank and calc. to Ca.

### 965.10★ Calcium (Acid-Soluble) in Fertilizers

**Titrimetric Method II**

**Final Action**
**Surplus 1980**

*See* **2.142**, 14th ed.

### 945.04 Calcium (Acid-Soluble) in Fertilizers

**Atomic Absorption Spectrometric Method**

**Final Action**

*See* **965.09**.

### 955.07★ Carbon (Carbonate) in Fertilizers

**Final Action**
**Surplus 1970**

*See* **2.107–2.108**, 11th ed.

### 928.02 Chlorine (Water-Soluble) in Fertilizers

**Final Action**

#### A. Reagents

**(a)** *Silver nitrate std soln.*—Dissolve ca 5 g recrystd $AgNO_3$ in $H_2O$ and dil. to 1 L. Stdze against pure, dry NaCl and adjust so that 1 mL soln = 0.001 g Cl.

**(b)** *Potassium chromate indicator.*—*See* **941.18B(b)**.

#### B. Determination

Place 2.5 g sample on 11 cm filter paper and wash with successive portions boiling $H_2O$ until washings total nearly 250 mL, collecting filtrate in 250 mL vol. flask. Cool, dil. to vol. with $H_2O$, and mix well. Pipet 50 mL into 150 mL beaker, add 1 mL $K_2CrO_4$ indicator, and titr. with std $AgNO_3$ soln to permanent red of $Ag_2CrO_4$.

Refs.: JAOAC **11**, 34,201(1928); **16**, 69(1933).

CAS-7782-50-5 (chlorine)

### 965.11 Cobalt in Fertilizers

**Colorimetric Method**

**First Action 1965**
**Final Action 1969**

(*Caution:* See safety notes on nitric acid and perchloric acid.)

#### A. Reagents

(Use $H_2O$ free of interfering elements. Check by shaking 2 drops 0.01% dithizone in $CCl_4$ with 10 mL $H_2O$. $CCl_4$ phase should remain green.)

**(a)** *Ternary acid mixture.*—*See* **960.03A(b)**.

**(b)** *Ammonium hydroxide.*—Use fresh stock. (Reagent becomes contaminated with heavy metals on prolonged storage in glass.)

**(c)** *Isoamyl acetate.*—Distd.

**(d)** *2-Nitroso-1-naphthol soln.*—0.05%. Dissolve 0.05 g 2-nitroso-1-naphthol in 8 drops 1*N* NaOH and 1 mL $H_2O$. Add 50–60 mL $H_2O$ and 6.5–7 mL $NH_4OH$, and dil. to 100 mL with $H_2O$. Divide into 2 ca equal parts and wash each part twice in 100 mL centrf. tube with 20 mL isoamyl acetate. Shake 30 sec and centrf. after each addn. (It may be necessary to remove part of aq. phase to ensure complete removal of foreign matter at interface.)

**(e)** *Cobalt std solns.*—(*1*) *Stock soln.*—200 μg Co/mL. Dissolve 0.0808 g $CoCl_2.6H_2O$ in $H_2O$ and dil. to 100 mL. (*2*) *Working soln.*—2 μg Co/mL. Dil. 1 mL stock soln to 100 mL with $H_2O$.

#### B. Determination

Slowly add 20 mL ternary acid mixt. to 2.00 g pulverized, mixed fertilizer in 150 mL beaker. Cover with watch glass and digest on steam bath overnight. Transfer to hot plate and heat covered until dense white fumes appear. (At this point $HNO_3$ will have been expelled. Take care not to lose significant amts of $HClO_4$.) Dil. sample contg undissolved residue with $H_2O$, transfer to 50 mL vol. flask, and dil. to vol. Transfer to 100 mL centrf. tube and centrf. 5 min at 2000 rpm. Transfer aliquot, contg 2–5 μg Co, to 50 mL g-s centrf. tube. Add 10 mL *20% diammonium citrate soln* and 2 drops phthln. Adjust pH carefully to distinct pink with $NH_4OH$ (1+1) and add successively 1 mL *10% $Na_2S_2O_3$ soln,* 2 mL 2-nitroso-1-naphthol soln, and 5 mL isoamyl acetate. (Only isoamyl acetate addn requires high degree of precision.) Shake mixt. 5 min and let sep. Centrf., if necessary. Draw off and discard aq. phase thru glass capillary tube attached to vac. Wash isoamyl acetate phase with two 5 mL portions 1*N* NaOH and one 5 mL portion 1*N* HCl. Shake 5 min after each addn, let layers sep., and draw off and discard aq. phase. Centrf. 2 min at 1500 rpm and measure *A* or %*T* at 530 nm against isoamyl acetate. Det. Co from calibration curve relating *A* or log %*T* to Co content of std solns contg 0, 2, 4, and 5 μg Co.

Refs: Anal. Chem. **30**, 1153(1958). JAOAC **48**, 412(1965).

CAS-7440-48-4 (cobalt)

## 975.01 Copper in Fertilizers
### Atomic Absorption Spectrometric Method
### Final Action

*See* **965.09**.

## 941.01★ Copper in Fertilizers
### Long Volumetric Method
### Final Action
### Surplus 1970

*See* **2.129–2.130**, 11th ed.

## 942.01 Copper in Fertilizers
### Short Volumetric Method
### Final Action 1960

***A. Reagents***

(**a**) *Sodium thiosulfate std soln*.—0.03*N*. Prep. daily by dilg 0.1*N* soln, **942.27**. 1 mL 0.03*N* $Na_2S_2O_3$ = 1.906 mg Cu.

(**b**) *Starch soln*.—Mix ca 1 g sol. starch with enough cold $H_2O$ to make thin paste, add 100 mL boiling $H_2O$, and boil ca 1 min while stirring.

(**c**) *Bromocresol green indicator*.—Dissolve 0.1 g tetrabromo-*m*-cresolsulfonphthalein in 1.5 mL 0.1*N* NaOH, and dil. to 100 mL with $H_2O$.

***B. Determination***

Place 2 g sample in 300 mL erlenmeyer and add 10 mL $HNO_3$ and 5 mL $H_2SO_4$. Digest on hot plate to white fumes. If soln darkens, owing to org. matter, cool slightly, add little more $HNO_3$, and digest again to white fumes, repeating operation if necessary until org. matter appears to be destroyed. Cool, add 50 mL $H_2O$, boil ca 1 min, and cool to room temp.

Add bromocresol green, then $NH_4OH$ until indicator changes to light green (pH 4). Cool again to room temp., and if indicator changes back to more acid color, add $NH_4OH$ dropwise until indicator becomes light green again, avoiding excess. Add 2 g *$NH_4HF_2$* (toxic; see safety notes on toxic dusts), mix well, and let stand ca 5 min. Add 8–10 g KI, mix well, and titr. with std $Na_2S_2O_3$ soln to light yellow. Add ca 1 mL starch soln and continue titrg slowly until color is nearly same as just before addn of the KI and becomes no darker on standing 20 sec. Report as % Cu.

Ref.: JAOAC **25,** 77,352(1942).

CAS-7440-50-8 (copper)

## 967.01 Iron in Fertilizers
### Titrimetric Method
### First Action 1967
### Final Action 1969

(*Note:* Diphenylamine may be harmful. See safety notes on mercury salts and toxic dusts.)

***A. Reagents***

(**a**) *Diphenylamine soln*.—Dissolve 1 g in 100 mL $H_2SO_4$.

(**b**) *Diphenylamine sulfonate soln*.—Dissolve 0.5 g in $H_2O$ in 100 mL vol. flask and dil. to vol.

(**c**) *Potassium dichromate std solns*.—0.1*N* and 0.01*N*. Prep. 0.1*N* $K_2Cr_2O_7$ as in **949.13C**. Prep. 0.01*N* soln by dilg 100 mL 0.1*N* soln to 1 L.

(**d**) *Mercuric chloride saturated soln*.—Shake $HgCl_2$ with $H_2O$ and let settle.

(**e**) *Stannous chloride soln*.—Dissolve 20 g $SnCl_2.2H_2O$ in 20 mL HCl, warming gently. Add 20 mL $H_2O$ and dil. to 100 mL with HCl (1+1). Keep warm until clear; then add few granules Sn. Dispense from dropping bottle.

***B. Preparation of Sample Solution***

(**a**) *Suitable for all fertilizers*.—Treat 1 g as in **957.02B(e)**, using 15 mL $HClO_4$. Hold ≥1 hr at ca 170° to remove $HNO_3$ completely. Dil. to 200 mL.

(**b**) *Suitable for soluble salts and oxides*.—Dissolve 1 g in 10 mL HCl, warming gently. Dil. to 200 mL.

***C. Reduction***

Heat aliquot of sample soln (100 mL and 50 mL, resp., for samples contg <0.5 and 0.5–4.0% Fe) to bp. Add few drops diphenylamine sulfonate soln; then $SnCl_2$ soln dropwise until violet color is discharged and 2 drops excess. (Usually 1–6 drops are required. Larger amt may be used with samples contg large amt of Fe.) If reduction does not occur, discard and proceed as follows with second aliquot:

Add few granules Zn, boil few min, and either filter off excess Zn, washing with hot $H_2O$, or let Zn dissolve. Heat to bp and finish reduction with $SnCl_2$ and diphenylamine sulfonate indicator as before. Add 10 mL HCl (1+1). Adjust vol. to 75–110 mL with $H_2O$. Cool rapidly in cold $H_2O$. Add 10 mL satd $HgCl_2$ soln, swirl gently, add 5 mL $H_3PO_4$, and titr. immediately. (Small amt of HgCl must ppt to ensure complete reduction.)

***D. Titration***

Add 1 drop diphenylamine indicator by pipet (no more; excess will interfere with end point if amt of Fe is small). Titr. with 0.01*N* $K_2Cr_2O_7$ soln. Since end point may be difficult to see with very small amt Fe, approach end point slowly, allowing few sec for color to develop. Titr. to permanent blue (sometimes green with very small amt Fe). For samples contg >4% Fe, use 0.1*N* $K_2Cr_2O_7$ for titrn. 1 mL 0.1*N* $K_2Cr_2O_7$ = 0.00558 g Fe; 1 mL 0.01*N* = 0.000558 g Fe.

Ref.: JAOAC **50,** 397(1967).

CAS-7439-89-6 (iron)

## 980.01 Iron in Fertilizers
### Atomic Absorption Spectrophotometric Method
### Final Action

*See* **965.09**.

## 983.03 Iron (Chelated) in Iron Chelate Concentrates
### Atomic Absorption Spectrophotometric Method
### First Action 1983

(Applicable to Fe ethylenediaminetetraacetate (EDTA), Fe hydroxyethylethylenediaminetriacetate (HEDTA), Fe diethylenetriaminepentaacetate (DTPA), Fe ethylenediaminedi-*o*-hydroxyphenylacetate (EDDHA), and Fe citrate. Not applicable to mixed fertilizers, or to samples contg non-chelated metals other than Fe.)

***A. Principle***

Sample is dissolved in $H_2O$, and non-chelated Fe is pptd as $FeOH_3$ at pH 8.5 and removed. Chelated Fe is detd by AAS, using std solns contg $Na_2H_2EDTA$.

### B. Apparatus and Reagents

(**a**) *Sodium hydroxide soln.*—0.5N. Dissolve 20 g NaOH in $H_2O$ and dil. to 1 L.

(**b**) *Disodium EDTA soln.*—0.66%. Dissolve 0.73 g $Na_2H_2EDTA.2H_2O$ in $H_2O$, and dil. to 100 mL.

(**c**) *Iron std solns.*—(*1*) *Stock soln.*—1000 μg Fe/mL. Dissolve 1.000 g pure Fe wire in ca 30 mL 6N HCl with boiling. Dil. to 1 L. (*2*) *Intermediate soln.*—100 μg Fe/mL. Pipet 10 mL Fe stock soln and 10 mL $Na_2H_2EDTA$ soln into 100 mL vol. flask and dil. to vol. with $H_2O$. (*3*) *Working solns.*—Dil. aliquots of intermediate soln with 0.5N HCl to make ≥4 std solns within range of detn (2–20 μg Fe/mL).

(**d**) *Atomic absorption spectrophotometer.*—With air-$C_2H_2$ flame. See **965.09A(a)**.

### C. Determination

Weigh sample contg ca 40 mg Fe into 200 mL tall-form beaker. Wet with 2–3 drops of alcohol and dissolve in 100 mL $H_2O$. Add 4 drops of 30% $H_2O_2$, mix and adjust pH of soln to 8.5 with 0.5N NaOH. If pH drifts above 8.8, discard soln and repeat analysis. Transfer soln to 200 mL vol. flask, dil. to vol. with $H_2O$, and mix. Filter soln thru quant. paper. Pipet 10 mL filtrate into 200 mL vol. flask and dil. to vol. with 0.5N HCl. Measure *A* of solns, using lean air-$C_2H_2$ flame as in **965.09D** and det. concn of Fe in sample (μg Fe/mL) from either calibration curve or digital concn readout. In same manner, det. Fe blank on all reagents used.

% Chelated iron = (μg Fe/mL in sample − μg Fe/mL in blank) × 0.4/g sample

Refs.: JAOAC **66,** 952(1983); **69,** 280(1986).

## 984.01 Magnesium (Acid-Soluble) in Fertilizers

**Atomic Absorption Spectrophotometric Method**

**Final Action**

*See* **965.09**.

## 964.01 Magnesium (Acid-Soluble) in Fertilizers

**EDTA Titration Method**

**First Action 1964**
**Final Action 1965**

(Applicable to samples contg ≤0.25% Mn or Zn)

### A. Reagents

Use reagents **962.01A(a)**, (**b**), (**c**), (**d**), (**f**) (1 mL = 1 mg Ca, equiv. to 0.6064 mg Mg), (**g**), (**h**) (stdzd as in **964.01B**), and in addn:

(**a**) *Triethanolamine soln.*—(1+1).

(**b**) *Potassium ferrocyanide soln.*—Dissolve 4 g $K_4Fe(CN)_6.3H_2O$ in 100 mL $H_2O$.

(**c**) *Ferric ammonium sulfate soln.*—Dissolve 136 g $FeNH_4(SO_4)_2.12H_2O$ in $H_2O$ contg 5 mL $H_2SO_4$, and dil. to 1 L. Filter if not clear.

### B. Standardization

Pipet 10 mL Ca std soln into 300 mL erlenmeyer. Add 100 mL $H_2O$, 10 mL KOH-KCN soln, 2 drops triethanolamine soln, 5 drops $K_4Fe(CN)_6$ soln, and 15±1 mg calcein indicator. Immediately place flask on mag. or other mech. stirrer in front of daylight fluorescent light and white background. While stirring, titr. with EDTA soln to disappearance of all fluorescent green and until soln remains pink. Titr. ≥3 aliquots. From av., calc. Ca titer in mg/mL EDTA soln. Ca titer × 0.6064 = Mg titer in mg/mL.

### C. Preparation of Solution

(*Caution*: See safety notes on wet oxidation and perchloric acid.)

(**a**) *Organic materials.*—Weigh 1 g sample into 250 mL boiling flask or erlenmeyer. Add 5 mL HCl and 10 mL $HNO_3$, and boil on hot plate or over low flame until easily oxidized org. matter is destroyed (ca 15 min). Cool, add 5 mL 70–72% $HClO_4$, and heat to appearance of copious fumes and momentary cessation of boiling, but not to dryness. Cool, and transfer to 250 mL beaker with ca 100 mL $H_2O$. Continue with pH adjustment, as in **964.01D**.

(**b**) *Inorganic materials and mixed fertilizers.*—Weigh 1 g sample into 250 mL beaker. Add 5 mL HCl and 10 mL $HNO_3$. Cover with watch glass and heat on asbestos mat on hot plate nearly to dryness (ca 30 min). If soln remains colored from org. residues, cool, add 5 mL $HClO_4$ (70–72%), and continue heating to copious fumes and momentary cessation of boiling, but not to dryness.

### D. Determination

Cool prepd soln to room temp. Wash watch glass and inside of beaker to ca 100 mL with $H_2O$. Using pH meter with glass electrode and mech. stirring, adjust to ca pH 3 with 30% KOH soln and finally to pH 4.0 with 10% KOH soln. Add $FeNH_4(SO_4)_2$ soln, 5 mL for sample <7% $P_2O_5$, 10 mL for sample 7–15% $P_2O_5$, 15 mL for sample 16–30% $P_2O_5$, and proportionate amts for samples >30% $P_2O_5$. Adjust to pH 5.0 with KOH solns as above, or with HCl (1+4) if pH is >5.0. Cool to room temp. and transfer to 250 mL vol. flask with $H_2O$. Dil. to vol. with $H_2O$ and mix. Let stand until ppt settles. Disturbing ppt as little as possible, filter enough soln for aliquots required for titrn thru dry 11 cm fluted paper, Whatman No. 1, or equiv.

Pipet two equal aliquots contg <15 mg Ca + Mg (usually 25 mL) into two 300 mL erlenmeyers and dil. each to 100 mL with $H_2O$. To one (titrn *1* for Ca + Mg) add 5 mL pH 10 buffer soln, 2 mL KCN soln, 2 drops triethanolamine soln, 5 drops $K_4Fe(CN)_6$ soln, and 8 drops eriochrome black T indicator. Titr. immediately with EDTA soln, stirring and lighting as in stdzn. Color changes are wine red, purple, dark blue, to clear pure blue end point, becoming green if overtitrd.

To second aliquot (titrn *2* for Ca) add 10 mL KOH-KCN soln, 2 drops triethanolamine soln, 5 drops $K_4Fe(CN)_6$ soln, and 15±1 mg calcein indicator. Titr. immediately with EDTA soln as in stdzn.

(Titrn *1* − Titrn *2*) × Mg titer EDTA × 100/mg sample in aliquot = % Mg

Titrn *2* × Ca titer EDTA × 10/mg sample in aliquot = % Ca

Ref.: JAOAC **47,** 450(1964).

CAS-7439-95-4 (magnesium)

## 937.01★ Magnesium (Acid-Soluble) in Fertilizers

**Gravimetric Method**

**Final Action**
**Surplus 1970**

*See* **2.123**, 11th ed.

## 940.01★ Magnesium (Acid-Soluble) in Fertilizers

### Volumetric Method

**Final Action**
**Surplus 1970**

*See* **2.124**, 11th ed.

## 937.02 Magnesium (Water-Soluble) in Fertilizers

**Final Action 1966**

(**a**) *In potassium-magnesium sulfate, magnesium sulfate, and kieserite*.—Weigh 1 g sample into 250 mL vol. flask, add 200 mL $H_2O$, and boil 30 min. Cool, dil. to vol. with $H_2O$, and mix. If detn is to be conducted gravimetrically, **937.01★**, or volumetrically, **940.01A★**, *see* **2.125**, 11th ed.

(**b**) *In other materials, including mixed fertilizers*.—Weigh 1 g sample into 500 mL vol. flask, add 350 mL $H_2O$, and boil 1 hr. Cool, dil. to vol., mix, and filter if necessary. If detn is to be conducted gravimetrically, **937.01★**, or volumetrically, **940.01A★**, *see* **2.125**, 11th ed.

(**c**) *By EDTA method*.—Transfer aliquot soln prepd as in (**a**) or (**b**) to beaker and det. Mg as in **964.01D**, using HCl or KOH to adjust pH.

Refs.: JAOAC **20,** 252(1937); **22,** 270(1939); **23,** 249(1940); **24,** 268(1941); **25,** 326(1942).

## 972.02 Manganese (Acid-Soluble) in Fertilizers

### Atomic Absorption Spectrophotometric Method

**First Action 1972**
**Final Action 1974**

(**a**) *Applicable to* $Mn^{+2}$ *only*.—Prep. sample soln as in **940.02**, omitting the 50 mL $H_3PO_4$ (1+9). Proceed as in **965.09D**, using std solns prepd as in **965.09B(f)** and (**h**), substituting 0.5$N$ $H_2SO_4$ for 0.5$N$ HCl in **965.09B(h)**.

(**b**) *Applicable to total* $Mn^{+2}$ *and* $Mn^{+4}$.—Prep. sample soln as in **965.09C**. Proceed as in **965.09D**, using std solns prepd as in **965.09(f)** and (**h**).

Ref.: JAOAC **55,** 695(1972).

## 940.02 Manganese (Acid-Soluble) in Fertilizers

### Colorimetric Method

**Final Action**

(Applicable to samples contg $Mn^{+2}$ only and with ≤5% Mn)

### *A. Reagent*

*Potassium permanganate std soln*.—500 ppm Mn. Prep. and stdze as in **940.35**, except use 1.4383 g $KMnO_4$ and 0.12 g Na oxalate. Transfer aliquot contg 20 mg Mn to beaker. Add 100 mL $H_2O$, 15 mL $H_3PO_4$, and 0.3 g $KIO_4$, and heat to bp. Cool, and dil. to 1 L. Protect from light. Dil. this soln contg 20 ppm Mn with $H_2O$ (previously boiled with 0.3 g $KIO_4$/L) to make convenient working stds in range of concns to be compared.

### *B. Determination*

Place 1 g sample in 200 mL wide-neck vol. flask or 250 mL beaker. Add 10 mL $H_2SO_4$ and 30 mL $HNO_3$. Heat gently until brown fumes diminish; then boil 30 min. If org. matter is not destroyed, cool, add 5 mL $HNO_3$, and boil. Repeat process until no org. matter remains, and boil until white fumes appear. Cool slightly, and add 50 mL $H_3PO_4$ (1+9). Boil few min. Cool, dil. to 200 mL in vol. flask, mix, and let stand to allow pptn of $CaSO_4$.

Pipet 50 mL clear soln into beaker. Heat nearly to bp. With stirring or swirling, add 0.3 g *$KIO_4$* for each 15 mg Mn present, and hold 30–60 min at 90–100°, or until color development is complete. Cool, and dil. to measured vol. that will provide satisfactory concn for colorimetric measurement by instrument chosen (usually <20 ppm Mn). Compare in colorimeter against std $KMnO_4$ soln, or in spectrophtr at 530 nm. Calc. to Mn.

Ref.: JAOAC **23,** 249(1940).

CAS-7439-96-5 (manganese)

## 941.02★ Manganese (Acid-Soluble) in Fertilizers

### Bismuthate Method

**Final Action**
**Surplus 1970**

(Applicable to $Mn^{+2}$ only)

*See* **2.127–2.128**, 11th ed.

## 972.03 Manganese (Water-Soluble) in Fertilizers

### Atomic Absorption Spectrophotometric Method

**First Action 1972**
**Final Action 1974**

(Applicable to $Mn^{+2}$ only)

Place 1 g sample in 50 mL beaker, wet with alcohol, add 20 mL $H_2O$, and let stand 15 min, stirring occasionally. Transfer to 9 cm Whatman No. 5 paper, and wash with small portions $H_2O$ until filtrate measures ca 230 mL. Let each portion pass thru paper before adding more. Add 3–4 mL $H_2SO_4$ to filtrate. Proceed as in **965.09D**, using std solns prepd as in **965.09B(f)** and (**h**), substituting 0.5$N$ $H_2SO_4$ for 0.5$N$ HCl in **965.09B(h)**.

Ref.: JAOAC **55,** 695(1972).

## 974.01 Sodium in Fertilizers

### Flame Photometric Method

**First Action 1974**

### *A. Reagents*

*See* **955.06★**, and in addn:
*Sodium chloride*.—Dry 2 hr at 105°.

### *B. Preparation of Solution*

Prep. soln as in **955.06★**, using 2.5 g sample (<4% Na) or 1.25 g (4–20% Na).

### *C. Preparation of Standard Curve*

(**a**) *Samples containing 1% or more sodium*.—Proceed as in **955.06★**, using 1.2711 g NaCl, range of diln 0–40 ppm Na, intervals ≤5 ppm, and full scale for 40 ppm Na.

(**b**) *Samples containing less than 1% sodium*.—Proceed as in **955.06★**, using 1.2711 g NaCl, range of diln 0–10 ppm Na, intervals 2 ppm, and full scale for 10 ppm Na.

#### D. Determination

Transfer 25 mL (<4% Na) or 10 mL (4–20% Na) sample soln to 250 mL vol. flask, dil. to vol. with $H_2O$, and mix (if internal std instrument is used, add required amt $LiNO_3$ before dilg to vol.). Atomize portions of sample several times to obtain reliable av. readings for each soln. Det. ppm Na from std curve (**a**) or (**b**). Calc. % Na as follows:

$$0\text{–}4\%: \text{ppm Na}/10 = \%\ \text{Na}$$
$$4\text{–}20\%: \text{ppm Na}/2 = \%\ \text{Na}$$

Refs.: JAOAC **55,** 986(1972); **56,** 859(1973); **57,** 1402(1974).

CAS-7440-23-5 (sodium)

## 983.04 Sodium in Fertilizers

### Atomic Absorption Spectrophotometric Method

### First Action 1983

#### A. Reagents and Apparatus

(**a**) *Ammonium oxalate soln.*—Dissolve 40 g $(NH_4)_2C_2O_4$ in 1 L $H_2O$.

(**b**) *Sodium chloride.*—Dry 2 h at 105°.

(**c**) *Atomic absorption spectrophotometer.*—Model AA6 (replacement model is Spectr-AA Series, Varian Instrument Group), or equiv.

#### B. Preparation of Solution

Weigh 2.5 g (<4% Na) or 1.25 g (4–20% Na) sample into 250 mL vol. flask, add 125 mL $H_2O$ and 50 mL $(NH_4)_2C_2O_4$ soln, and boil 30 min. Cool, dil. to vol., mix, and pass thru dry filter. For samples contg <1% Na, use this soln for detn. For samples contg 1–20% Na, place 20 mL in 100 mL vol. flask, dil. to vol. with $H_2O$, and mix.

#### C. Preparation of Standard Curve

Dissolve 2.5421 g dried NaCl in $H_2O$ and dil. to 1 L (1000 ppm Na). Prep. std solns to cover range 0–200 ppm at intervals ≤20 ppm Na.

#### D. Determination

Set wavelength at 330.3 nm using air-$C_2H_2$ flame. Aspirate stds and samples. Plot curve from std values and det. Na content of sample solns from plot of *A* against ppm Na. Calc. % Na as follows:

$$\leq 1\%: \text{ppm Na} \times 25/M = \%\text{Na}$$
$$1\text{–}20\%: \text{ppm Na} \times 125/M = \%\text{Na}$$

where $M$ = wt of sample (mg).

Ref.: JAOAC **66,** 1234(1983).

CAS-7440-23-5 (sodium)

## 980.02 Sulfur in Fertilizers

### Gravimetric Method

### First Action 1980
### Final Action 1985

(*Caution:* See safety notes on sodium hydroxide and peroxides.)

(**a**) *Total sulfur (sulfate and elemental) in dry fertilizers.*—Accurately weigh sample contg 100–150 mg S into 400 mL beaker, add 200 mL $H_2O$, 15 mL HCl, heat to bp, and boil gently ca 10 min. Filter thru gooch crucible contg glass fiber paper and wash with hot $H_2O$. Set washed crucible aside.

Quant. transfer filtrate back to beaker and bring nearly to bp. Add slowly, with const stirring, slight excess (ca 15 mL) 10% $BaCl_2$ soln. Digest on low temp. hot plate adjusted so that soln does not boil, or on steam bath 1 hr, and let stand at room temp. overnight. Filter thru gooch crucible contg glass fiber paper previously dried at 250°, cooled, and weighed. Wash with ten portions hot $H_2O$, dry crucible and contents 1 hr at 250°, cool to room temp., and weigh.

$$\%\ \text{Sulfate S} = \text{g } BaSO_4 \times 0.1374 \times 100/\text{g sample}$$

Wash insol. residue with five 10 mL portions acetone satd with S, dry 1 hr at 100°, cool, and weigh. Wash residue with three 5 mL portions $CS_2$, drain, dry 1 hr at 100°, cool in desiccator, and weigh. Difference in wt = elemental S ($S^0$).

Perform blank on $S^0$ portion of method by weighing 5.0 g fertilizer sample contg no $S^0$ into 400 mL beaker and proceeding as above, beginning "add 200 mL $H_2O$, . . . . Set washed crucible aside." Omit sulfate S detn and continue, beginning "Wash insol. residue with five. . . ." Correct difference in wt (= elemental S) for blank found.

$$\%\ S^0 = \text{g S} \times 100/\text{g sample}$$
$$\%\ \text{Total S} = \%\ \text{Sulfate S} + \%\ S^0$$

(If $S^0$ is ≤5%, use 5 g sample and repeat $S^0$ portion of detn.)

(**b**) *Total sulfur (sulfate, sulfite, thiosulfate, and sulfide) in liquid fertilizers.*—Accurately weigh sample contg 100–150 mg S into 400 mL beaker. Add 50 mL $H_2O$, 2 mL 50% NaOH, and 2 mL 30% $H_2O_2$. Cover with watch glass and reflux 1 hr, adding 1 mL increments $H_2O_2$ (≤5 mL total) as reaction subsides. After 1 hr, wash watch glass and remove. Dil. to ca 175 mL with $H_2O$, acidify with ca 10 mL HCl (1+1), and bring to bp. Proceed as in (**a**), beginning "Add slowly, with constant stirring, . . ."

$$\%\ \text{Total S} = \text{g } BaSO_4 \times 0.1374 \times 100/\text{g sample}$$

(**c**) *Total sulfur in sulfur-coated urea and elemental sulfur formulations.*—Accurately weigh sample contg 200–300 mg S into 125 mL g-s erlenmeyer, add ca 50 mL $H_2O$, stopper, and shake vigorously 30 sec. Filter quant. with suction thru gooch crucible contg glass fiber paper and wash with $H_2O$. Proceed as in (**a**), beginning "Wash insol. residue with five 10 mL portions . . ."

$$\%\ S^0 = \text{g S} \times 100/\text{g sample}$$

Refs.: JAOAC **63,** 854(1980); **64,** 420(1981).

CAS-7704-34-9 (sulfur)

## 942.02★ Zinc in Fertilizers

### Gravimetric Method

### Final Action 1976
### Surplus 1976

(For samples contg ≥0.1% Zn)

*See* **2.138**, 12th ed.

## 942.03★ Zinc in Fertilizers

### Colorimetric Method

### Final Action 1976
### Surplus 1976

(For samples contg <4% Zn)

*See* **2.139**, 12th ed.

## 975.02 Zinc in Fertilizers

**Atomic Absorption Spectrophotometric Method**
**Final Action 1976**

*See* **965.09**.

## 973.01 Zinc in Fertilizers

**Zincon Ion Exchange Method**
**First Action 1973**
**Final Action 1976**

(Clean all glassware with hot chromic acid or $HNO_3$ (1 + 1). Rinse thoroly with $H_2O$. *Caution*: See safety notes on chromic and perchromic acids and nitric acid.)

### *A. Reagents*

(**a**) *Anion exchange resin*.—100–200 mesh, strong base, polystyrene alkyl quaternary amine, 7% cross linkage.

(**b**) *Zincon indicator*.—Dissolve 0.12 g zincon (*o*-[[α-[(2-hydroxy-5-sulfophenyl)azo]benzylidene]hydrazino]benzoic acid, Na salt) (J.T. Baker Inc., No. X690) in 5 mL 0.3*N* NaOH and dil. to 100 mL with $H_2O$. Prep. fresh weekly.

(**c**) *Hydrochloric acid solns*.—(*1*) *0.5N*.—Dil. 20 mL HCl to 500 mL with $H_2O$. (*2*) *0.25N*.—Dil. 2 mL HCl to 100 mL with $H_2O$. (*3*) *0.005N*.—Dil. 2.5 mL HCl to 6 L with $H_2O$.

(**d**) *Borate buffer soln*.—pH 9.8. Dissolve 4 g $H_3BO_3$ in 140 mL $H_2O$. Add 5 mL $NH_4OH$ by pipet and then dropwise to pH 9.8. Check daily.

(**e**) *Ammonium thiocyanate*.—1*M*. Dissolve 0.76 g $NH_4CNS$ in 10 mL $H_2O$.

(**f**) *Zinc std solns*.—(*1*) *Stock soln*.—1000 ppm. Dissolve 1.000 g pure Zn metal in small amt HCl-$HNO_3$ (1+1). Evap. to small vol., add 3 mL HCl, and heat. Dil. to 1 L with $H_2O$. (*2*) *Working soln*.—10 ppm. Dil. 10 mL stock soln to 1 L with $H_2O$.

(**g**) *Sodium hydroxide soln*.—0.3*N*. Dissolve ca 1.25 g NaOH in 100 mL $H_2O$.

### *B. Preparation of Resin Column*

Wash 12 g new resin in 250 mL beaker with $H_2O$ until washings are neut. Introduce resin as slurry into 25 × 2.2 cm chromatgc tube with fritted glass disk and stopcock at bottom. Mark vol. levels on column at 10, 40, and 50 mL above packed resin and on 250 mL separator at 90 mL. (Keep resin wet and store under liq. when not in use.) Connect separator to top of column thru stopper. Attach inverted U-shaped glass dispensing tube to 250 mL vol. flask thru vented stopper or cork and connect with Zn-free plastic tubing to stopcock of column with stopcock grease. *See* Fig. **973.01**.

Mount reservoir (aspirator bottle or carboy) contg ≥ 1 L 0.005*N* HCl high enough to effect backwashing. Attach Zn-free tubing and pinch clamp.

### *C. Flow Calibration*

Use sweep sec hand of watch or stopwatch to establish flow rates. Det. number drops/mL leaving dispensing tube. Remove separator and vol. flask; drain and then backwash resin (*see* **973.01E**). Remove reservoir tubing, open stopcock, elute 40 drops from dispensing tube, and measure vol. Use this factor to convert 0.5 mL/min (required in Zn elution, **973.01E**) to drops/sec.

### *D. Preparation of Sample*

Remove separator and elution tubing from column. Activate resin by draining column and adding 50 mL 0.5*N* HCl. Drain column to 40 mL mark.

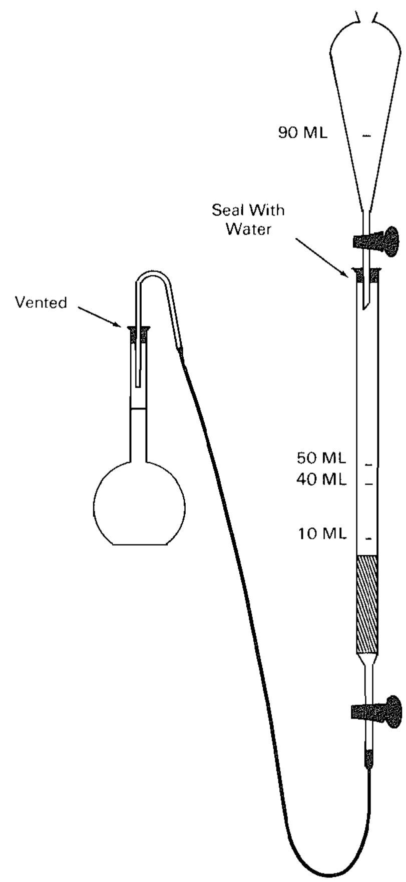


**Fig. 973.01—Apparatus for elution of resin column**

(**a**) *Samples containing 0.14% or more zinc*.—Dissolve 1.000 g well-ground sample in 10 mL HCl and 5 mL $HNO_3$ in 250 mL beaker. Evap. to near dryness on hot plate. (*Caution: Do not* bake.) Redissolve residue in ca 40 mL 0.5*N* HCl, boiling gently if necessary. Filter thru Whatman No. 41 paper into 100 mL vol. flask. Thoroly wash residue and dil. filtrate to vol. with 0.5*N* HCl. Drain column to 10 mL mark. Tap column to pack resin. Pipet aliquot contg 0.7–0.8 mg Zn onto column. Elute sample soln at ca 5 sec/drop.

(**b**) *Samples containing less than 0.14% zinc*.—Weigh, to nearest mg, sample contg 0.7–0.8 mg Zn into 250 mL beaker. Digest and filter sample and prep. column as in (**a**). Tightly attach open separator to column. Close stopcock. Transfer entire sample soln to separator, rinsing with two 10 mL portions 0.5*N* HCl. Open stopcock. Elute sample soln at ca 5 sec/drop. Remove empty separator, rinse twice with 20 mL 0.5*N* HCl, and add rinses to remaining soln in column.

### *E. Elution of Zinc*

After sample soln passes thru resin, immediately rinse column with 0.5*N* HCl at ca 1.5 sec./drop until 1 mL eluate gives clear, colorless soln with 1*M* $NH_4SCN$. If $Fe^{+3}$ is present, soln will turn brown. Drain resin and backwash with 0.005*N* HCl from reservoir thru elution tubing, forcing out air bubbles from tubing and column. Simultaneously, tap resin into suspension as it is forced up. Close column stopcock when liq. reaches 50 mL mark on column. Attach dispensing tube to vol. flask. Reopen stopcock, and raise flask until flow just stops. Continue ht adjustment until a drop remains in equilibrium at tip of dispensing tube and neither rises nor falls. Secure flask. Attach open separator with $H_2O$ seal to column and close stopcock. Add 240 mL 0.005*N* HCl to separator and reopen stopcock. Open column stopcock until rate of ca 0.5 mL/min is sustained 10 min. If rate decreases, increase rate slightly until nearly const. Let elution continue overnight. Then, if >90 mL remains in separator, readjust rate as above and continue elution to 90 mL mark. Finally, lower flask, fill to 250 mL mark at convenient rate from dispensing tip, and mix. Detach hose and separator from column, and drain all 3. Reactive resin, and stopper column as in **973.01D**.

***F. Determination***

Pipet 20 mL eluate into 50 mL vol. flask contg small piece litmus paper. Make alk. with 0.3*N* NaOH, then just acidic with 0.25*N* HCl. Pipet in 2 mL more acid, 5 mL buffer, and 3 mL zincon soln. Dil. to vol. with $H_2O$. Similarly prep. 0, 1, 2, and 3 ppm std solns, using 0, 5, 10, and 15 mL std Zn working soln, resp. Using 0 ppm std soln as blank, det. *A* at 620 nm 15–45 min after zincon addn. Plot std curve of ppm against *A*.

$$\% \text{ Zn} = (C \times F)/W$$

where $C$ = ppm from std curve; $W$ = g sample; and $F$ = 0.0625 for samples contg <0.14% Zn or 6.25/mL aliquot pipetted onto resin for samples contg ≥0.14% Zn.

Ref.: JAOAC **56,** 846(1973).

CAS-7440-66-6 (zinc)

## 936.01★ Acid-Forming or Nonacid-Forming Quality of Fertilizers

**Final Action**
**Surplus 1970**

*See* **2.141–2.142**, 11th ed.

## 983.05 Aluminum in Aluminum Sulfate-type Soil Acidifiers

**Atomic Absorption Spectrophotometric Method**
**First Action 1983**

***A. Apparatus and Reagents***

(**a**) *Atomic absorption spectrophotometer*.—Perkin-Elmer Model 303, or equiv. See **972.06A** for typical operating parameters.

(**b**) *Diluting soln*.—Add 20 mL $H_2SO_4$ and 2.5 g NaCl to 500 mL $H_2O$. Dil. to 1 L with $H_2O$.

(**c**) *Aluminum std solns*.—(*1*) *Stock soln*.—1 mg Al/mL (1000 ppm). Accurately weigh 1.000 g pure Al and dissolve in ca 25 mL HCl. Evap. almost to dryness, add 500 mL $H_2O$, 20 mL $H_2SO_4$, and 2.5 g NaCl, and dil. to 1 L with $H_2O$. (*2*) *Working solns*.—Dil. aliquots of stock soln with dilg soln, (**b**), to make 4 std solns within range 50–150 ppm.

***B. Preparation of Sample***

Accurately weigh ca 1 g sample into 500 mL screw-cap erlenmeyer, add 250 mL $H_2O$, and shake on wrist-action shaker ca 15 min. Quant. transfer to 500 mL vol. flask and dil. to vol. with $H_2O$. Filter thru Whatman No. 2 paper. Dil. aliquot as necessary (diln factor = *F*) with dilg soln, (**b**), for concn range 50–150 ppm.

***C. Determination***

Set up app. as shown in Table **983.05**, or use previously established optimum settings for app. Zero app. while aspirating dilg soln, (**b**). Det. *A* of std solns within 50–150 ppm range, alternating with sample soln readings. Flush burner with dilg soln, (**b**), and check zero point between readings.

**Table 983.05 Operating Parameters**

| | |
|---|---|
| Wavelength, nm | 309.3 |
| Slit width, mm | 1 |
| Source, ma | 30 |
| $N_2O$, aspirating | 4.5 (scale divisions) |
| $N_2O$, auxiliary and aspirating | 5.5 (scale divisions) |
| $C_2H_2$ fuel | 6 (metal ball scale division) |
| Flame | reducing |
| Ht, burner to light path, in. | 1/8 |
| Sample uptake, mL/min | 4 |
| Optimum concn range, μg/mL | 50–150 |

Det. Al content from std curve of *A* against μg Al/mL.

$$\% \text{ Al} = (\mu g \text{ Al/mL}) \times F \times (500/\text{g sample}) \times 10^{-4}$$

Ref.: JAOAC **66,** 946(1983).

CAS-7429-90-5 (aluminum)

# PEAT

## 973.02 Sampling of Peat

**Procedure 1973**

(Moss, humus, and reed-sedge types)

Use slotted single or double tube or slotted tube and rod, all with pointed ends and min. 1″ diam. for loose materials. Use cutting type core sampler, with plunger, for compressed materials. Pennsylvania State Forage Sampler (NASCO, 901 Janesville Ave, Fort Atkinson, WI 53538) is satisfactory core sampler.

Take representative sample from lot or shipment as follows:

(**a**) *Packaged or baled peats*.—Lay bag or bale horizontally and remove core diagonally from end to end. From lots of 1–10 bags, sample all bags; from lots of ≥11, sample 10 bags. Take 1 core from each bag sampled; except for lots of 1–4 bags, take diagonal cores from each bag and addnl cores to total ≥5 cores.

(**b**) *Bulk samples*.—Draw ≥10 cores from different regions.

(**c**) *Small containers (10 lb or less)*.—Take entire package. Working rapidly to prevent moisture losses, reduce composite sample to ≤500 g (by wt) or 2 L (by vol.) by mixing on clean plastic or paper and quartering. Place sample in air-tight container.

Sampling by random "grab" procedure is necessary if particle size range is to be detd or if representative sample cannot be taken with core sampler as above.

Refs.: Book of ASTM Stds (1971) Pts 11, 22, and 30, ASTM D2973-D2978, D2980, and D2944. JAOAC **56,** 154(1973).

## 967.02 Preparation of Peat Sample

**First Action 1967**
**Final Action 1978**

Place representative field sample on square rubber sheet, paper, or plastic. Reduce sample to amt required by quartering and place in moisture-proof container. *Work rapidly to prevent moisture losses.*

Ref.: JAOAC **50,** 394(1967).

## 967.03 Moisture in Peat

**First Action 1967**
**Final Action 1978**

### *A. Method I*

Mix sample thoroly and place 10–12 g in ignited and weighed (with fitted heavy-duty Al foil cover) Vycor or porcelain evapg dish, ≥75 mL capacity. Crush soft lumps with spoon or spatula. Cover immediately with Al foil cover and weigh to nearest mg. Dry, uncovered, 16 hr at 105°. Remove from oven, cover tightly, cool, and weigh.

% Moisture (report to nearest 0.1%)
= (g as-received sample − g oven-dried sample) × 100/g as-received sample

### *B. Method II*

(Use when pH, N, fiber, etc., are to be detd.)

Mix thoroly and weigh 100–300 g representative sample, **967.02**, and spread evenly on large flat pan. Crush soft lumps with spoon or spatula and let come to moisture equilibrium with room air ≥24 hr. Stir occasionally to maintain max. air exposure of entire sample. When wt is const, calc. loss in wt as % moisture removed by air drying. Grind representative portion air-dried sample 1–2 min in high-speed blender; use for moisture, ash, and N detns.

Mix air-dried, ground sample and weigh, to nearest mg, equiv. of 10 g sample on as-received basis (g air-dried sample equiv. to 10.0 g as-received sample = 10.0 − [(10.0 × % moisture removed)/100]). Place weighed sample in ignited and weighed (with fitted heavy duty Al foil cover) Vycor or porcelain evapg dish and proceed as in **967.03A**.

% Moisture (report to nearest 0.1%)
= (10.0 − g oven-dried sample) × 10.0

Ref.: JAOAC **50,** 394(1967).

## 973.03 Particle Size Range of Peat

**Mechanical Analysis**
**First Action 1973**

### *A. Apparatus*

*Mechanical sieve shaker.*—With 8″ diam., Nos. 8 and 20 sieves equipped with cover and bottom pan.

### *B. Preparation of Sample*

Air-dry as in **967.03B**.

### *C. Determination*

Mix thoroly and place 20.0 g air-dried sample on No. 8 sieve nested on No. 20 sieve. Secure sieves and shake at suitable speed 10 min. Remove and weigh foreign matter, such as sticks, stones, and glass, from No. 8 fraction. Weigh fractions of peat retained on Nos. 8 and 20 sieves and portion collected in bottom pan. Convert fraction and sample wts to as-received basis and calc. in terms of %. (If foreign matter is absent, conversion to as-received basis is not necessary.)

% Foreign matter = fraction removed from No. 8 sieve × 100
% Coarse fiber = fraction retained on No. 8 sieve × 100
% Medium fiber = fraction retained on No. 20 sieve × 100
% Fines = fraction collected in pan × 100

If mech. sieve shaker is not available, use hand sieving. Conduct sieving by appropriate lateral and vertical motions accompanied by jarring action. Continue until no appreciable change is noted in sieve fraction.

Refs.: Book of ASTM Stds(1971)Pts 11, 22, and 30, ASTM D2973–D2978, D2980, and D2944. JAOAC **56,** 154(1973).

## 973.04 pH of Peat

### *A. Apparatus and Reagents*

(**a**) *pH meter.*—Battery-operated or on elec. line with voltage regulator.
(**b**) *Carbon dioxide-free water.—See* **964.24**.
(**c**) *Acid potassium phthalate buffer soln.*—0.05*m*. *See* **964.24(c)**.
(**d**) *Phosphate buffer soln.*—0.025*m*. *See* **964.24(d)**.
(**e**) *Calcium chloride solns (Method II only).—(1) Stock soln.*—1.0*M*. Dissolve 147 g $CaCl_2.2H_2O$ in $H_2O$ in 1 L vol. flask, cool, dil. to vol., and mix. Dil. 15 mL of this soln to 200 mL with $H_2O$ in vol. flask and stdze by titrg 25 mL aliquot dild soln. with std 0.1*N* $AgNO_3$, **941.18C**, using 1 mL 5% $K_2CrO_4$ as indicator. (*2*) *Working soln.*—0.01*M* (pH 5.0–6.5). Dil. 20 mL stock soln. to 2 L with $H_2O$.

### *B. Determination*

(**a**) *Method I (in distilled water).*—Weigh ca 3.0 g air-dried peat or equiv. amt moist material into 100 mL beaker. Add 50 mL $H_2O$. (Addnl $H_2O$ may be needed for very fibrous materials such as sphagnum moss peat.) Let soak 30 min, with occasional stirring. Read on pH meter.
(**b**) *Method II (in 0.01M calcium chloride soln).*— Weigh ca 3.0 g air-dried peat or equiv. amt moist material into 100 mL beaker. Add 50 mL 0.01*M* $CaCl_2$. Let soak 30 min, with occasional stirring. Read on pH meter. Report results as pH in 0.01*M* $CaCl_2$ soln. (pH values in $CaCl_2$ soln are usually ca 0.5–0.8 units lower than those in $H_2O$. Observed pH in $CaCl_2$ soln is virtually independent of initial amt salt present in soil, whereas pH readings in $H_2O$ can be modified by salts such as fertilizer material.)

Refs.: Book of ASTM Stds(1971)Pts 11, 22, and 30, ASTM D2973–D2978, D2980, and D2944. JAOAC **56,** 154(1973).

## 967.04 Ash of Peat

**First Action 1967**
**Final Action 1978**

Place uncovered (retain cover for weighing) Vycor or porcelain dish contg dried sample from moisture detn in furnace. Gradually bring to 550° and hold until completely ashed. Cover with retained Al foil cover, cool, and weigh.

% Ash (report to nearest 0.1%) = g ash × 100/g as-received sample taken for moisture detn. (If moisture Method II was used, g as-received sample = 10.0.)

Ref.: JAOAC **50,** 394(1967).

## 973.05 Sand in Peat

**First Action 1973**

### *A. Preparation of Sample*

Air-dry as in **967.03B**.

### *B. Determination*

(*Caution:* See safety notes on chloroform.)

Place 25 g air-dried, ground sample into 125 mL tall-form beaker, or equiv. Nearly fill beaker with $CHCl_3$, stir briefly, and let settle ca 1 min. With spoon, discard most floating org. material, decant remaining org. material and $CHCl_3$, taking care not to disturb settled portion (sand), and air-dry to remove residual $CHCl_3$. (Stirring aids drying.)

When dry, weigh settled portion and calc. as % sand (includes other minerals present such as limestone, etc.).

% Sand = (g air-dried settled residue × 100)/g air-dried sample

Refs.: Book of ASTM Stds (1971) Pts 11, 22, and 20, ASTM D2973–D2978, D2980, and D2944. JAOAC **56,** 154(1973).

## 967.05 Organic Matter in Peat

**First Action 1967**
**Final Action 1978**

% Org. matter = 100.0 − (% moisture + % ash)

Ref.: JAOAC **50,** 394(1967).

## 973.06 Nitrogen (Total) in Peat

**First Action 1973**

Det. N as in **955.04**, using well mixed, air-dried, ground sample equiv. to 10.0 g sample on as-received basis.

Det. g air-dried sample equiv. to 10.0 g as-received sample as in **967.03B**.

Refs.: Book of ASTM Stds (1971) Pts 11, 22, and 30, ASTM D2973-2978, D2980, and D2944. JAOAC **56,** 154(1973).

## 969.05 Water Capacity and Volumes for Peat

**First Action 1969**
**Final Action 1978**

### A. Apparatus

*Dispensing apparatus.*—2 dispensing burets, 250 mL in 1 mL subdivisions, ±2 mL tolerance, pinchcock type; 1-hole No. 6 rubber stopper; straight polyethylene drying tube with serrated rubber tubing fittings, 15 cm long, $^3/_4$″ od, $^5/_8$″ id (No. 14782-2, Cenco, Inc., 2600 Kostner Ave, Chicago, IL 60623); and stainless steel screen circle, ca 16 mesh and 28.7 mm diam.

Assemble dispensing app. as follows: Discard serrated rubber tubing fittings from polyethylene drying tube and use tube only. Center stainless steel screen on one end of tube and seal. (Soldering iron is useful.) Adjust length of tube to match convenient graduation of buret; then scallop end without screen to allow for $H_2O$ drainage, and insert into dispensing buret with screen side up.

### B. Preparation of Sample

*See* **967.02**.

### C. Determination

Det. moisture content on sep. sample by **967.03A** or **B**.

Weigh buret fitted with plastic tube and screen. Working rapidly to prevent moisture losses, mix sample thoroly, place on top of No. 4 screen, and shake until sieving is complete. Use only portion that has passed thru sieve for detn. Firmly pack buret with 25 cm (10″) of 4 mesh sample as follows: Attach rubber stopper to delivery end of buret. Add ca 20 mL portions, *firmly* tapping 3 times vertically from ht of 15 cm (6″) on rubber stopper, for final ht of 25 cm. (This will ensure that ht of final wet vol. is 19–25 cm.) Remove stopper; weigh buret to nearest g. Position buret to use sink as drain. Place $H_2O$ source (19 L (5 gal.) bottle) equipped with siphon device above level of buret. Connect clamped rubber tubing of siphon device to buret with glass tubing (ca 13 cm (5″) long, constricted at one end) inserted into one-hole rubber stopper *fitting tightly* into top of buret. Attach rubber tubing with pinch clamp to delivery end of buret. Open both clamps and pass $H_2O$ thru sample ≥24 hr, maintaining water reservoir over sample at all times. (Moss-type samples may float but gradually settle as sample becomes wet.) After initial soaking, regulate $H_2O$ flow thru column by adjusting screw clamp at delivery end of buret. (In-flow of $H_2O$ should be ca equal to out-flow; flow of ca 1 drop/sec is suitable.) When sample is *supersatd,* close both clamps and let sample settle in $H_2O$ ca 5 min. Top surface of sample should be as level as possible.

Raise buret and replace rubber tubing on delivery end of buret with 250 mL dispensing buret filled with $H_2O$, using rubber stopper for connection. Connect two burets tightly, *with no air leaks*. Remove siphon device and open outlet clamps of both burets to empty. (Suction created is equiv. to ca 38 cm (15″) $H_2O$. Check for air leaks to ensure that std suction is exerted on sample. It is important to remove excess $H_2O$ as described.) Measure ht of wet peat. Ht should be 19–25 cm. Record vol. in mL and weigh buret, plastic tube with screen, and wet peat to nearest g.

Wet sample again as above ≥1 hr, drain by suction, record vol., and weigh. Repeat until consistent results are obtained.

### D. Calculations

(**a**) *Saturated Volume Weights, g/mL*

As-recd = g as-recd sample/mL wet vol.

Oven-dried = g dried sample/mL wet vol., where g dried sample = g as-recd sample × [(100 − % moisture)/100]

Wet = g wet sample/mL wet vol.

(**b**) *Water-Holding Capacity, %*

(*1*) *Weight basis:*

As-recd
= [(g wet sample − g as-recd sample) × 100]/g as-recd sample

Oven-dried
= [(g wet sample − g dried sample) × 100]/g dried sample

(*2*) *Volume basis:*

Water vol.
= [(g wet sample − g dried sample) × 100]/(mL wet vol. × 1.0)

(**c**) *Dry Peat Volume, %*

Dry peat vol. = (g dried sample × 100)/(mL wet vol. × 1.5)

(**d**) *Air Volume, %*

Air vol. = 100 − (% water vol. + % dry peat vol.)

Refs.: JAOAC **51,** 1296(1968); **52,** 384(1969).

## 973.07 Volume, Peat

**Alternative Method**
**First Action 1973**

### A. Principle

Method consists of dividing particles of peat from original container by passing them thru 12.7 mm (0.5″) sieve and allowing them to fall into vol.-measuring container.

### B. Apparatus

(**a**) *Sieve.*—No. 1/2″ (12.7 mm).

(**b**) *Measuring box.*—Steel or wood, bound with metal having one of the following sets of inner dimensions: (*1*) 1/2 cu. ft. = 12 × 12 × 12″ with line scribed 6″ from bottom; (*2*) 3/4 cu. ft. = 12 × 12 × 12″ with line scribed 9″ from bottom; (*3*) 1 cu. ft. = 12 × 12 × 12″ box, 2 cu. ft. = 16 × 16 base × 13.5″ ht, 5 cu. ft = 16 × 16 base × 33.75″ ht.

### C. Determination

(**a**) *Loose peat.*—Remove material from bag or container, pass it thru 1/2″ sieve, and place directly into measuring box. Pour contents from ca 60 cm (2′) into measuring box. Det. contents of bag or container only once. Fill corners of measuring box by shaking with rotary motion, 1 rotation/sec for 5 sec, without lifting box from floor or surface. When filled, level off by straightedge. Use ht of box to calc. vol. in cu. ft.

(**b**) *Baled peat.*—Vol. baled material = ht × area of base. Correct measurements for outside wrappers. Det. amt loose peat in bale by passing thru 1/2″ sieve and measuring amt loose peat, using 12 × 12 × 12″ box as in (**a**). Report vol. of peat in cu. ft.

Report total vol. of sieved peat from original container.

Refs.: Book of ASTM Stds(1971)Pts 11, 22, and 30, ASTM D2973-D2978, D2980, and D2944. JAOAC **56,** 154(1973).

## 973.08 Volume Weight, Water-Holding Capacity, and Air Capacity of Water-Saturated Peat Materials

**First Action 1973**

### A. Apparatus

(**a**) *Hollow spray nozzle.*—Monarch F-97-W, nozzle No. 4.6160 (Monarch Mfg. Works Inc., 2501 E Ontario St, Philadelphia, PA 19134), or equiv.

(**b**) *Pipe connection.*—For installation of nozzle on $H_2O$ faucet in sink.

(**c**) *Containers.*—Approx. 2 L (2 lb coffee cans are suitable) fitted with plastic covers. Replace metal bottom of one with No. 20 Cu screen (test container).

(**d**) *Aluminum pie pans.*—20 cm (8″) diam. Drill holes in side walls of pan so that $H_2O$ depth in pan remains ca 1.3 cm (0.5″).

### B. Preparation of Sample

*See* **967.02**.

### C. Determination

Det. moisture content on sep. sample by **967.03A** or **B**.

Weigh test container fitted with plastic cover, screen, and circle of filter paper (12.5 cm Whatman No. 4, or equiv.) which is placed on screen. Thoroly mix equal wts of $H_2O$ and peat and place in container without pressure to ht of 10 cm (4″); record wt in g. (If peat is dried out, mix 1 part peat with 2 parts $H_2O$. If wet, mix 2 parts peat with 1 part $H_2O$.)

Place test container in Al pan filled with $H_2O$ in sink $\geq$30 cm (12″) directly under spray nozzle. Water ca 24 hr as mist to prevent compression of peat. Place cover on container, seal (tape is suitable) to prevent evapn, and let stand in Al pan, maintaining 1.3 cm $H_2O$ head 2 days. Remove from pan and drain 2 hr with container at 45° angle. Remove seal on cover, and record wt and vol. Vol. can be detd by using container identical to test container not fitted with screen, filling $H_2O$ to same ht as sample in test container, and transferring to graduate with mL markings.

### D. Calculations

(**a**) *Saturated Volume Weights, g/mL*

(*1*) *As-recd* = g as-recd sample/mL wet vol., where g as-recd sample = g total sample/2; or g total sample/3 if 2 parts $H_2O$ used; or (g total sample × 2)/3 if 2 parts peat used.

(*2*) *Oven-dried.*—*See* **969.05D**.

(*3*) *Wet.*—*See* **969.05D**.

(**b**) *Water-Holding Capacity, %.*—*See* **969.05D**.

(**c**) *Dry Peat Volume, %.*—*See* **969.05D**.

(**d**) *Air Volume, %.*—*See* **969.05D**.

Refs.: Book of ASTM Stds(1971)Pts 11, 22, and 30, ASTM D2973–D2978, D2980, and D2944. JAOAC **56,** 154(1973).

## 973.09 Cation Exchange Capacity for Peat

**Titrimetric Method**

**First Action 1973**
**Final Action 1978**

***AOAC-ASTM Method***

### A. Principle

Cation exchange capacity is measure of total amt exchangeable cations that can be held by peat, expressed as mequiv./100 g air-dried peat. Peat sample is shaken with 0.5*N* HCl to remove bases and to sat. sorption complex with $H^+$. Excess acid is removed; absorbed $H^+$ is replaced with $Ba^{+2}$, titrd with 0.1*N* NaOH, using phthln indicator, and calcd to mequiv./100 g air-dried peat.

### B. Reagents

(**a**) *Dilute hydrochloric acid.*—0.5*N*. Dil. 42 mL HCl to 1 L with $H_2O$.

(**b**) *Barium acetate soln.*—0.5*N*. Dissolve 64 g $Ba(OAc)_2$ in $H_2O$ and dil. to 1 L.

(**c**) *Silver nitrate soln.*—1%. Dissolve 1 g $AgNO_3$ in 100 mL $H_2O$.

(**d**) *Sodium hydroxide std soln.*—0.1*N*. Prep. and stdze as in **936.16**.

### C. Preparation of Sample

*See* **967.02**.

### D. Determination

Thoroly mix air-dried ground peat sample and place 2.00 g in 300 mL erlenmeyer. Add ca 100 mL 0.5*N* HCl; stopper flask and shake vigorously periodically during 2 hr (or shake mech. 30 min). Filter thru rapid paper (24 cm fluted, or equiv.) in large powder funnel. Wash with 100 mL portions $H_2O$ until 10 mL wash shows no ppt with ca 3 mL 1% $AgNO_3$. Discard filtrate. Immediately transfer moist peat to 300 mL erlenmeyer, by puncturing apex of paper and forcing moist peat thru funnel stem into erlenmeyer, using spray from wash bottle contg ca 100 mL 0.5*N* $Ba(OAc)_2$. Stopper flask and shake vigorously periodically during 1 hr (or shake mech. 15 min). Filter, and wash with three 100 mL portions $H_2O$. Discard peat, and titr. washings with 0.1*N* NaOH, using 5 drops phthln, to first pink.

Calc. mequiv./100 g air-dried peat
= (mL × normality NaOH × 100)/g sample.

Ref.: JAOAC **56,** 154(1973).

# 3. Plants

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### 922.01 Sampling of Plants
**Final Action**

When more than one plant is sampled, include enough plants in sample to ensure that it adequately represents av. composition of entire lot of plants sampled. (This number depends upon variability in composition of the plants.) Det. details of sampling by purpose for which sample is taken.

Refs.: Botan. Gaz. **73,** 44(1922). Proc. Am. Soc. Hort. Sci. 1927, p. 191. JAOAC **13,** 224(1930); **16,** 71(1933); **19,** 70(1936).

### 922.02 Plants
**Preparation of Sample**
**Final Action**

(**a**) *For mineral constituents.*—Thoroly remove all foreign matter from material, especially adhering soil or sand, but to prevent leaching, avoid excessive washing. Air- or oven-dry as rapidly as possible to prevent decomposition or wt loss by respiration, grind, and store in tightly stoppered bottles. If results are to be expressed on fresh wt basis, record sample wts before and after drying. When Cu, Mn, Zn, Fe, Al, etc. are to be detd, avoid contaminating sample by dust during drying and from grinding and sieving machinery.
(**b**) *For carbohydrates.*—Thoroly remove all foreign matter and rapidly grind or chop material into fine pieces. Add weighed sample to hot redistd alcohol to which enough pptd $CaCO_3$ has been added to neutze acidity, using enough alcohol so that final concn, allowing for $H_2O$ content of sample, is ca 80%. Heat nearly to bp on steam or $H_2O$ bath 30 min, stirring frequently. (Samples may be stored until needed for analysis.)

Refs.: Botan. Gaz. **73,** 44(1922). Proc. Am. Soc. Hort. Sci. 1927, p. 191. JAOAC **13,** 224(1930); **16,** 71(1933); **19,** 70(1936).

### 930.04 Moisture in Plants
**Final Action 1965**

*See* **934.01**, **920.36★**, or **930.15**.

### 930.05 Ash of Plants
**Final Action 1965**

*See* **900.02A**, **900.02B**, or **942.05**.

### 920.08★ Sand and Silica in Plants
**Gravimetric Method**
**Final Action**
**Surplus 1989**

*See* **3.005**, 14th ed.

## METALS

### 953.01 Metals in Plants
**General Recommendations for Emission Spectrographic Methods**
**Final Action 1988**

(Applicable to aluminum, barium, boron, calcium, copper, iron, magnesium, manganese, molybdenum, phosphorus, potassium, sodium, strontium, and zinc)

(**a**) *Instrumental technic.*—If, because of equipment limitations, described methods cannot be followed in detail, or if detn of other elements is desired, following protocol is recommended: Det. experimentally, with available facilities, potentials of various sample prepns and excitation conditions with relation to element detectability and general concn requirements. Select analysis lines on basis of desirable intensity and freedom from spectral interference by other elements, as detd by prepg spectrum of each component element at av. concn at which it occurs in samples to be analyzed. Line and phototube characteristics are usually detd by instrument manufacturer.
(**b**) *Precision.*—Stdze all conditions of technic and det. reproducibility of results by making ca 20 successive exposures on sample of representative composition. For each element, calc. std deviation of single exposure and divide by square root of number of individual exposures that will be averaged in practice to constitute 1 detn. From this est. of std deviation of single detn, calc. coefficient of variation for each element. Following upper limits for precision error of spectrographic detns in analysis of plant material are satisfactory in relation to other routine methods or to practical requirements: Coefficients of variation (%)—Ca, Mg, Mn, and Mo, 3–7; B, Ba, Cu, K, P, and Zn, 7–15; and Al, Fe, and Na, >15. Coefficients of variation vary from instrument to instrument for each element; above values were obtained from std plant tissue by 11 different instruments.
(**c**) *Accuracy.*—Precise technic is essential but not only factor involved in accuracy. Reliability and appropriateness of stds and judgment used in ref. method are of utmost importance. Failure in any of these respects can result in serious calibration error for otherwise satisfactory method.
Carefully prep. synthetic stds from highest grade $H_2O$-free analyzed chems, collectively blanked for minor and trace elements. Preferably confirm values assigned to natural stds by results of >1 laboratory.
Matrix similarity between stds and samples, or closely controlled correction system for matrix differences, is essential. Check correction scales frequently against stds which closely match particular types of plant materials being analyzed.
Precision error of technic applies to ref. exposures as well as to samples. For this reason, base fiducial adjustments on as many ref. exposures as may be feasibly included in each series of samples.

Refs.: JAOAC **36,** 411(1953); **37,** 721(1954); **58,** 764(1975).

## 980.03 Metals in Plants
### Direct Reading Spectrographic Method
### Final Action 1988

**A. Apparatus**

(**a**) *Spark excitation source.*

(**b**) *Spectrograph.*—1.5 m grating spectrograph with spark stand and disk attachment rotating at 30 rpm.

(**c**) *Electrode sharpener.*

(**d**) *Disk electrode.*—High purity graphite disk 0.492" diam. and 0.200" thick.

(**e**) *Upper* (*pin*) *electrode.*—Point appropriate lengths of std grade spectrographic C rods, 0.180" diam., in pencil sharpener equipped with pin stop to produce $^1/_{16}$" diam. flat tip.

(**f**) *Porcelain boat.*—60 mm long, 10 mm wide, and 8 mm high (Coors No. 2, or equiv.).

**B. Reagents**

(**a**) *Buffer.*—Dissolve 50 g $Li_2CO_3$ in 200 mL $HNO_3$, and dil. to 1 L with $H_2O$. (*Caution:* See safety notes on nitric acid.)

(**b**) *Element stock std solns.*—On basis of expected sample concn range, prep. stock solns from individual pure nitrates, chlorides, or carbonate salts, or metal of resp. elements, as indicated in Table **980.03A**.

(**c**) *Mixed element std solns.*—Prep. 5 std solns contg % or ppm element indicated in Table **980.03B** as follows: Dissolve 50 g $Li_2CO_3$ in 200 mL $HNO_3$, pipet in indicated aliquots, and dil. to 1 L with $H_2O$. Prep. fresh every 6 months.

**C. Preparation of Sample**

Dry plant material 24 hr at 80° and grind in Wiley mill with No. 20 stainless steel sieve. Store in air-tight containers or in coin envelopes in dry atm.

Weigh 1.0 g prepd sample into 30 mL high form crucible (porcelain is satisfactory). Ash ≥4 hr at 500° with crucible resting on asbestos plate rather than on floor of furnace. Cool, add 5.0 mL buffer soln, (**a**), stir, and let stand 30 min.

**D. Determination**

(**a**) *Excitation.*—Align and space electrodes 4 mm apart in holders; position pin electrode over disk electrode. Set source parameters to give uniform breakdown voltage at tandem air gap with operating parameters at 4 breaks/cycle and 4 amp (parameters may vary with source for best operating efficiency).

Place aliquot of prepd soln in porcelain boat, set boat on arc stand, and raise to immerse $^1/_{16}$" of disc in soln. Spark 10 sec to condition electrodes and photomultiplier tubes and then spark addnl 30 sec for integration.

(**b**) *Calibration.*—Calibration technic varies with instrument. Use mixed element std solns and known plant tissue stds to calibrate spectrograph by same technic as for samples. Prep. std curves to cover desired concn range, using ratio to internal std and background correction for best results.

**Table 980.03A Preparation of Stock Standard Solutions**

| Element | Salt | Element, g/L | Salt, g/L | Solvent |
|---|---|---|---|---|
| K | KCl | 125 | 238.36 | $H_2O$ |
| Ca | $CaCO_3$ | 40 | 99.89 | 1 *N* $HNO_3$ |
| Mg | MgO | 20 | 33.16 | 1 *N* $HNO_3$ |
| P | $H_3PO_4$ | 10 | 31.64 | $H_2O$ |
| Na | NaCl | 10 | 25.42 | $H_2O$ |
| Fe | Fe metal | 1 | 1.00 | 1 *N* $HNO_3$ |
| Mn | MnO | 1 | 1.29 | 1 *N* $HNO_3$ |
| Al | Al metal | 1 | 1.00 | 1 *N* $HNO_3$ |
| Zn | Zn metal | 1 | 1.00 | 1 *N* $HNO_3$ |
| Cu | Cu metal | 1 | 1.00 | 1 *N* $HNO_3$ |
| Ba | $BaCl_2$ | 1 | 1.52 | $H_2O$ |
| Sr | $SrCO_3$ | 1 | 1.68 | 1 *N* $HNO_3$ |
| B | $H_3BO_3$ | 1 | 5.72 | $H_2O$ |
| Mo | $(NH_4)_6Mo_7O_{24}.H_2O$ | 0.1 | 1.29 | $H_2O$ |

## 955.08★ Metals in Plants
### Direct Current Arc Excitation Method
### Surplus 1970

*See* **44.003–44.006,** 11th ed.

## 955.09★ Metals in Plants
### Alternating Current Spark Excitation Method
### Surplus 1970

*See* **44.007–44.011**, 11th ed.

**Table 980.03B Preparation of Mixed Element Standard Solutions**

| Element | Element, g/L | Standard Solution Number 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|
| | | | | mL to give (%)[a] | | |
| K (CAS-7440-09-7) | 125 | 2(0.5) | 4(1.0) | 8(2.0) | 12(3.0) | 20(5.0) |
| Ca (CAS-7440-70-2) | 40 | 100(2.0) | 50(1.0) | 30(0.6) | 10(0.2) | 15(0.3) |
| Mg (CAS-7439-95-4) | 20 | 100(1.0) | 70(0.7) | 50(0.5) | 20(0.2) | 10(0.1) |
| P (CAS-7723-14-0) | 10 | 2(0.1) | 4(0.2) | 6(0.3) | 10(0.5) | 14(0.7) |
| | | | | mL to give (ppm)[a] | | |
| Na (CAS-7440-23-5) | 10 | 1(50) | 2(100) | 10(500) | 20(1000) | 40(2000) |
| Fe (CAS-7439-89-6) | 10 | 10(500) | 4(200) | 2(100) | 1(50) | 6(300) |
| Mn (CAS-7439-96-5) | 10 | 0.4(20) | 1(50) | 2(100) | 4(200) | 10(500) |
| Al (CAS-7429-90-5) | 10 | 0.6(30) | 1(50) | 2(100) | 4(200) | 10(500) |
| Zn (CAS-7440-66-6) | 1 | 2(10) | 4(20) | 6(30) | 10(50) | 20(100) |
| Cu (CAS-7440-50-8) | 1 | 1(5) | 2(10) | 4(20) | 10(50) | 14(70) |
| Ba (CAS-7440-39-3) | 1 | 20(100) | 10(50) | 4(20) | 2(10) | 1(5) |
| Sr (CAS-7440-24-6) | 1 | 40(200) | 20(100) | 10(50) | 6(30) | 3(15) |
| B (CAS-7440-42-8) | 1 | 1(5) | 2(10) | 4(20) | 10(50) | 14(70) |
| Mo (CAS-7439-98-7) | 0.1 | 2(1) | 4(2) | 8(4) | 12(6) | 20(10) |

[a] Concn based on 1 g sample taken up in 5 mL buffer.

## 975.03 Metals in Plants

**Atomic Absorption Spectrophotometric Method**
**First Action 1975**
**Final Action 1988**

(Applicable to calcium, copper, iron, magnesium, manganese, potassium, and zinc)

### A. Apparatus and Reagents

Deionized $H_2O$ may be used. *See* **965.09A** and **B**, and following:

(**a**) *Potassium stock soln.*—1000 μg K/mL. Dissolve 1.9068 g dried (2 hr at 105°) KCl in $H_2O$ and dil. to 1 L. Use following parameters for Table **965.09**: 7665 A, air-$C_2H_2$ flame, and 0.04–2 μg/mL range.

(**b**) *Calcium stock solns.*—Prep. Ca stock soln and working stds as in **965.09B**.

(**c**) *Cu, Fe, Mg, Mn, and Zn stock solns.*—Prep. as in **965.09B(b)**, (**c**), (**e**), (**f**), and (**g**).

(**d**) *Working std solns.*—Dil aliquots of solns (**c**) with 10% HCl to make ≥ 4 std solns of each element within range of detn.

### B. Preparation of Sample

(**a**) *Dry ashing.*—Accurately weigh 1 g sample, dried and ground as in **922.02(a)**, into glazed, high-form porcelain crucible. Ash 2 hr at 500°, and let cool. Wet ash with 10 drops $H_2O$, and carefully add 3–4 mL $HNO_3$ (1+1). Evap. excess $HNO_3$ on hot plate set at 100–120°. Return crucible to furnace and ash addnl 1 hr at 500°. Cool crucible, dissolve ash in 10 mL HCl (1+1), and transfer quant. to 50 mL vol. flask.

(**b**) *Wet ashing.*—Accurately weigh 1 g sample, dried and ground as in **922.02(a)**, into 150 mL Pyrex beaker. Add 10 mL $HNO_3$ and let soak thoroly. Add 3 mL 60% $HClO_4$ and heat on hot plate, slowly at first, until frothing ceases. (*Caution*: See safety notes on wet oxidation.) Heat until $HNO_3$ is almost evapd. If charring occurs, cool, add 10 mL $HNO_3$, and continue heating. Heat to white fumes of $HClO_4$. Cool, add 10 mL HCl (1+1), and transfer quant. to 50 mL vol. flask.

### C. Determination

To soln in 50 mL vol. flask, add 10 mL 5% La soln, and dil. to vol. Let silica settle, decant supernate, and proceed as in **965.09D**.

Make necessary dilns with 10% HCl to obtain solns within ranges of instrument.

### D. Calculations

$$\text{ppm Element} = (\mu g/mL) \times F/\text{g sample}$$
$$\%\ \text{Element} = \text{ppm} \times 10^{-4}$$

where $F$ = (mL original diln × mL final diln)/mL aliquot if original 50 mL is dild.

Ref.: JAOAC **58,** 436(1975).

## 985.01 Metals and Other Elements in Plants

**Inductively Coupled Plasma Spectroscopic Method**
**First Action 1985**
**Final Action 1988**

(Applicable to B, Ca, Cu, K, Mg, Mn, P, and Zn)

### A. Principle

Sample is dry-ashed, treated with $HNO_3$, and dissolved in HCl; elements are detd by ICP emission spectroscopy.

### B. Reagents and Apparatus

(**a**) *Stock solns.*—1000 μg/mL. Weigh designated reagent into sep. 1 L vol. flasks, dissolve in min. amt of dissolving reagent, and dil. to vol. with $H_2O$.

| Element | Reagent | g | Dissolving Reagent |
|---|---|---|---|
| B | $H_3BO_3$ | 5.7192 | $H_2O$ |
| Ca | $CaCO_3$ | 2.4973 | 6N HCl |
| Cu | pure metal | 1.0000 | $HNO_3$ |
| K | KCl | 1.9067 | $H_2O$ |
| Mg | $MgSO_4.7H_2O$ | 10.1382 | $H_2O$ |
| Mn | $MnO_2$ | 1.5825 | 6N HCl |
| P | $NH_4H_2PO_4$ | 3.7138 | $H_2O$ |
| Zn | pure metal | 1.0000 | 6N HCl |

(**b**) *Std solns.*—Pipet following vols of stock soln into 1 L vol. flasks. Add 100 mL HCl and dil. to vol. with $H_2O$.

| | Std Soln 1 | | Std Soln 2 | |
|---|---|---|---|---|
| Element | Stock Soln, mL | Final Concn, μg/mL | Stock Soln, mL | Final Concn, μg/mL |
| B | 0 | 0 | 10 | 10 |
| Ca | 5 | 5 | 60 | 60 |
| Cu | 0 | 0 | 1 | 1 |
| K | 5 | 5 | 60 | 60 |
| Mg | 1 | 1 | 20 | 20 |
| Mn | 0 | 0 | 10 | 10 |
| P | 5 | 5 | 60 | 60 |
| Zn | 0 | 0 | 10 | 10 |

Make any needed subsequent dilns with 10% HCl (1 + 9).

(**c**) *ICP emission spectrometer.*—Suggested operating parameters: forward power, 1.1 kilowatts; reflected power, <10 watts; aspiration rate, 0.85–3.5 mL/min; flush between samples, 15–45 s; integration time, 1–10 s.

| Element | Wavelength, Å |
|---|---|
| B (CAS-7440-42-8) | 2496 |
| Ca (CAS-7440-70-2) | 3179 |
| Cu (CAS-7440-50-8) | 3247 |
| K (CAS-7440-09-7) | 7665 |
| Mg (CAS-7439-95-4) | 2795 |
| Mn (CAS-7439-96-5) | 2576 |
| P (CAS-7723-14-0) | 2149 |
| Zn (CAS-7440-66-6) | 2138 |

### C. Dry Ashing

Accurately weigh 1 g sample, dried and ground as in **922.02(a)**, into glazed, high-form porcelain crucible. Ash 2 h at 500°, and let cool. Wet ash with 10 drops of $H_2O$, and carefully add 3–4 mL $HNO_3$ (1 + 1). Evap. excess $HNO_3$ on hot plate set at 100–120°. Return crucible to furnace and ash addnl 1 h at 500°. Cool crucible, dissolve ash in 10 mL HCl (1 + 1), and transfer quant. to 50 mL vol. flask. Dil. to vol. with $H_2O$.

### D. Determination

Elemental detn is accomplished by inductively coupled plasma emission spectroscopy. Calibration of instrument is done thru use of known calibration stds. After calibration is complete, samples can be analyzed. Check calibration after every 10 samples. If instrument has drifted out of calibration (>3% of original values), recalibrate.

Calc. concn for each element of each dild sample as μg/mL.

Ref.: JAOAC **68,** 499(1985).

## INDIVIDUAL METALS

### 928.03 Aluminum and Iron in Plants

**Titrimetric Method**

**Final Action**

(*Caution*: See safety notes on sulfuric acid.)

Take aliquot of *Soln I*, **920.08**, contg enough Fe and Al to form ca 40 mg Fe- and $AlPO_4$. Add few drops $HNO_3$, Br-$H_2O$, or $H_2O_2$ to oxidize Fe. If soln does not already contain excess phosphate, add 0.5 g $(NH_4)_2HPO_4$, stir until dissolved, and dil. to 50 mL with $H_2O$. Add few drops thymol blue soln (0.1%: dissolve 0.1 g thymol blue in $H_2O$, add enough 0.1N NaOH to change color to blue, and dil. to 100 mL), and then add $NH_4OH$ until soln just turns yellow. Add 0.5 mL HCl and 25 mL 25% $NH_4OAc$ soln, and stir. Let stand at room temp. until ppt settles (ca 1 hr). Filter, and wash 10 times with hot 5% $NH_4NO_3$ soln. Ignite at 500–550° and weigh as $FePO_4$ and $AlPO_4$.

Fuse ignited residue in Pt crucible with ca 4 g $Na_2CO_3$-$K_2CO_3$ (1+1) mixt. When fusion is complete, let crucible cool, add 5 mL $H_2SO_4$, and heat until copious fumes of $SO_3$ are evolved. Cool, transfer to flask, add $H_2O$, and digest until soln is clear. Reduce Fe with Zn, cool, and titr. with 0.1*N* $KMnO_4$. Correct for blank and calc. as % Fe or % $Fe_2O_3$. Calc. to $FePO_4$ and subtract from total Fe- and $AlPO_4$ to obtain $AlPO_4$. Correct for blank and report as $Al_2O_3$.

Refs.: JAOAC **11,** 203(1928); **16,** 70(1933); **19,** 70(1936).

CAS-7784-30-7 (aluminum phosphate)
CAS-10045-86-0 (ferric phosphate)

### 910.01 Calcium in Plants

**Titrimetric Macro Method**

**Final Action**

Transfer aliquot of *Soln I*, **920.08**, to 200 mL beaker, add $H_2O$ if necessary to vol. of 50 mL, heat to bp, and add 10 mL satd $(NH_4)_2C_2O_4$ soln and drop Me red (dissolve 1 g Me red in 200 mL alcohol). Almost neutze with $NH_4OH$ and boil until ppt is coarsely granular. Cool, add $NH_4OH$ (1+4) until color is faint pink (pH 5.0), and let stand ≥4 hr. Filter, and wash with $H_2O$ at room temp. until filtrate is oxalate-free. (Reserve filtrate and washings for Mg detn, **920.09**.)

Break point of filter with Pt wire, and wash ppt into beaker in which Ca was pptd, using stream of hot $H_2O$. Add ca 10 mL $H_2SO_4$ (1+4), heat to ca 90°, add ca 50 mL hot $H_2O$, and titr. with 0.05*N* $KMnO_4$. Finally add filter paper to soln and complete titrn.

Refs.: J. Biol. Chem. **7,** 83(1910). JAOAC **4,** 392(1921); **16,** 70(1933).

CAS-7440-70-2 (calcium)

### 921.01 Calcium in Plants

**Titrimetric Micro Method**

**Final Action**

Weigh 2 g sample into small crucible and ignite in furnace at 500–550°. Dissolve ash in HCl (1+4) and transfer to 100 mL beaker. Add 5 mL HCl and evap. to dryness on steam bath to dehydrate $SiO_2$. Moisten residue with 5 mL HCl, add ca 50 mL $H_2O$, heat few min on steam bath, transfer to 100 mL vol. flask, cool quickly to room temp., dil. to vol., shake, and filter, discarding first portion of filtrate.

Pipet 15 mL aliquot into conical-tip centrf. tube contg 2 mL satd $(NH_4)_2C_2O_4$ soln and 2 drops Me red (dissolve 1 g Me red in 200 mL alcohol). Add 2 mL HOAc (1+4), rotating tube to mix contents thoroly. Add $NH_4OH$ (1+4), while intermittently rotating tube, until soln is faintly alk.; then add few drops of the HOAc until color is faint pink (pH 5.0). (It is important at this point to rotate tube so that last bit of liq. in conical tip has required color.) Let stand ≥4 hr; then centrf. 15 min. (Ppt should be in firm lump in tip of tube.) Remove supernate, using suction device, Fig. **921.01**, taking care not to disturb ppt. Wash ppt by adding 2 mL $NH_4OH$ (1+49), rotating tube to break up ppt. (It may be necessary to jar tube sharply.) Centrf. 10 min, again remove supernate, and wash with reagent as before. Repeat washing of ppt 3 times.

After removing last supernate, add 2 mL $H_2SO_4$ (1+4) to tube, break up ppt as before, heat on steam bath to 80–90°, and titr. in tube with 0.02*N* $KMnO_4$, rotating liq. during titrn to attain proper end point. If tube cools to <60° during titrn, as indicated by slow reduction of $KMnO_4$, reheat in steam bath few min and complete titrn. Perform blank on identical vol. $H_2SO_4$ in similar tube heated to same temp. to det. vol. $KMnO_4$ soln necessary to give end point color. Subtract this value from buret reading. 1 mL 0.02*N* $KMnO_4$ = 0.000400 g Ca. Report as % Ca.

Refs.: J. Biol. Chem. **47,** 475(1921); **50,** 527, 537(1922). JAOAC **14,** 216(1931); **16,** 71(1933); **19,** 71(1936).

CAS-7440-70-2 (calcium)

### 951.01 Cobalt in Plants

**Nitrosocresol Method**

**Final Action 1965**

(*Caution*: See safety notes on distillation, toxic solvents, carbon tetrachloride, and nitroaromatics.)

***A. Reagents***

(Make all distns in Pyrex stills with ₮ joints. Store reagents in g-s Pyrex bottles.)

(**a**) *Redistilled water*.—Distil twice, or pass thru column of ion exchange resin (IR-100A, H-form, or equiv.) to remove heavy metals.

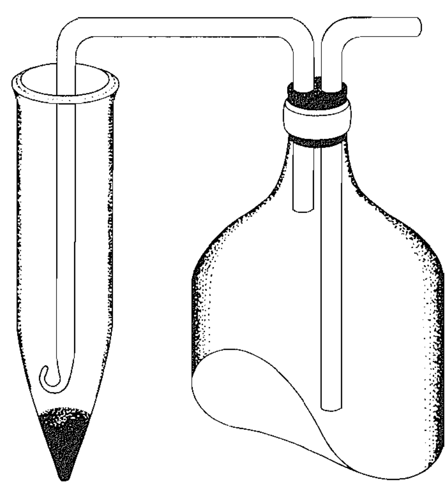

**FIG. 921.01—Suction device used in micro method for determining calcium**

(**b**) *Hydrofluoric acid*.—48%. Procurement in vinyl plastic bottles is advantageous.

(**c**) *Perchloric acid*.—60%. No further purification necessary.

(**d**) *Hydrochloric acid*.—(1+1). Add equal vol. HCl to distd $H_2O$ and distil.

(**e**) *Ammonium hydroxide*.—(1 + 1). Distil concd $NH_4OH$ into equal vol. redistd $H_2O$.

(**f**) *Ammonium hydroxide*.—0.02*N*. Add 7 mL of the $NH_4OH$ (1+1) to 2.5 L redistd $H_2O$.

(**g**) *Carbon tetrachloride*.—Distil over CaO, passing distillate thru dry, acid-washed filter paper. Used $CCl_4$ may be recovered as in **941.03A(a)**.

(**h**) *Dithizone*.—Dissolve 0.5 g dithizone in 600–700 mL $CCl_4$ (tech. grade is satisfactory). Filter into 5 L separator contg 2.5–3.0 L 0.02 *N* $NH_4OH$, shake well, and discard $CCl_4$ layer. Shake with 50 mL portions redistd $CCl_4$ until $CCl_4$ phase as it seps is pure green. Add 1 L redistd $CCl_4$ and acidify slightly with the HCl (1+1). Shake the dithizone into $CCl_4$ layer and discard aq. layer. Store in cool, dark place, preferably in refrigerator.

(**i**) *Ammonium citrate soln*.—40%. Dissolve 800 g citric acid in 600 mL distd $H_2O$, and, while stirring, slowly add 900 mL $NH_4OH$. Reaction is exothermic; take care to prevent spattering. Adjust pH to 8.5, if necessary. Dil. to 2 L and ext with 25 mL portions dithizone soln until aq. phase stays orange and $CCl_4$ remains predominantly green. Then ext soln with $CCl_4$ until all orange is removed.

(**j**) *Hydrochloric acid*.—0.1*N*. Dil. 16.6 mL of the HCl (1+1) to 1 L with redistd $H_2O$.

(**k**) *Hydrochloric acid*.—0.01*N*. Dil. 100 mL of the 0.1*N* HCl to 1 L with redistd $H_2O$.

(**l**) *Sodium hydroxide soln*.—1*N*. Dissolve 40 g NaOH in 1 L redistd $H_2O$.

(**m**) *Borate buffer*.—pH 7.8. Dissolve 20 g $H_3BO_3$ in 1 L redistd $H_2O$. Add 50 mL 1*N* NaOH and adjust pH, if necessary. Equal vols borate buffer and 0.01*N* HCl should give soln of pH 7.9.

(**n**) *Borate buffer*.—pH 9.1. To 1 L borate buffer, pH 7.8, add 120 mL 1*N* NaOH and adjust pH, if necessary.

(**o**) *Skellysolve B*.—Essentially *n*-hexane. Purify by adding 20–30 g silica gel/L, let stand several days, and distil. Available from Getty Refining and Marketing Co., PO Box 1650, Tulsa, OK 74102.

(**p**) *Cupric acetate soln*.—Dissolve 10 g $Cu(OAc)_2.H_2O$ in 1 L redistd $H_2O$.

(**q**) *o-Nitrosocresol soln*.—Dissolve 8.4 g anhyd. $CuCl_2$ and 8.4 g $NH_2OH.HCl$ in 900 mL $H_2O$. Add 8 mL *m*-cresol (Eastman Kodak Co., practical grade) and stir vigorously while slowly adding 24 mL 30% $H_2O_2$. Stir mech. 2 hr at room temp. (Standing for longer periods results in excessive decomposition.) Add 25 mL HCl and ext *o*-nitrosocresol with four 150 mL portions Skellysolve B, (**o**), in large separator. Then add addnl 25 mL HCl and again ext with four 150 mL portions Skellysolve B. Wash combined Skellysolve B exts twice with 50–100 mL portions 0.1*N* HCl and twice with 50–100 mL portions redistd $H_2O$. Shake *o*-nitrosocresol soln with successive 50–100 mL portions 1% $Cu(OAc)_2$ soln until aq. phase is no longer deep blood-red. When light purple is evident, extn is complete. Discard Skellysolve B phase, acidify aq. soln of Cu salt with 25 mL HCl, and ext reagent with two 500 mL portions Skellysolve B; wash twice with 150–200 mL portions 0.1*N* HCl and several times with 150–200 mL portions redistd $H_2O$. Store *o*-nitrosocresol soln in refrigerator at ca 4°. Reagent is stable ≥6 months.

(**r**) *Sodium o-nitrosocresol soln*.—Ext 100 mL *o*-nitrosocresol by shaking with two 50 mL portions borate buffer, pH 9.1, in separator. (If this is carried out as 2 extns, resulting reagent is more concd. It is important that total vol. *o*-nitrosocresol soln equal total vol. buffer.)

(**s**) *Cobalt std solns*.—(*1*) *Stock soln*.—Heat $CoSO_4.7H_2O$ in oven at 250–300° to const wt (6–8 hr). Weigh exactly 0.263 g of the $CoSO_4$ and dissolve in 50 mL redistd $H_2O$ and 1 mL $H_2SO_4$. Dil. to 1 L. (*2*) *Working soln*.—0.5μg/mL. Transfer 5 mL stock soln to 1 L vol. flask and dil. to vol. with redistd $H_2O$.

(**t**) *Hydroxylamine acetate buffer*.—pH 5.1±0.1. Dissolve 10 g $NH_2OH.HCl$ and 9.5 g anhyd. NaOAc in 500 mL redistd $H_2O$.

### *B. Apparatus*

(**a**) *Platinum dishes*.—Approx. 70 mL; for ashing.

(**b**) *Automatic dispensing burets*.—100 mL; type that can be fitted to ordinary 5 lb reagent bottle and filled by means of aspirator bulb is most convenient.

(**c**) *Wooden separator rack*.—Twelve-unit 125 mL separator size is convenient for dithizone extns. Rack is fitted across top with removable bar padded with sponge rubber so all 12 separators can be shaken as unit.

(**d**) *Racks*.—Consisting of 5 × 5 × 65 cm (2 × 2 × 25″) wooden bars with holes drilled at close intervals to take 50 mL centrf. tubes fitted with No. 13 ₮ glass stoppers. To make these tubes, ream out necks of heavy-wall Pyrex centrf. tubes (Rockefeller Institute type) with ₮ C rod and grind to take ₮ stopper. Place tubes upright in one section, and place other section (fitted with sponge rubber disks 13 mm thick in bottom of holes) across their tops. Fasten 2 sections at ends with removable rubber connectors made from ordinary tubing of convenient size, so that any number of tubes can be shaken as unit. Use these tubes for reaction of Co with nitrosocresol, extn of complex into Skellysolve B, and washing of Skellysolve B soln.

(**e**) *Shaking machine*.—Mech. shaker giving longitudinal stroke of 5 cm at ca 180 strokes/min; use to make dithizone extns and to ext Co complex, or shake by hand.

### *C. Cleaning of Glassware*

Clean 120 mL Pyrex separators for dithizone extns by initially soaking 30 min in hot $HNO_3$ and rinsing several times with $H_2O$. As added precaution, shake with several portions dithizone in $CCl_4$. After use, clean by rinsing with $H_2O$, drain, and stopper to avoid contamination. It is not necessary to clean every time with acid. Repeat $HNO_3$ cleaning if blanks are unusually high.

Clean 50 mL g-s Pyrex centrf. tubes by soaking 30 min in $HNO_3$ followed by several rinsings in $H_2O$.

Completely submerge pipets in cylinder of chromic acid cleaning soln overnight, rinse several times with $H_2O$, and suspend upright in rack to dry.

Wash all other glassware thoroly in detergent and rinse well with tap $H_2O$ followed by dip in chromic acid cleaning soln. Rinse off cleaning soln with tap $H_2O$ followed by several distd $H_2O$ rinses.

Clean Pt by scrubbing with sea sand followed by boiling in HCl (1+2) 30 min, and rinse several times with $H_2O$.

### *D. Preparation of Sample*

*See* **922.02(a)**. Oven-dry all plant material 48 hr and prep. for ashing by either of following methods:

(**a**) Grind material in Wiley mill equipped with stainless steel sieve, mix thoroly by rolling, and sample by quartering.

(**b**) Using stainless steel shears, cut material by hand fine enough for convenient sampling.

### *E. Ashing of Samples*

(*Caution*: See safety notes on distillation, hydrofluoric acid, and perchloric acid.)

Weigh 6 g dry plant tissue into clean Pt dish. Cover with Pyrex watch glass and place in cool furnace; heat slowly to 500° and hold at this temp. overnight. Remove sample and cool. Wet down ash carefully with fine stream redistd $H_2O$. From dispensing buret, slowly add 2–5 mL $HClO_4$, dropwise at first to prevent spattering. Add ca 5 mL HF, evap. on steam bath, transfer to sand bath, and keep at medium heat until fuming ceases.

Cover with Pyrex watch glass, return to partially cooled furnace, heat gradually to 600°, and keep at this temp. 1 hr. Remove sample and cool. Add 5 mL HCl (1+1) and ca 10 mL redistd $H_2O$. Replace cover glass and warm on steam bath to dissolve. (Usually clear soln essentially free of insol. material is obtained.) Transfer sample to 50 mL vol. flask, washing dish several times with redistd $H_2O$, dil. to vol., and mix thoroly. (Pt dishes can ordinarily be used several times between sand and acid cleanings.)

### F. Dithizone Extraction

(*Caution*: See safety notes on distillation, perchloric acid, and carbon tetrachloride.)

Transfer suitable aliquot (2–3 g dry material) to 120 mL separator (use petroleum jelly as stopcock lubricant). Add 5 mL $NH_4$ citrate soln and 1 drop phthln; adjust to pH 8.5 with $NH_4OH$ (1+1). If ppt forms, add addnl $NH_4$ citrate. Add 10 mL dithizone in $CCl_4$ and shake 5 min. Drain $CCl_4$ phase into 100 mL beaker. Repeat as many times as necessary, using 5 mL dithizone soln and shaking 5 min each time. Extn is complete when aq. phase remains orange and $CCl_4$ phase remains predominantly green. Then add 10 mL $CCl_4$, shake 5 min, and combine with $CCl_4$ ext. Final 10 mL $CCl_4$ should be pure green. If not, extn was incomplete and must be repeated.

Add 2 mL $HClO_4$ to combined $CCl_4$ exts, cover beaker with Pyrex watch glass, and digest on hot plate until colorless. Remove cover glass and evap. slowly to dryness. (If sample is heated any length of time at high temp. when dry, losses of Co may occur. Heat only enough to evap. completely to dryness. If free acid remains, it interferes with next step where pH control is important.)

Add 5 mL 0.01$N$ HCl to residue. Heat slightly to assure soln. If Cu is to be detd, transfer with redistd $H_2O$ to 25 mL vol. flask, and dil. to vol. Transfer 20 mL aliquot to 50 mL g-s centrf. tube or 60 mL separator and reserve remainder for Cu detn, **953.03B**. If Cu is not to be detd, transfer entire acid soln with redistd $H_2O$ to centrf. tube or separator.

### G. Determination

Add 5 mL borate buffer, pH 7.8, and 2 mL freshly prepd Na *o*-nitrosocresol soln to sample soln. Add exactly 5 mL Skellysolve B and shake 10 min. Remove aq. phase by moderate suction thru finely-drawn glass tube. To Skellysolve B layer add 5 mL $Cu(OAc)_2$ soln and shake 1 min to remove excess reagent. Again remove and discard aq. phase. Wash Skellysolve B by shaking 1 min with 5 mL redistd $H_2O$, removing aq. layer as before; finally shake Skellysolve B 1 min with 5 mL $NH_2OH$-NaOAc buffer to reduce Fe. Transfer Skellysolve B soln of the Co complex to 5 cm cell and read in spectrophtr as close as possible to point of max. $A$, 360 nm.

### H. Blanks and Standards

With each set of detns include ashing blank and Co stds of 0.0, 0.5, and 1.0 μg. Beer's law holds for this range. $A$ of 0.0 μg point should be <0.05. If above, repurify *o*-nitrosocresol by transferring alternately to aq. phase as Cu salt and to Skellysolve B phase as free compd after acidifying aq. phase.

It is also advisable to include std sample with each set of samples to detect contamination or unusual losses of Co in method. Com. buckwheat flour contg 0.05 ppm Co has proved satisfactory for this purpose.

### I. Calculations

Express results in terms of ppm Co, based upon dry wt of sample.

$$\text{ppm Co} = (\mu g\ \text{Co/mL dithizone aliquot}) \times (\text{mL total soln/g dry sample})$$

Value for μg Co is obtained from curve minus ashing blank.

Refs.: JAOAC **34,** 710(1951); **36,** 405(1953).

CAS-7440-48-4 (cobalt)

## 953.02 Cobalt in Plants
**Nitroso-R-Salt Method**
**Final Action 1965**

### A. Reagents

Those listed in **951.01A**, and following:

(**a**) *Nitroso-R-salt soln.*—0.2%. Dissolve 2 g powd nitroso-R-salt (Eastman Kodak Co., No. 1124) in redistd $H_2O$, **951.01A(a)**, and dil. to 1 L.

(**b**) *Dilute nitric acid.*—(1+1). Dil. $HNO_3$ with equal vol. $H_2O$ and redistil in Pyrex app. Store in Pyrex bottles.

(**c**) *Bromine water.*—Satd soln of Br in redistd $H_2O$, **951.01A(a)**.

(**d**) *Citric acid soln.*—0.2 $N$. Use special reagent grade Pb-free citric acid.

### B. Preparation and Ashing of Samples

Proceed as in nitrosocresol method, **951.01D** and **E**, thru "(Usually clear soln essentially free of insol. material is obtained.)" except use 10 g instead of 6 g dry plant tissue.

### C. Dithizone Extraction

Transfer entire soln to 120 mL separator, and proceed as in **951.01F**, thru "If free acid remains . . . pH control is important.)" Dissolve in 1 mL citric acid soln, (**d**), transfer to 25 mL vol. flask, and dil. to vol. with redistd $H_2O$, **951.01A(a)**.

### D. Determination

Transfer suitable aliquot (ca 8 g dry material) of citric acid soln, **953.02C**, to 50 mL beaker. Evap. to 1–2 mL. Add 3 mL borate buffer, **951.01A(n)**, and adjust pH to 8.0–8.5 with NaOH (check externally with *phenol red*). (Vol. ≤5 mL.) Add 1 mL nitroso-R-salt soln *slowly with mixing*. Boil 1–2 min and add 2 mL dil. $HNO_3$. Boil 1–2 min, add 0.5–1.0 mL Br-$H_2O$, cover with watch glass, and let stand warm 5 min. Boil 2–3 min to remove excess Br (use effective fume removal device). Cool, and dil. to 10 or 25 mL (depending on length of light path in absorption cell). Transfer to cell and read at 500 nm within 1 hr. Prep. stds contg 0.5, 1, 2, 3, and 4 μg Co and add 1 mL citric acid soln, **352.03A(d)**, to each. Proceed as for unknowns, beginning "Evap. to 1–2 mL."

Ref.: JAOAC **36,** 405(1953).

CAS-7440-48-4 (cobalt)

## 953.03 Copper in Plants
**Colorimetric Method**
**Final Action 1965**

### A. Reagents

Those listed in **951.01A**, and following:

(**a**) *Sodium diethyldithiocarbamate soln.*—0.1%. Freshly prepd in redistd $H_2O$, **951.01A(a)**.

(**b**) *Copper std soln.*—1 μg/mL. Dissolve 0.3929 g $CuSO_4.5H_2O$ in redistd $H_2O$, **951.01A(a)**, add 5 mL $H_2SO_4$, dil. to 1 L, and mix. Take 10 mL aliquot, add 5 mL $H_2SO_4$, dil to 1 L, and mix.

### *B. Determination*

Transfer aliquot (0.5–1 g dry material) from soln obtained from **951.01F** or **953.02C** to 125 mL separator. Add 2 mL $NH_4$ citrate soln, 1 drop phthln, 5 mL Na diethyldithiocarbamate soln, and $NH_4OH$ (1+1), **951.01A(e)**, until pink. Add 10 mL $CCl_4$ and shake 5 min. Drain $CCl_4$, centrf. 5 min, transfer to absorption cell, and read at max. *A*, ca 430 nm.

Prep. std curve with 0, 1, 5, 10, 15, and 20 μg Cu treated as above.

Ref.: JAOAC **36,** 405(1953).

CAS-7440-50-8 (copper)

## 937.03 Iron in Plants
### Colorimetric Method
### Final Action

### *A. Reagents*

(**a**) *Acetic acid.*—2*M*. Dil. 120 g HOAc to 1 L with $H_2O$.

(**b**) *Ammonium citrate soln.*—1%. Dissolve 1 g $NH_4$ citrate in $H_2O$ and dil. to 100 mL.

(**c**) *Bromophenol blue indicator.*—0.04%. Grind 0.1 g bromophenol blue in mortar with 3 mL 0.05*N* NaOH, transfer to vol. flask, and dil. to 250 mL with $H_2O$.

(**d**) *Buffer solns.*—*(1) pH 3.5.*—Mix 6.4 mL 2*M* NaOAc with 93.6 mL 2*M* HOAc and dil. to 1 L. (*2*) *pH 4.5.*—Mix 43 mL 2*M* NaOAc with 57 mL 2*M* HOAc and dil. to 1 L.

(**e**) *Hydroquinone soln.*—Dissolve 1 g hydroquinone in 100 mL pH 4.5 buffer, (**d**)(*2*). Keep in refrigerator, and discard when any color develops.

(**f**) *o-Phenanthroline soln.*—Dissolve 1 g *o*-phenanthroline.$H_2O$ in $H_2O$, warming if necessary, and dil. to 400 mL.

(**g**) *Sodium acetate soln.*—2*M*. Dissolve 272 g $NaOAc.3H_2O$ in $H_2O$ and dil. to 1 L.

(**h**) *Iron std soln.*—1 mg/mL. Dissolve 1 g electrolytic Fe in 50 mL $H_2SO_4$ (1+9), warming if necessary to hasten reaction. Cool, and dil. to 1 L with $H_2O$.

### *B. Preparation of Sample*

(*Caution:* See safety notes on distillation, hydrofluoric acid, and sulfuric acid.)

Use *Soln I,* **920.08**, or if *Soln I* is not available, weigh samples of finely ground plant material (1–5 g, depending on Fe content) into porcelain crucibles with smooth inner surfaces, and ash overnight at 500–550° in furnace. Cool, add 5 mL HCl (1+1), and heat on steam bath 15 min to dissolve Fe and to hydrolyze pyrophosphate. Filter into 100 mL vol. flask. Transfer insol. residue to filter and wash 5 times with 3 mL portions hot HCl (1+100), then with hot $H_2O$ until washings are Cl-free. Ignite paper and any remaining C in Fe-free Pt crucible. Cool, add 2 drops $H_2SO_4$ and 1 mL HF, and carefully evap. to $SO_3$ fumes. Cool, add few drops HCl (1+1), and warm. Filter and wash as before into same vol. flask, dil. to vol., and mix.

### *C. Determination*

Pipet identical aliquots of *Soln I,* **920.08**, or sample soln, **937.03B**, into 25 mL vol. flask and into test tube or small erlenmeyer. Add 5 drops bromophenol blue indicator to aliquot in test tube, and titr. with 2*M* NaOAc soln until color matches that of equal vol. of pH 3.5 buffer contg same amt of indicator. Add 1 mL hydroquinone soln and 2 mL *o*-phenanthroline soln to aliquot in vol. flask, and adjust pH to 3.5 by adding same vol. NaOAc soln found necessary for aliquot in test tube. If turbidity develops upon adjusting pH of aliquot in test tube, add 1 mL $NH_4$ citrate soln to vol. flask before adding the NaOAc soln. Dil. to vol., mix, and let stand 1 hr for complete color development, and measure *A* at max., ca 510 nm.

Prep. curve relating *A* to mg Fe in 25 mL by treating series of solns contg amts of Fe that cover usable range of instrument exactly as described for unknowns, detg their respective readings at max. *A*, ca 510 nm, and plotting these against corresponding concns of Fe. $H_2O$ may be used as ref., and blanks detd to correct for amt Fe in reagents used, or blank soln itself may be made basis of comparison.

Refs.: Ind. Eng. Chem. Anal. Ed. **9,** 67(1937); **10,** 13(1938). JAOAC **25,** 555(1942); **27,** 526(1944).

CAS-7439-89-6 (iron)

## 936.02 Iron in Plants
### Titrimetric Method
### Final Action

Take appropriate aliquot of *Soln I* or of soln prepd as in **937.03B**, and oxidize Fe by adding soln of $KMnO_4$ (1+1000) dropwise until very faint permanganate color persists. Add 5 mL 10% $NH_4SCN$ and titr. with *dil. $TiCl_3$ soln* until red color disappears. (To prep. appropriate $TiCl_3$ soln, boil 5–10 mL 20% $TiCl_3$ with 50 mL HCl few min, cool, and dil. to 1 L. Stdze against std Fe soln, keep in dark in well-filled container, and restdze each time it is used, or every few hr when many detns are being made. Discard when decomposition is indicated by loss of color and increased titer against std.)

Refs.: JAOAC **19,** 359(1936); **27,** 526(1944).

CAS-7439-89-6 (iron)

## 920.09★ Magnesium in Plants
### Gravimetric Method
### Final Action
### Surplus 1989

*See* **3.039**, 14th ed.

## 921.02 Manganese in Plants
### Colorimetric Method
### Final Action

To aliquot of *Soln I,* **920.08**, contg 0.2–0.5 g ash, add 15 mL $H_2SO_4$ and evap. to ca 30 mL. Add 5–10 mL $HNO_3$ and continue evapn. (Do not evap. until dense fumes appear, because $Fe_2(SO_4)_3$ then dissolves with difficulty. $HNO_3$ may be present, but not HCl.) Add $H_2O$, little at time, heat until Fe salts dissolve, and dil. to ca 150 mL. Add 0.3 g *$KIO_4$*, or its equiv. in $HIO_4$, in small portions, boil few min or until color of $KMnO_4$ shows no further increase in intensity, and let cool.

Prep. std as follows: To vol. $H_2O$ equal to sample add 15 mL $H_2SO_4$ and enough pure $Fe(NO_3)_3$, free from Mn, to equal approx. amt of Fe in sample. Add measured vol. 0.1 *N* $KMnO_4$ until color is slightly darker than sample, then add 0.3 g $KIO_4$, and boil few min. When cool, transfer sample and std to 250 mL vol. flasks and dil. to vol. with $H_2O$. (If color is weak, it may be necessary to dil. to <250 mL.) Measure $A$ with photometer or spectrophtr set at max., ca 530 nm. Report as % Mn.

Ref.: JAOAC **4,** 393(1921).

CAS-7439-96-5 (manganese)

## 960.05 Molybdenum in Plants

### Colorimetric Method

**First Action 1960**
**Final Action 1965**

### A. Apparatus

*Photoelectric colorimeter or spectrophotometer.*—Capable of isolating band at ca 465 nm. (Photometer equipped with filter with max. $T$ at 440–460 nm and 1 cm cells of 10 mL capacity is suitable.)

### B. Reagents

(**a**) *Isoamyl alcohol.*—Reagent grade 3-methyl-1-butanol, bp 128–132°.

(**b**) *Dilute hydrochloric acid.*—(*1*) *20% soln.*—Dil. concd HCl to ca 20% HCl (1+1.85). (*2*) *6N soln.*—Stdze to second decimal place.

(**c**) *Iron std soln.*—100 µg/mL. Dissolve 0.7022 g $Fe(NH_4)_2(SO_4)_2.6H_2O$ in $H_2O$, add 1 mL $H_2SO_4$, and dil. to 1 L.

(**d**) *Molybdenum std solns.*—(*1*) *Stock soln.*—100 µg/mL. Dissolve 0.0920 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in $H_2O$ and dil. to 500 mL. (*2*) *Working soln.*—5 µg/mL. Dil. 25 mL stock soln to 500 mL.

(**e**) *Potassium thiocyanate soln.*—20%. Dissolve 50 g KSCN in $H_2O$ and dil. to 250 mL.

(**f**) *Sodium fluoride saturated soln.*—Add 200 mL $H_2O$ to ca 10 g NaF. Stir until satd and filter.

(**g**) *Stannous chloride solns.*—(*1*) *20% soln.*—Weigh 10 g $SnCl_2.2H_2O$ into beaker, add 10 mL 20% HCl, (**b**)(*1*), and heat until completely dissolved. Cool, add granule of metallic Sn, dil. to 50 mL with $H_2O$, and store in g-s bottle. (*2*) *0.8% wash soln.*—Dil. 4 mL 20% soln to 100 mL with $H_2O$.

### C. Determination

(*Caution*: See safety notes on wet oxidation, nitric acid, and perchloric acid.)

Weigh 1–5 g finely ground sample, contg ≤35 µg Mo, into 200 mL tall-form Pyrex beaker. To 1, 2, or 5 g samples add 10, 15, or 35 mL $HNO_3$, resp. Include 2 beakers for blanks. Cover beaker with cover glass, and let stand ca 15 min; then heat cautiously on steam bath or hot plate at ca 100°, avoiding frothing over top. If froth approaches cover glass, remove beaker from heat until frothing subsides; then continue heating. Digest, usually ca 2 hr, until most of solids disappear.

Cool to room temp. If contents should go to dryness, add few mL $HNO_3$. Add 6 mL 70–72% $HClO_4$, cover beaker, place on hot plate, and gradually raise temp. so that contents boil vigorously but do not bump. Continue heating until digestion is complete as indicated by liq. becoming colorless or pale yellow. If necessary, make repeated addns of $HNO_3$ and $HClO_4$ and continue to digest until C is completely oxidized.

After digestion is complete, place cover glass slightly to one side of top of beaker, or replace it with elevated watch glass, and evap. just to dryness or until residue appears only slightly moist. Remove beaker from hot plate, and cool. Wash down sides of beaker and underside of cover glass with few mL $H_2O$, return to hot plate, and boil few min. Remove from hot plate, cool, and again rinse sides of beaker and cover glass with small amt $H_2O$.

Add 2 drops Me orange and neutze with $NH_4OH$. Add 6*N* HCl, dropwise with stirring, until soln is just acid; then add 8.2 mL excess to give final concn of ca 3% HCl. Add 2 mL satd NaF soln, and 1 mL Fe soln, if sample contains <100 µg Fe.

Transfer soln to 125 mL separator and dil. to 50 mL with $H_2O$. Add 4 mL 20% KSCN soln, mix thoroly, and add 1.5 mL 20% $SnCl_2$ soln. Mix again, and from buret or pipet, add exactly 15 mL isoamyl alcohol. Stopper separator and shake vigorously 1 min, let phases sep., and drain and discard aq. layer. Ext into alcohol without delay, since colored complex is somewhat unstable in aq. soln.

Add 25 mL freshly prepd 0.8% $SnCl_2$ wash soln, and shake gently 15 sec. Let phases sep., and drain and discard aq. layer. Transfer isoamyl alcohol soln to centrf. tube, and centrf. 5 min at ca 2000 rpm to remove $H_2O$ droplets. If alcohol layer is not clear, recentrf. Stopper tubes to prevent evapn, if $A$ readings cannot be made immediately.

Compare unknown solns with isoamyl alcohol at ca 465 nm in spectrophtr, and make appropriate corrections in $A$ readings for Mo in blanks. Obtain Mo concn from calibration curve relating $A$ readings to concns of series of solns of known Mo content.

Prep. calibration curve for instrument used, as follows: Place aliquots of working std soln contg 0, 5, 10, 15, 20, 25, 30, and 35 µg Mo, resp., into 200 mL tall-form beakers and carry them thru entire detn, beginning with digestion with $HNO_3$ and $HClO_4$. Plot $A$ against corresponding Mo concns.

Refs.: JAOAC **36,** 412(1956); **41,** 309(1958); **43,** 510(1960).

CAS-7439-98-7 (molybdenum)

## 925.01★ Potassium and Sodium in Plants

### Gravimetric Method

**Final Action**
**Surplus 1974**

*See* **3.015**, 11th ed.

## 956.01 Potassium and/or Sodium in Plants

### Flame Photometric Method

**Final Action 1965**

### A. Reagents

(**a**) *Potassium stock soln.*—1000 ppm K. Dissolve 1.907 g dry KCl in $H_2O$ and dil. to 1 L.

(**b**) *Sodium stock soln.*—1000 ppm Na. Dissolve 2.542 g dry NaCl in $H_2O$ and dil. to 1 L.

(**c**) *Lithium stock soln.*—1000 ppm Li. Dissolve 6.108 g LiCl in $H_2O$ and dil. to 1 L. (Needed only if internal std method of evaluation is to be used.)

(**d**) *Ammonium oxalate stock soln.*—0.24*N*. Dissolve 17.0 g $(NH_4)_2C_2O_4.H_2O$ in $H_2O$ and dil. to 1 L.

(**e**) *Extracting solns.*—(*1*) *For potassium.*—For internal std method, dil. required vol. LiCl stock soln to 1 L; otherwise use $H_2O$. (*2*) *For sodium.*—To 250 mL $NH_4$ oxalate stock soln add required vol. LiCl stock soln (if internal std method is used) and dil. to 1 L. If internal std requirements are same for both Na and K detns, this reagent may be used as common extg soln.

### *B. Preparation of Standard Solutions*

Dil. appropriate aliquots of stock solns to prep. series of stds contg K and/or Na in stepped amts (including 0) to cover instrument range, and Li and $NH_4$ oxalate (if required) in same concns as in corresponding extg solns. (If common extg soln is used, 1 set of stds contg both K and Na suffices.)

### *C. Sample Extraction*

Transfer weighed portion of finely ground and well mixed sample to erlenmeyer of at least twice capacity of vol. of extg soln to be used. Add measured vol. extg soln, stopper flask, and shake vigorously at frequent intervals during $\geq$15 min. Filter thru dry, fast paper. If paper clogs, pour contents onto addnl fresh paper and combine filtrates. Use filtrate for detn.

*Note:* Do not make exts more concd than required for instrument because there is tendency toward incomplete extn as ratio of sample wt to vol. extg soln increases. Prep. sep. exts for K and Na when their concns in sample differ greatly. For K, use wt sample $\leq$0.1 g/50 mL extg soln; for low Na concns use $\geq$1.0 g/50 mL extg soln; and for higher concns, prep. weaker exts by reducing ratio of sample to extg soln rather than by dilg stronger exts.

### *D. Determination*

(*Caution:* See safety notes on flame photometer.)

Rinse all glassware used in Na detn with dil. $HNO_3$, followed by several portions $H_2O$. Protect solns from air-borne Na contamination.

Operate instrument according to manufacturer's instructions. Permit instrument to reach operating equilibrium before use. Aspirate portions of std solns toward end of warm-up period until reproducible readings for series are obtained.

Run stds, covering concn range of samples involved, at frequent intervals within series of sample soln detns. Repeat this operation with both std and sample solns enough times to result in reliable av. reading for each soln. Plot curves from readings of stds, and calc. % K and/or Na in samples.

Ref.: JAOAC **39,** 419(1956).

CAS-7440-09-7 (potassium)
CAS-7440-23-5 (sodium)

## 935.03★ Potassium in Plants

**Platinic Chloride Method**

**Final Action**
**Surplus 1974**

*See* **3.020**, 11th ed.

## 936.03★ Potassium in Plants

**Perchloric Acid Method**

**Surplus 1974**

*See* **3.021**, 11th ed.

## 935.04★ Potassium in Plants

**Rapid Method for Potassium Only**

**Surplus 1974**

*See* **3.022**, 11th ed.

## 929.03★ Sodium in Plants

**Uranyl Acetate Method**

**Final Action**
**Surplus 1989**

*See* **3.052–3.053**, 14th ed.

## 941.03 Zinc in Plants

**Mixed Color Method**

**Final Action 1965**

### *A. Reagents*

(Redistil all $H_2O$ from Pyrex. Treat all glassware with $HNO_3$ (1+1) or fresh chromic acid cleaning soln. Rinse repeatedly with ordinary distd $H_2O$ and finally with Zn-free $H_2O$.)

**(a)** *Carbon tetrachloride.*—Use ACS grade without purification. If tech. grade is used, dry with anhyd. $CaCl_2$ and redistil in presence of small amt CaO. (Used $CCl_4$ may be reclaimed by distn in presence of NaOH (1+100) contg small amts of $Na_2S_2O_3$, followed by drying with anhyd. $CaCl_2$ and fractional distn in presence of small amts of CaO.) (*Caution:* See safety notes on distillation and carbon tetrachloride.)

**(b)** *Zinc std solns.*—*(1) Stock soln.*—1 mg/mL. Place 0.25 g pure Zn in 250 mL vol. flask. Add ca 50 mL $H_2O$ and 1 mL $H_2SO_4$; heat on steam bath until all Zn dissolves. Dil. to vol. and store in Pyrex vessel. *(2) Working soln.*—10 μg/mL. Dil. 10 mL stock soln to 1 L. Store in Pyrex vessel.

**(c)** *Ammonium hydroxide soln.*—1*N*. With all-Pyrex app. distil $NH_4OH$ into $H_2O$, stopping distn when half has distd. Dil. distillate to proper concn. Store in g-s Pyrex vessel.

**(d)** *Hydrochloric acid.*—1*N*. Displace HCl gas from HCl in glass flask by slowly adding equal vol. $H_2SO_4$ from dropping funnel that extends below surface of the HCl. Conduct displaced HCl gas thru delivery tube to surface of $H_2O$ in receiving flask (no heat is necessary). Dil. to proper concn. Use of 150 mL each of HCl and $H_2SO_4$ will yield 1 L purified HCl soln of concn >1*N*.

**(e)** *Diphenylthiocarbazone* (*dithizone*) *soln.*—Dissolve 0.20 g dithizone in 500 mL $CCl_4$, and filter to remove insol. matter. Place soln in g-s bottle or large separator, add 2 L 0.02*N* $NH_4OH$ (40 mL 1*N* $NH_4OH$ dild to 2 L), and shake to ext dithizone into aq. phase. Sep. phases, discard $CCl_4$, and ext ammoniacal soln of dithizone with 100 mL portions $CCl_4$ until $CCl_4$ ext is pure green. Discard $CCl_4$ after each extn. Add 500 mL $CCl_4$ and 45 mL 1*N* HCl, and shake to ext dithizone into $CCl_4$. Sep. phases and discard aq. phase. Dil. $CCl_4$ soln of dithizone to 2 L with $CCl_4$. Store in brown bottle in dark, cool place.

**(f)** *Ammonium citrate soln.*—0.5*M*. Dissolve 226 g $(NH_4)_2HC_6H_5O_7$ in 2 L $H_2O$. Add $NH_4OH$ (80–85 mL) to pH of 8.5–8.7. Add excess dithizone soln (aq. phase is orange-yellow after phases have been shaken and sepd), and ext with 100 mL portions $CCl_4$ until ext is full green. Add more dithizone if necessary. Sep. aq. phase from $CCl_4$ and store in Pyrex vessel.

**(g)** *Carbamate soln.*—Dissolve 0.25 g Na diethyldithiocarbamate in $H_2O$ and dil to 100 mL with $H_2O$. Store in refrigerator in Pyrex bottle. Prep. fresh after 2 weeks.

**(h)** *Dilute hydrochloric acid.*—0.02*N*. Dil. 100 mL 1*N* HCl to 5 L.

### *B. Preparation of Solutions*

To reduce measuring out reagents and minimize errors due to variations in composition, prep. 3 solns in appropriate amts from reagents and store in Pyrex vessels, taking care to avoid loss of $NH_3$ from *Solns 1* and *2*. Discard solns after 6–8 weeks because Zn increases slowly with storage. Det. std curve for each new set of reagents. Following amts of *Solns 1* and *2* and 2 L dithizone soln are enough for 100 detns:

(1) *Soln 1*.—Dil. 1 L 0.5*M* $NH_4$ citrate and 140 mL 1*N* $NH_4OH$ to 4 L.

(2) *Soln 2*.—Dil. 1 L 0.5 *M* $NH_4$ citrate and 300 mL 1 *N* $NH_4OH$ to 4.5 L. Just before using, add 1 vol. carbamate soln to 9 vols $NH_3$-$NH_4$ citrate soln to obtain vol. of *Soln 2* immediately required.

*Note:* If Zn-free reagents have been prepd, they can be used to test chemicals for Zn. Certain lots of $NH_4OH$ and HCl are sufficiently free of Zn to be used without purification.

### C. Ashing

Ash 5 g finely ground, air-dried plant material in Pt dish in furnace at 500–550°. Include blank detn. Moisten ash with little $H_2O$; then add 10 mL 1*N* HCl (more if necessary) and heat on steam bath until all substances sol. in HCl are dissolved. Add 5–10 mL hot $H_2O$. Filter off insol. matter on 7 cm paper (Whatman No. 42, or equiv., previously washed with two 5 mL portions hot 1*N* HCl, then washed with hot $H_2O$ until HCl-free), and collect filtrate in 100 mL vol. flask. Wash filter with hot $H_2O$ until washings are not acid to Me red. Add 1 drop Me red (dissolve 1 g Me red in 200 mL alcohol), to filtrate in 100 mL flask; neutze with 1*N* $NH_4OH$ and add 4 mL 1*N* HCl. Cool, and dil. to vol. with $H_2O$.

### D. First Extraction

(Sepn of dithizone complex-forming metals from ash soln)

Pipet aliquot of ash soln contg ≤30 μg Zn into 125 mL Squibb separator. Add 1 mL 0.2 *N* HCl for each 5 mL ash soln <10 mL taken, or 1 mL 0.2*N* $NH_4OH$ for each 5 mL >10 mL taken. (10 mL aliquot is usually satisfactory in analysis of plant materials.) Add 40 mL *Soln 1* and 10 mL dithizone reagent. Shake vigorously 30 sec to ext from aq. phase the Zn and other dithizone complex-forming metals that may be present; then let layers sep. At this point excess dithizone (indicated by orange or yellow-orange aq. phase) must be present. If excess dithizone is not present, add more reagent until, after shaking, excess is indicated. Shake down the drop of $CCl_4$ ext from surface, and drain $CCl_4$ ext into second separator as completely as possible without letting any aq. layer enter stopcock bore. Rinse down $CCl_4$ ext from surface of aq. layer with 1–2 mL clear $CCl_4$; then drain this $CCl_4$ into second separator without letting aq. phase enter stopcock bore. Repeat rinsing process as often as necessary to flush ext completely into second separator. Add 5 mL clear $CCl_4$ to first separator, shake 30 sec, and let layers sep. ($CCl_4$ layer at this point will appear clear green if metals that form dithizone complexes have been completely extd from aq. phase by previous extn.) Drain $CCl_4$ layer into second separator and flush ext down from surface and out of separator as directed previously. If last ext does not possess distinct clear color, repeat extn with 5 mL clear $CCl_4$ and flushing-out process until complete extn of dithizone complex-forming metals is assured; then discard aq. phase.

### E. Second Extraction

(Sepn of Cu by extn of Zn into 0.02 *N* HCl)

Pipet 50 mL 0.02*N* HCl into separator contg $CCl_4$ soln of metal dithizonates. Shake vigorously 1.5 min, and let layers sep. Shake down drop from surface of aq. phase, and as completely as possible drain $CCl_4$ phase contg all Cu as dithizonate, without letting any aq. phase, which contains all the Zn, enter stopcock bore. Rinse down $CCl_4$ ext from surface of aq. phase, and rinse out stopcock bore with 1–2 mL portions clear $CCl_4$ (same as in first extn) until all traces of green dithizone have been washed out of separator. Shake down drop of $CCl_4$ from surface of aq. phase, and drain $CCl_4$ as completely as possible without letting any aq. phase enter stopcock bore. Remove stopper from separator and lay it across neck until small amt of $CCl_4$ on surface of aq. phase evaps.

### F. Final Extraction

(Extn of Zn in presence of carbamate reagent)

Pipet 50 mL *Soln 2* and 10 mL dithizone soln into 50 mL 0.02*N* HCl soln contg the Zn. Shake 1 min and let phases sep. Flush out stopcock and stem of separator with ca 1 mL $CCl_4$ ext; then collect remainder in test tube. Pipet 5 mL ext into 25 mL vol. flask, dil. to vol. with clear $CCl_4$, and measure *A* with spectrophtr set at absorption max., ca 525 nm. (*Caution:* Protect final ext from sunlight as much as possible and read within 2 hr.)

Det. Zn present in aliquot from curve relating *A* and concn, correct for Zn in blank, and calc. % Zn in sample.

### G. Standard Curve

Place 0, 5, 10, 15, 20, 25, 30, and 35 mL Zn working std soln in 100 mL vol. flasks. To each flask add 1 drop Me red and neutze with 1 *N* $NH_4OH$; then add 4 mL 1 *N* HCl and dil. to vol. Proceed exactly as for ash solns, beginning with first extn, and using 10 mL aliquots of each of the Zn solns (0, 5, 10, 15, 20, 25, 30, and 35 μg Zn, resp.). Construct std curve by plotting μg Zn against *A*.

Refs.: Ind. Eng. Chem. Anal. Ed. **13,** 145(1941). JAOAC **24,** 520(1941).

CAS-7440-66-6 (zinc)

## 953.04 Zinc in Plants
### Single Color Method
### Final Action 1965

### A. Reagents

*See* **941.03A** and **B** plus following:

(**a**) *Dilute dithizone soln.*—Dil. 1 vol. dithizone soln, **941.03A(e)**, with 4 vols $CCl_4$.

(**b**) *Carbamate soln.*—Dissolve 1.25 g Na diethyldithiocarbamate in $H_2O$ and dil. to 1 L. Store in refrigerator and prep. fresh after long periods of storage.

(**c**) *Dilute ammonium hydroxide.*—Dil. 20 mL 1*N* $NH_4OH$, **941.03A(c)**, to 2 L.

### B. Ashing

Weigh 2 g sample finely ground plant material into well-glazed porcelain, Vycor, or Pt crucible, include crucible for blank detn, and heat in furnace at 500–550° until ashing is complete. Cool, moisten ash with little $H_2O$, add 10 mL 1*N* HCl (more if necessary to ensure excess of acid), and heat on steam bath until all sol. material dissolves. Add few mL hot $H_2O$ and filter thru quant. paper into 200 mL vol. flask. Wash paper with hot $H_2O$ until washings are not acid to Me red. Add 2 drops Me red soln to filtrate, neutze with 1*N* $NH_4OH$, add exactly 3.2 mL 1*N* HCl, dil. to vol. with $H_2O$, and mix.

### C. Formation of Zinc Dithizonate

(Removal of interferences and sepn of excess dithizone)

Pipet aliquot of ash soln contg ≤15 μg Zn into 125 mL amber glass separator. (25 mL aliquot is usually satisfactory.) If necessary to use different vol., add 0.4 mL 0.2*N* HCl for each 5 mL less, or 0.4 mL 0.2 $N$ $NH_4OH$ for each 5 mL more, than 25 mL taken. If <25 mL of the soln is taken, add $H_2O$ to 25 mL.

Add 10 mL dithizone reagent, **941.03A(e)**, to aliquot in separator and shake vigorously 1 min. Let layers sep. and discard $CCl_4$ layer. Add 2 mL $CCl_4$ to aq. soln, let layers sep., and discard $CCl_4$. Repeat this rinsing once. Then add 5 mL $CCl_4$, shake vigorously 15 sec, let layers sep., and discard $CCl_4$. Rinse once more with 2 mL $CCl_4$ as above. Discard $CCl_4$ layer and let $CCl_4$ remaining on surface of soln in funnel evap. before proceeding.

Add 40 mL $NH_4$ citrate *Soln 1*, **941.03B(l)**, 5 mL carbamate soln, **954.04A(b)**, and 25 mL dil. dithizone reagent, **954.04A(a)**. Accurately add carbamate and dithizone reagents from pipet or buret. Shake vigorously 1 min. Let layers sep. and draw off aq. layer thru fine tip glass tube connected to aspirator with rubber tubing. To remove excess dithizone from $CCl_4$ layer, add 50 mL 0.01$N$ $NH_4OH$ and shake vigorously 30 sec.

### D. Determination

Dry funnel stem with pipestem cleaner and flush out with ca 2 mL of the Zn dithizonate soln. Collect adequate portion of remaining soln in 25 mL erlenmeyer, or other suitable container, and stopper tightly. (Amber glass containers are convenient, but colorless glassware will suffice if solns are kept in dark until $A$ readings are made.)

Measure $A$ of each soln against $CCl_4$ with spectrophtr set at absorption max., ca 535 nm. Correct for Zn in blank detns. Calc. amt Zn present in soln from curve relating concn and $A$.

### E. Standard Curve

Into 200 mL vol. flasks place 0, 2, 4, 6, 8, 10, 12, and 14 mL, resp., Zn working std soln. To each flask add 2 drops Me red soln, neutze with 1$N$ $NH_4OH$, add 3.2 mL 1$N$ HCl, and dil. to vol. with $H_2O$. Pipet 25 mL aliquots of each of these solns, contg 0, 2.5, 5, 7.5, 10, 12.5, 15, and 17.5 $\mu$g Zn, resp., into amber glass separators, and proceed as for ash solns, **954.04C**, beginning with second par. Det. $A$ of each soln and plot values against corresponding amts Zn.

Ref.: JAOAC **36,** 397(1953).

CAS-7440-66-6 (zinc)

## NONMETALS

## 930.06 Arsenic in Plants
**Titrimetric Method**
**Final Action**

Prep. soln as in **963.21C**. Proceed as in **963.21D**, or take aliquot and det. as in **925.02**, beginning ". . . add 3 mL $H_2SO_4$ . . ."

CAS-7440-38-2 (arsenic)

## 958.03 Boron in Plants
**Quinalizarin Method**
**First Action 1958**
**Final Action 1965**

### A. Reagents

(**a**) *Dilute sulfuric acid.*—0.36 $N$. Dil. 10 mL $H_2SO_4$ to 1 L.

(**b**) *Calcium hydroxide saturated soln.*—Filter before use.

(**c**) *Quinalizarin soln.*—Dissolve 45 mg quinalizarin in 1 L 95–96% $H_2SO_4$.

(**d**) *Boron std soln.*—0.5 mg B/mL. Dissolve 2.860 g $H_3BO_3$ and dil. to 1 L with $H_2O$. Prep. working stds by further diln with $H_2O$.

### B. Determination

Place 1.00–2.00 g dry, ground plant material in Pt or $SiO_2$ dish. Add 5 mL satd $Ca(OH)_2$ soln and dry at 105°. Carefully volatilize over burner, ash in furnace 1 hr at 600°, and cool. Add exactly 10 or 15 mL 0.36 $N$ $H_2SO_4$, break up ash with glass rod, stir gently, and filter. Transfer 2 mL filtrate to colorimeter tube, add an exact amt (e.g., 15 mL) quinalizarin reagent, stopper, and mix by swirling gently. Let stand at room temp. 24 hr (or until both unknowns and stds have cooled to same temp.). Shake tube again immediately before reading in photoelec. colorimeter (620 nm filter).

Adjust colorimeter to 100% $T$ with blank soln prepd as above but using 2 mL $H_2O$ in place of sample soln. Prep. std curve with series of stds contg 0.5–10 $\mu$g B/mL.

Ref.: JAOAC **41,** 304(1958).

CAS-7440-42-8 (boron)

## 928.04★ Chloride in Plants
**Gravimetric Method**
**Final Action**
**Surplus 1989**

*See* **3.069–3.070**, 14th ed.

## 915.01 Chloride in Plants
**Volumetric Method I**
**Final Action**

(Since precision of this titrn is considered to be ±0.2 mg Cl, accuracy of 1.0% requires samples contg ≥20 mg.)

### A. Reagents

(**a**) *Silver nitrate std soln.*—1 mL = 0.00355 g Cl. Prep. soln slightly stronger than 0.1$N$, stdze as in **941.18E**, and adjust to exactly 0.1$N$.

(**b**) *Ammonium or potassium thiocyanate std soln.*—0.1$N$. Prep. soln slightly stronger than 0.1 $N$, stdze as in **941.18D(b)**, and adjust to exactly 0.1 $N$.

(**c**) *Ferric indicator.*—Satd soln of $FeNH_4(SO_4)_2.12H_2O$.

(**d**) *Nitric acid.*—Free from lower oxides of N by dilg $HNO_3$ with ca 1/4 vol. $H_2O$, and boiling until perfectly colorless.

### B. Determination

To prepd soln, **928.04A**, add known vol. std $AgNO_3$ soln in slight excess. Stir well, filter, and wash AgCl ppt thoroly. To combined filtrate and washings add 5 mL ferric indicator and few mL $HNO_3$, and titr. excess Ag with thiocyanate std soln to permanent light brown. From mL $AgNO_3$ used, calc. % Cl.

Refs.: Sutton, "Systematic Handbook of Volumetric Analysis," 11th ed., 1924, p. 146. J. Am. Chem. Soc. **37,** 1128(1915).

CAS-7782-50-5 (chlorine)

## 935.05 Chloride in Plants
### Volumetric Method II
### Final Action

#### A. Reagents

(**a**) *Potassium iodide std soln.*—1 mL = 1 mg Cl. Weigh 4.6824 g pure (ACS) KI, dried to const wt at 105–150°, dissolve in $H_2O$, and dil. to 1 L.

(**b**) *Silver nitrate stock soln.*—Approx. 0.3*N*. 1 mL = ca 10 mg Cl. Dissolve 48 g $AgNO_3$ in $H_2O$, filter, and dil. to 1 L.

(**c**) *Silver nitrate std soln.*—Dil. 100 mL reagent (**b**) to ca 900 mL and adjust by stdzg against reagent (**a**) so that 1 mL = 1 mg Cl.

(**d**) *Chloride-free starch indicator.*—For each 100 mL final soln take 2.5 g sol. starch and make to paste with cold $H_2O$. Stir out lumps, add 25–50 mL more cold $H_2O$, and stir or shake 5 min. Centrf., decant, and discard liq. Repeat extn 3 times and finally transfer residue to flask contg proper amt of boiling $H_2O$. Stir again, heat to bp, cover with small beaker, and cool under tap, shaking occasionally.

(**e**) *Dilute sulfuric acid.*—Add 35 mL $H_2SO_4$ to each 1 L $H_2O$, boil 5–10 min, and cool to room temp.

(**f**) *Iodine indicator.*—To ca 20 g I in 500 mL g-s bottle add 400 mL dil. $H_2SO_4$, (**e**), and shake 10 min. Decant and discard first soln, since it may contain iodides. Repeat process and store soln in small g-s bottles.

(**g**) *Potassium permanganate soln.*—Dissolve 60 g $KMnO_4$ in 400 mL warm $H_2O$ (ca 50°) and dil. to 1 L.

(**h**) *Potassium sulfate-copper sulfate mixture.*—Thoroly mix 16 parts $K_2SO_4$ and 1 part $CuSO_4.5H_2O$.

(**i**) *Wash soln.*—Mix 980 mL $H_2O$ and 20 mL $HNO_3$.

#### B. Determination

(*Caution:* See safety notes on wet oxidation, nitric acid, and permanganates.)

Weigh sample contg 10–40 mg Cl into beaker. (If >4 g is taken, use proportionately more $HNO_3$ and $KMnO_4$ soln.) Add 10 mL 0.3*N* $AgNO_3$ and stir until sample is thoroly soaked, adding little $H_2O$ or warming if necessary. Add 25 mL $HNO_3$, stir, add 5 mL $KMnO_4$ soln, and stir until frothing stops. Place mixt. in $H_2O$ bath or on hot plate and keep just below bp. Stir, and wash down sides of beaker at intervals with min. $H_2O$. After 20 min, or when reaction stops, add addnl $KMnO_4$ soln, little at time, until color begins to fade slowly. Dil. to ca 125 mL with boiling $H_2O$ and heat 10 min longer. (Beaker may stand in bath or on hot plate until ready to filter.)

Filter while hot thru Whatman No. 5, or equiv. paper, with suction as follows: Place disk of 30-mesh stainless steel wire gauze or No. 40 filter cloth in bottom of 3″ (7.6 cm) Hirsch funnel. Fold 9 cm paper over bottom of No. 11 rubber stopper, shaping it to funnel by making 9–10 folds up side of stopper. Place paper in funnel and apply strong suction. Wet paper and keep wet while fitting into funnel so as to avoid double thicknesses of paper. Wash paper thoroly, first with $H_2O$ and then with wash soln. Discard washings and rinse out flask. Decant thru filter and transfer ppt and sample residue to filter. If filtrate is not turbid, or if it is only slightly opalescent, wash ppt thoroly, applying wash soln very gently, but keeping strong suction on filter. If combined filtrate and washings are clear, test for Ag. If turbid, reheat and pass thru filter, repeating until clear, and finally wash as above. If filtrate does not give definite test for Ag, repeat detn on smaller sample.

Place paper and contents in Kjeldahl flask and add such amts of $K_2SO_4$-$CuSO_4$ mixt. and $H_2SO_4$ as would be appropriate for protein detn on same kind and amt of sample, and digest similarly. (For 2 g grass, 8 g sulfate mixt. and 20 mL acid are enough.) When digest is cool, add 175 mL $H_2O$, boil 5–10 min, and cool to room temp. Titr. the $Ag_2SO_4$ in Kjeldahl flask with KI std soln, using 5 mL starch indicator and 30 mL I indicator. (Add latter just before titrn.) Rinse neck of flask after each addn of KI when near end point and titr. until soln stays blue after shaking. If <30 mg Cl is present, add starch and I solns at beginning. If larger but unknown amt is present, add 2 mL starch and 10 mL I indicator at beginning and titr. until end point approaches. Shake vigorously to coagulate ppt, add rest of starch and I solns, and proceed to end point. If known large amt is present, titr. to within 2 mL of end point, shake as above, add indicator reagents, and continue titrn. If end point is overrun, add 5 mL std $AgNO_3$ soln and titr. again.

Blank detns are not necessary after testing reagents. If blank made by using pure sugar as sample is >0.05 mg, examine filter paper, distd $H_2O$, and various reagents for Cl.

Refs.: JAOAC **18,** 379(1935); **19,** 72(1936).

CAS-7782-50-5 (chlorine)

## 975.04 Fluoride in Plants
### Potentiometric Method
### First Action 1975
### Final Action 1988

(Rinse all plastic and glass containers with HCl (1+3) and $H_2O$ before use. Perform analyses in laboratory free from F; prep. samples in another laboratory.)

#### A. Principle

F is extd from dry, pulverized foliage with $HNO_3$ followed by aq. KOH. Slurry is adjusted to pH 5.5, and complexing agent and background F are added. Potential is measured with ion selective electrode and compared against calibration curve. Method is applicable to 10–2000 μg F/g dry wt leaf tissue not exposed to unusual amts of Al or other F-binding agents; it is not applicable to insol. inorg. F or F in org. combinations. Between-laboratory precision of individual analyses is ±20% at 30 ppm F; ±10%, ≥100 ppm F. Accuracy is 90–100%.

#### B. Apparatus

*Electrometer.*—Range ±200 mv with readability of 0.1 mv (Model 701 or 701A (replacement model SA720) digital pH/mv meter, Orion Research Inc., or equiv.) or expanded scale pH meter with mv mode of operation, with F ion selective electrode (No. 94–09 single electrode, Orion Research Inc., or equiv.) and reference electrode (No. 90–01 single junction, Orion Research Inc., or equiv.). Check system at intervals to assure adherence to following performance criteria: Using technic of **975.04D**, system should reach equilibrium ($\Delta$ E <0.2 mv/min) within 5 min with each F working std soln, checked in following sequence: 0.1, 0.2, 0.5, 2.0, and 10.0 ppm F. Replicate std solns should differ by ≤1 mv. Calibration curve should be linear between 0.2 and 10.0 ppm and slope should be 57±2 mv per 10-fold change in F concn. If any parameter is not obtained, check electrodes, reagents, and electrometer. Maintain temp. control of ±1°.

#### C. Reagents

(Store all solns in tightly closed, plastic bottles.)

(**a**) *Nitric acid.*—(*1*) *10N.*—Add 63 mL $HNO_3$ to $H_2O$, cool, and dil. to 100 mL. (*2*) *0.2N.*—Dil. 5.0 mL 10*N* to 250 mL. (*3*) *0.05N.*—Dil. 5.0 mL 10*N* to 1 L.

(**b**) *Potassium nitrate soln.*—0.4*M*. Dissolve 4.0 g $KNO_3$ in $H_2O$ and dil. to 100 mL.

(**c**) *Sodium citrate soln.*—0.8*M*. Dissolve 58.8 g Na citrate.$2H_2O$ in 200 mL $H_2O$, adjust to pH 5.5 by dropwise addn of 10*N* $HNO_3$, using pH meter, and dil. to 250 mL with $H_2O$.

(**d**) *Sodium citrate with fluoride soln.*—0.4*M* citrate with 1 ppm F. Dil. 125 mL 0.8*M* Na citrate soln and 25.0 mL 10 ppm F std soln to 250 mL with $H_2O$.

(**e**) *Fluoride std solns.*—(*1*) *Stock soln.*—100 ppm F. Dry ca 1 g NaF 2 hr at 110°. Accurately weigh 0.221 g NaF, dissolve in $H_2O$, and dil. to 1 L. (*2*) *Intermediate soln.*—10 ppm F. Dil. 10.0 mL stock soln to 100 mL with $H_2O$. (*3*) *Working soln.*—Prep. as in Table **975.04** in 100 mL vol. flasks. Prep. 0.2 and 0.1 ppm solns fresh as needed.

### *D. Preparation of Calibration Curve*

Place 25.0 mL 0.1 ppm F working std soln into plastic container contg stirring bar. Insert electrodes ca 12 mm into soln and stir mag. Record mv readings at 1 min intervals until change is <0.2 mv/min. Remove electrodes, blot lightly with absorbent paper, and repeat reading with 0.2, 0.5, 2.0, and 10.0 ppm std solns. Place electrodes in 0.2 ppm std soln until samples are analyzed. (10 ppm std soln may be omitted if samples are known to contain <400 ppm F.)

Plot potential (mv) on vertical arithmetic axis and F concn (μg/mL; ppm) on horizontal (logarithmic) axis of 2-cycle semilog graph paper.

### *E. Preparation of Sample*

Dry foliage 48 hr at 80°. Grind to pass No. 40 sieve and store in clean, dry, tightly closed plastic bottle. Rotate bottle to mix sample thoroly before removing aliquots.

### *F. Determination*

Accurately weigh ca 0.25 g powd sample, and place in 75–100 mL wide-mouth plastic container. Add 20 mL 0.05*N* $HNO_3$ and place on rotating shaker or stir mag. 20 min. Add 20 mL 0.1*N* KOH (5.6 g/L) and agitate addnl 20 min. Add 5.0 mL Na citrate soln contg 1 ppm F, adjusted to pH 5.5, and 5.0 mL 0.2*N* $HNO_3$. (Samples may be stored covered ≤4 hr at this point.) Det. mv readings as in **975.04D** and prep. calibration curve before and after each series of samples.

If sample series contains mixt. of high and low samples, make preliminary estn of F content after 2 min. Then det. F concn in samples contg <40 ppm first and in higher ones last.

$$\text{ppm F } (\mu g/g) = (C - 0.10) \times 50/w$$

where $C$ = ppm F from curve; 0.10 = ppm background F in final soln; 50 = mL final soln; and $w$ = g sample.

Ref.: JAOAC **58,** 1129(1975).

CAS-7782-41-4 (fluorine)

## 984.02 Fluoride in Plants
### Willard–Winter Distillation Method
### Final Action

*See* **944.08**.

## 978.03 Fluoride in Plants
### Semiautomated Method
### First Action 1978
### *ASTM-Intersociety Committee-AOAC Method*

### *A. Principle*

Dried and ground plant material is ashed, fused with alkali, and dild to vol. In case of leaf samples, F on external surfaces may be washed off sep. Digest and $H_2SO_4$ are pumped into microdistn app. maintained at 170°. Stream of air carries acidified sample to fractionation column where F and $H_2O$ are distd into condenser, and condensate passes into small collector. Distillate is mixed continuously with alizarin F blue-lanthanum reagent, colored stream passes thru tubular flowcell of colorimeter, and $A$ is measured at 624 nm.

Interfering metal cations and inorg. phosphate are not distd, and org. substances are destroyed by ashing. Interference from remaining volatile inorg. anions is reduced with high concn acetate buffer with some reduction in sensitivity. Very large amts solid matter, particularly silicates, retard distn. Therefore, smallest sample consistent with obtaining suitable amt F should be used. Conditions must be carefully controlled, since accurate results depend upon obtaining same degree of efficiency of distn from samples as from std F solns used for calibration.

Acid concn during distn is maintained at const value by using specific amts CaO and NaOH for ashing and fusion and $HClO_4$ for transfer of fused samples. Any marked change in vac. (>0.2″ Hg or 5 torr) over short time indicates either leak or block in system. Distil at same vac. each day and maintain proper ratio between air flow on line drawing liq. and solid wastes from distn coil and on line drawing HF and $H_2O$ vapor from distn unit (Fig. **978.03A**). Adjust flowmeters to keep this ratio const and to maintain higher vac. on HF line, $C_1$, so that min. is diverted to waste line.

Method can detect 0.1 μg F/mL. Normal range is 0.1–4.0 μg F/mL. Dil. higher concns with NaOH-$HClO_4$ soln, (**k**). If digested samples routinely exceed 4.0 μg/mL, modify anal. portion of pump manifold to reduce sensitivity, or use smaller sample aliquot (preferred). Most accurate results are obtained in middle or upper part of calibration curve. For example, to decrease sensitivity, pump sample thru 0.081″ tube (2.5 mL/min) and dil. with $H_2O$ pumped thru 0.065″ tube (1.6 mL/min) before sample enters distn app. Total vol. sample and diluent should approx. original vol. used (4.1 mL/min).

If air-borne contaminants are present in laboratory, attach small drying bulb contg $CaCO_3$ granules to air inlet tube of microdistn unit. Teflon distn coil of microdistn unit must be cleaned periodically to avoid accumulation of solids which reduce sensitivity.

Coefficient of variation of 20–100 ppm F is generally ≤10%. Samples with large amt of Si (orchard grass) or Al may present special difficulties. There should be no significant deviation from linearity with different amts sample and with different amts added F.

### *B. Apparatus*

(Cat. Nos refer to current Technicon equipment, except where indicated. Corresponding equipment under previous Cat. Nos is satisfactory.)

(**a**) *Automatic analyzer.*—(Fig. **978.03B**) AutoAnalyzer, Technicon Instruments Corp., or equiv. (*1*) *Sampler.*—Sampler IV with rotary stirrer and 8.5 mL plastic sample cups. Use 10 or 20/hr cam with 1:3 sample-to-wash ratio (No. 171-

**Table 975.04 Preparation of Working Standard Solutions**

| Concn, ppm | mL soln to be dild to 100 mL: 0.4 *M* $KNO_3$ | 0.8 *M* Na citrate | 100 ppm F soln | 10 ppm F soln |
|---|---|---|---|---|
| 10 | 10.0 | 5.0 | 10.0 | 0.0 |
| 2 | 10.0 | 5.0 | 2.0 | 0.0 |
| 0.5 | 10.0 | 5.0 | 0.0 | 5.0 |
| 0.2 | 10.0 | 5.0 | 0.0 | 2.0 |
| 0.1 | 10.0 | 10.0 mL Na citrate soln contg 1 ppm F | | |

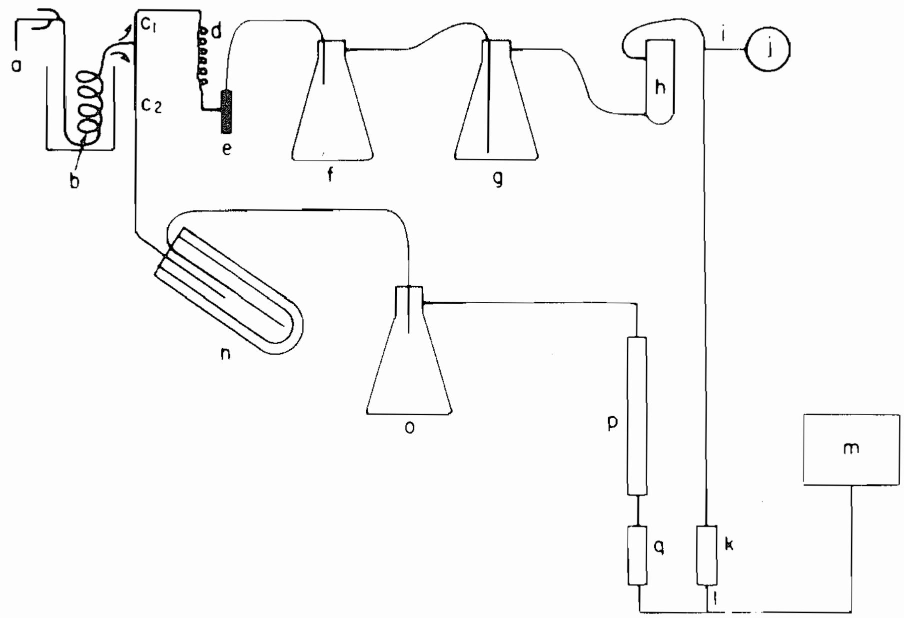


**FIG. 978.03A—Schematic drawing of air flow system used in semiautomated analysis for fluoride**

A015-07). (*2*) *Colorimeter.*—With 15 mm tubular flowcell and 624 nm interference filter (199-A001-05). (*3*) *Recorder.*—Ratio type with 2–100 mv full scale range (011-A115-01). (*4*) *Multichannel proportioning pump and manifold cartridge.*—With assorted pump tubes, nipple connectors, and glass connectors (pump III 113-A014-08; cartridge 116-8340-01).

(**b**) *Pulse suppressors.*—For sample and color reagent streams. Coil 10′ length of 0.035″ id Teflon std tubing around 2.5″ diam. tube. Force outlet end into short length of 0.045″ id Tygon tubing which is then sleeved with piece of 0.081″ Tygon tubing. Slip sleeved end over "h" fitting which joins sample and reagent streams. (Pulse suppressor included with manifold cartridge.)

(**c**) *Voltage stabilizer.*—161-A007-01 (also part of 199-A001-05).

(**d**) *Rotary vacuum and pressure pump.*—With continuous oiler.

(**e**) *Microdistillation apparatus.*—(Fig. **978.03C**) Major components are (Cat. Nos. are those of Lurex Scientific, except as noted); (*1*) Bottom only of jacketed 1 L resin reaction flask with conical flange (JR-5130), modified by evacuating space between inner and outer walls and sealing off port (*a*); (*2*) resin reaction flask top with conical flange (JR-7935) modified to have one ₮ 29/42 center joint and four ₮ 24/40 side joints; (*3*) resin reaction flask clamp (JR-9210-0000); (*4*) variable high-speed stirrer (S-6362) (*d*); (*5*) stainless steel, heavy

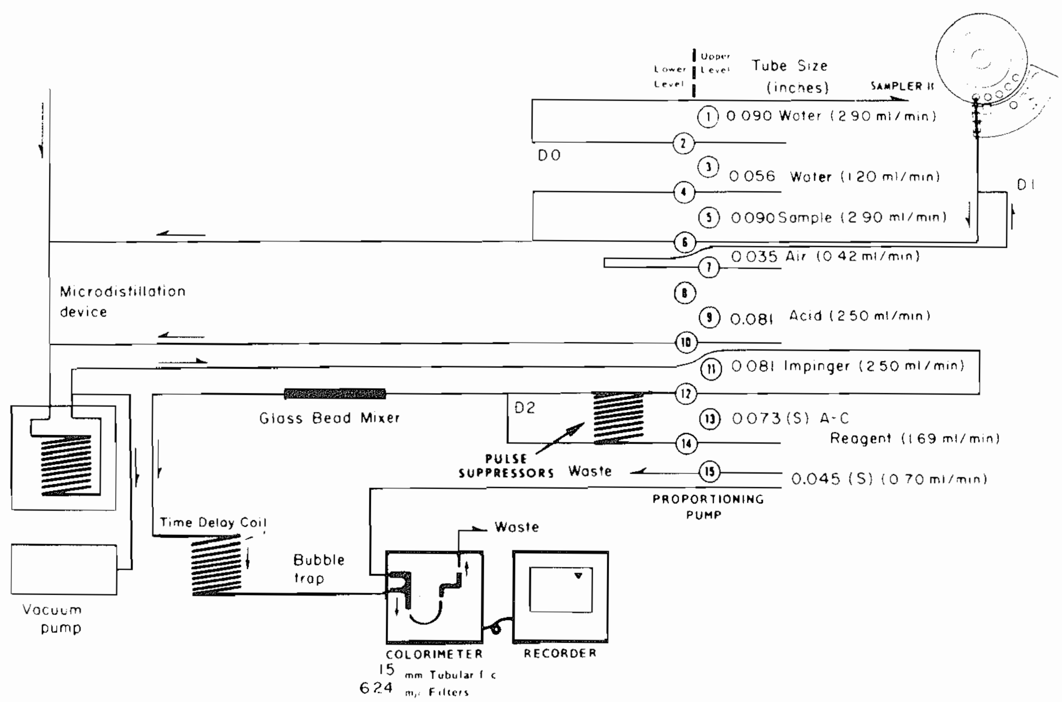


**FIG. 978.03B—Flow diagram for semiautomated analysis for fluoride**

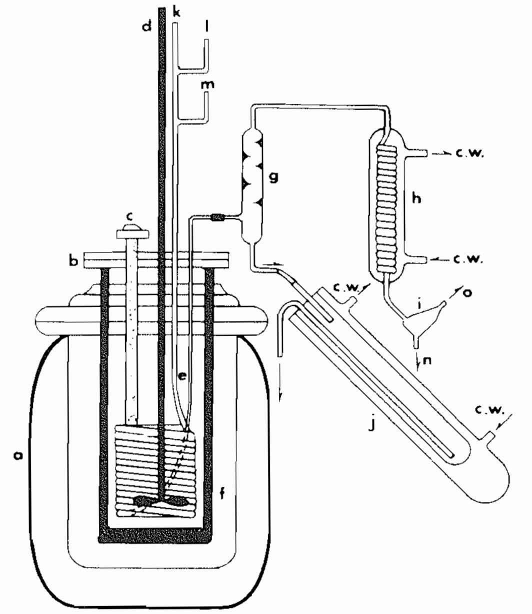


**FIG. 978.03C—Schematic drawing of microdistillation apparatus**

duty stirrer stuffing box with ⊤ 29/42 and shredded Teflon packing (JS-1160-0102 and JS-3050-0000); (*6*) 10 mm diam. stainless steel stirrer rod with propeller to fit stuffing box; (*7*) thermometer-thermoregulator, range 0–200° (*c*); (*8*) electronic relay control box; (*9*) low drift immersion heater, 750 watts (*b*); (*10*) 30′ length coil of flexible Teflon TFE tubing, $^1/_8$″ id, $^3/_{16}$″ od, 0.030″ wall, on rigid support of such diam. that completed coil will fit into resin reaction flask (avoid kinking of tubing) (*e*); (*11*) 2 flowmeters with ranges 0.15–1.00 and 0.6–5.0 L/min, both with needle valve controls (Dwyer Instruments, Inc., PO Box 373, Michigan City, IN 46360); (*12*) vac. gage with range 0–10″ Hg or 0–254 torr (mm Hg); (*13*) fractionation column of borosilicate glass (*g; see also* Fig. **978.03D**; (*14*) distillate collector; (*15*) $H_2O$-jacketed condenser (*h*); (*16*) Dow-Corning 200 fluid (100 centistokes at 25°) (*f*); and (*17*) condenser (*j*).

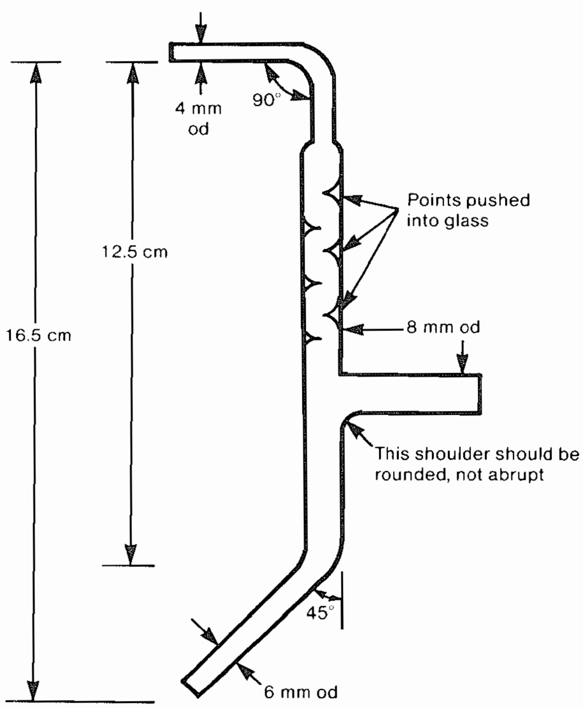


**FIG. 978.03D—Microdistillation column**

(**f**) *Crucibles*.—Inconel, Ni, or Pt, 40–50 mL.

(**g**) *Air flow system*.—(Fig. **978.03A**) Draw air thru air inlet tube, (*a*), before Teflon microdistn coil, (*b*). Air sweeps thru (*b*) to fractionation column, and is diverted into 2 channels. In channel $c_1$, air passes thru $H_2O$-jacketed condenser, (*d*), sample trap, (*e*), to waste bottle, (*f*). Air then passes thru $^1/_8$″ id glass tube directed against surface of $H_2SO_4$ in waste bottle, (*g*). Partially dehydrated air passes thru gas drying tower, (*h*), contg 450 g indicating silica gel. Emerging air passes thru T-tube, (*i*), connected to vac. gage, (*j*) (0–10″ Hg or 0–254 torr), thru flowmeter, (*k*) (0–5 L/min), thru T-tube, (*l*), and then to vac. pump, (*m*).

In channel $c_2$, air passes thru $H_2O$-jacketed waste trap, (*n*), to waste bottle, (*o*). Air leaving waste bottle flows thru drying bulb, (*p*), filled with indicating silica gel, and the dry air then passes thru flowmeter, (*q*) (0–1 L/min). Air stream then connects thru T-tube, (*l*), with air from first channel.

### *C. Reagents*

(*Caution:* See safety notes on perchloric acid and sulfuric acid.)

(Deionized $H_2O$ may be used. CaO for ashing and NaOH for fusion must be low in F.)

(**a**) *Sulfuric acid*.—(1+1). Mix 500 mL $H_2SO_4$ with 500 mL $H_2O$ and cool before use.

(**b**) *Acetate buffer*.—2.14*M* (pH 4.0). Dissolve 60 g $NaOAc.3H_2O$ in 500 mL $H_2O$, add 100 mL HOAc, and dil. to 1 L with $H_2O$. Stable at 25°.

(**c**) *Alizarin fluorine blue color reagent stock soln*.—0.01*M*. Suspend 0.9634 g reagent (alizarin complexone, alizarin complexan; 3-amino-ethylalizarin-*N,N*-diacetic acid; Burdick & Jackson Laboratories, Inc.) in ca 100 mL $H_2O$ in 250 mL vol. flask. Add 2 mL $NH_4OH$ and shake until completely dissolved. Add 2 mL HOAc and dil. to vol. with $H_2O$. Stable indefinitely at 4°.

(**d**) *Lanthanum nitrate stock soln*.—0.02*M*. Dissolve 8.6608 g $La(NO_3)_3.6H_2O$ in $H_2O$ and dil. to 1 L in vol. flask.

(**e**) *Wetting soln*.—30% soln (w/v) polyoxyethylene lauryl ether in $H_2O$ (Brij-35, Technicon No. T21-0110). Soln is stable at 25°.

(**f**) *Working reagent*.—Mix, in order listed, 300 mL acetate buffer, 244 mL $H_2O$, 300 mL acetone, 100 mL *tert*-butanol, 36 mL alizarin fluorine blue stock soln, 20 mL La $(NO_3)_3$ stock soln, and 2 mL wetting soln. Unused reagent is stable 7 days at 4°. Before using reagent, place under vac. 10 min to remove air bubbles from soln.

(**g**) *Fluoride std solns*.—(*1*) *Stock soln*.—100 μg F/mL. Dissolve 0.2207 g NaF in $H_2O$ and dil. to 1 L. (*2*) *Working solns*.—Prep. 7 solns contg 0.2, 0.4, 0.8, 1.6, 2.4, 3.2, and 4.0 μg F/mL. Before dilg to vol., add 6 g NaOH and 20 mL 70% $HClO_4$ for each 100 mL final working soln so that stds have same composition as sample solns. Dil. with $H_2O$ only for analysis of $H_2O$ samples or air samples absorbed in $H_2O$. Store working solns at 4° in polyethylene bottles; stable in presence of NaOH.

(**h**) *EDTA solns*.—1%. Dissolve 1 g $Na_4EDTA$ in 99 mL $H_2O$. Prep. 0.05% and 0.01% solns by mixing 5 mL 1% soln with 95 mL $H_2O$ and 1 mL 1% soln with 99 mL $H_2O$, resp.

(**i**) *Phenolphthalein soln*.—Dissolve 1 g phthln in 50 mL absolute alcohol or isopropanol and add 50 mL $H_2O$.

(j) *Detergent*.—Alconox (Alconox, Inc., 215 Park Ave S, New York, NY 10003); available from laboratory supply firms.

(k) *Sodium hydroxide-perchloric acid soln*.—Dissolve 6 g NaOH in $H_2O$, add 40 mL 70% $HClO_4$ (1+1), and dil. to 100 mL with $H_2O$. Use to dil. samples when F in unknown sample exceeds std curve.

### D. Preparation of Sample

(a) *Leaves*.—If it is necessary to remove surface F, wash sample with aq. soln contg 0.05% detergent and 0.05% $Na_4EDTA$ in polyethylene container 30 sec with gentle agitation. Remove, drain 3–4 sec, and rinse 10 sec in each of 3 beakers of $H_2O$. Discard solns after use.

(b) *Fresh plant tissues*.—Dry 24–48 hr in 80° forced-draft oven, and grind as in (c).

(c) *Dry plant tissues*.—Grind in semimicro Wiley mill to pass No. 40 sieve, and store in plastic container.

### E. Ashing and Fusion

Accurately weigh 0.1–2.0 g well mixed dried plant tissue into crucible. Add 100±10 mg low-F CaO, enough $H_2O$ to make loose slurry, and 4 drops phthln soln. Mix thoroly with polyethylene policeman. Final mixt. should be purple and remain purple during evapn to dryness.

Place crucible on cold hot plate and under IR lamp. Evap. under lamp to dryness, turn on hot plate, and char 1 hr. Transfer crucible to furnace at 600° and ash 2 hr. (*Caution:* To avoid flaming, place crucibles at front of furnace with door open ca 5 min to further char samples; then reposition in furnace.)

Remove crucibles, add 3.0±0.1 g NaOH pellets, and replace in furnace with door closed to melt NaOH. (*Caution:* Avoid creeping of molten NaOH.) Remove crucibles individually and swirl to suspend particulate matter until melt is partially solidified. Let cool until addn of small amt $H_2O$ does not cause spattering. Wash down inner walls with 10–15 mL $H_2O$. Suspend melt with polyethylene policeman and transfer with $H_2O$ to plastic tube graduated at 50 mL. Rinse crucible with 20.0 mL 70% $HClO_4$ (1+1), add rinse to tube, and dil. to 50 mL with $H_2O$. Solns can be stored at this point if tightly capped.

Analyze blank contg all reagents with each set of ca 10 samples.

Clean crucibles as soon as possible after each use. Boil Inconel crucibles 1 hr in 10% NaOH soln. Rinse with hot tap $H_2O$, detergent, and then distd $H_2O$. Immerse crucibles which held samples contg >100 μg F in 4*N* HCl 45 min before boiling in NaOH soln. Perform blank analyses on these crucibles before addnl use to check for contamination. Scrub Ni and Pt crucibles with detergent and hot $H_2O$ and rinse thoroly with $H_2O$. Briefly rinse crucibles which held samples contg >100 μg F in 4*N* HCl before rinsing with $H_2O$.

### F. Analytical System

Place F std solns, ashed and fused samples, or impinged air samples in 8.5 mL plastic cups in sample module. Actuate sampler and pump from cup at net rate of 2.48 mL/min with air segmentation of 0.42 mL/min after sampler crook, and pump into microdistn device thru sample inlet (*l*, Fig. **978.03C**), using 0.051″ id Teflon tubing. Pump $H_2SO_4$ at 2.5 mL/min thru acid inlet (*m*, Fig. **978.03C**). Cool and discard acid and solids. Pump distillate from sample trap at 2.0 mL/min thru 0.051″ Teflon tubing, add color reagent at 1.69 mL/min, and mix in 4″ length of 1/8″ id glass tubing packed with pieces of 20 mesh broken Pyrex glass. Pass colored stream thru time delay coil of 15′ of 0.035″ Teflon spaghetti tubing, thru debubbler fitting where small portion of stream and bubbles are removed to waste bottle at rate of 0.70 mL/min, and thru 15 mm tubular flowcell of colorimeter. *A* is measured at 624 nm and plotted on recorder. Lag time from sampling to appearance of peak is ca 5 min. Time between samples is 6 min with sampling rate of 10/hr and 3 min at 20/hr.

### G. Start-Up

Turn on $H_2O$ to condenser and cooling jacket. Turn on colorimeter. Engage manifold on proportioning pump and start pump. Turn on stirring motor of microdistn unit, vac. pump adjusted for full vac., and heater of microdistn unit. Connect lines to $H_2SO_4$, color reagent, and $H_2O$ bottles. Sampling tube of sampler unit should be in $H_2O$ reservoir. Equilibrate app. until silicone oil in microdistn unit reaches 170±2°. Check that all connections are secure. Adjust distn flowmeter (*k*, Fig. **978.03C**) to 2.5–3 L/min; adjust waste flowmeter (*q*) to 0.3 L/min. Distillate should now fill sample trap. Readjust flowmeter (*k*) to give reading on vac. gage of 5–6″ Hg (127–150 torr). (Satisfactory setting for app. must be detd by trial and error. Once detd, use each day.) No air bubbles should be in anal. system beyond point where color reagent and distillate streams are joined. Turn on recorder, adjust baseline to desired level, and run several min to assure that all components are operating properly. Baseline should be reasonably smooth and straight.

Transfer F std solns to 8.5 mL plastic cups and place in sampler. Sep. last std soln from sample solns with cup of $H_2O$. Program sampler for 10 samples/hr (90 sec sampling period, 270 sec washout period) or 20 samples/hr (45 sec sampling period, 135 sec washout period).

Prep. std curve, **978.03J**, before and after each day's set of samples. Net *A* of 0.7–0.9 should be obtained with std soln contg 4 μg F/mL. *A* of each std soln should be reproducible within 10% from day to day and std curve should be linear from 0.2 to ≥3.2 μg/mL.

### H. Shut-Down

Turn off chart recorder. Disconnect $H_2SO_4$ line and place in $H_2O$. Disconnect color reagent line and place in 0.01% EDTA soln ca 1 min; then transfer line to $H_2O$ and let $H_2O$ pass thru system ca 5 min. Clean Teflon distn coil as in **978.03I(a)**. Turn off heater and stirrer of microdistn unit. Turn off vac. pump. Release pump tube manifold. Turn off $H_2O$ to condenser and cooling trap.

### I. Maintenance

(a) *Cleaning of Teflon distillation coil*.—(After use with samples contg particulate matter.) Briefly insert Tygon tube connected to air inlet line of microdistn unit into 0.01% EDTA soln. After all deposited material has been removed, wash with 3–4 five mL portions distd $H_2O$.

(b) *Pump tubes*.—Replace after 200 working hr or earlier if hard and inflexible or flattened. Always leave in relaxed position when not in use. Remove dirt and grease from pump plates and rollers after each day of use.

(c) *Indicating silica gel*.—Regenerate when ca 2/3 has lost normal blue color.

(d) *Cleaning tubing*.—Clean tubing contg reagent after each daily run with 0.01% EDTA soln followed by distd $H_2O$.

(e) *Monthly checks*.—Oil proportioning pumps monthly. Check gain on recorder monthly and adjust.

### J. Calibration and Standards

Before and after each day's set of samples, prep. std curve by transferring aliquots of each working std soln to 8.5 mL sample cups and proceed with analysis. Draw straight line connecting baseline before and after analysis. Record *A* of each peak and subtract *A* of baseline at peak. Plot net *A* against μg F/mL.

### K. Calculations

$$\text{ppm F in sample} = (F \times V \times D)/W$$

where $F$ = µg F/mL sample from std curve; $V$ = mL sample, usually 50; $D$ = diln factor used only when F of sample exceeds std curve = mL final vol. to which original aliquot was dild with NaOH-$HClO_4$ soln, (**k**)/mL original aliquot taken; and $W$ = g sample taken for analysis.

### L. Check Procedure

(**a**) *Contamination.*—Perform reagent and equipment blank with crucibles and reagents but without sample to detect contamination from previous samples, contaminated furnace, and reagents. Blank values >5 µg F are evidence of contamination. Perform 2 blank detns with each set of 20–40 samples. Usual blanks are 1–3 µg.

(**b**) *Recoveries.*—Occasionally add known amts F std soln from microburet to aliquots of low F tissue. Recovery of added F should be 100±10%. Low values indicate loss of F, possibly during pretreatment; high values indicate contamination.

(**c**) *Linearity.*—Occasionally analyze different amts (0.1–2.0 g) plant sample contg 50–65 ppm F. Linear relationship should exist between F found and amt tissue taken. Nonlinearity may indicate that some component of tissue is retarding distn or interfering with color development.

(**d**) *Calibration curves.*—Prep. at least twice daily.

### M. Trouble Shooting

(**a**) *Irregular baseline.*—May result from: (*1*) excessive pulse pressures—check for faulty pump tubes, absence of surge suppressors, or improperly made or placed suppressors; (*2*) air bubbles in flowcell—check for absence of debubbler bypass, blockage in reagent pump tube, or periodic emptying of sample trap (latter results if air flow to distn trap becomes too great); (*3*) excessive $H_2SO_4$ carry-over—check for too high temp. in oil bath, improper $H_2SO_4$ concn, or too high vac. on system; (*4*) air flow imbalances—check flowmeter settings, trapped air in tubing, or leak or block in system; (*5*) high F content in samples (baseline may not return to normal between samples)—dil. or check sampling speed and sample-to-wash ratio.

(**b**) *Irregular peaks.*—Asymmetrical or double peaks or peaks with shoulders may result from: (*1*) baseline irregularities; (*2*) interfering substances from sample or impure reagents; (*3*) inadequate buffer concn; or (*4*) excessive amts solids in distn coil. Presence or accumulation of solids may be due to insufficient flow of $H_2SO_4$, too large sample, excess CaO or NaOH in sample, inadequate suspension of particles in samples, or lack of proper air segmentation in sample tubing.

(**c**) *Poor reproducibility.*—Check for: improper sample pickup; faulty pump tubes; inadequate washing of distn coil between samples; large deviations in acid concn, temp., or air flow in distn coil; or changes in vac. on waste system.

Refs.: JAOAC **55,** 991(1972); **61,** 150(1978).

CAS-7782-41-4 (fluorine)

## 966.01 Phosphorus in Plants

**Gravimetric Quinolinium Molybdophosphate Method**
**Final Action 1974**

### A. Preparation of Solution

Accurately weigh ca 2 g plant sample in porcelain dish, and add 7.5 mL $Mg(NO_3)_2$ soln (dissolve 950 g P-free $Mg(NO_3)_2.6H_2O$ in $H_2O$ and dil. to 1 L). Dry in oven 2 hr at 110–115° (or until dry). Ignite carefully over Fisher burner, or equiv., until bubbling and smoking cease. Complete ashing in furnace 4 hr at 550–600°. Dissolve ash in few mL HCl (2+1) and evap. to dryness on steam bath. Take up residue in 10–15 mL HCl (1+9) and filter thru coarse paper into 200 mL vol. flask. Wash paper thoroly with $H_2O$ and let filtrate cool to room temp. Dil. to vol. with $H_2O$.

### B. Determination

Pipet 40 mL aliquot into 300 or 500 mL erlenmeyer and proceed as in **962.02C**.

Ref.: JAOAC **49,** 284(1966).

## 933.01★ Phosphorus in Plants

**Macro Method**
**Final Action**
**Surplus 1974**

*See* **3.065**, 13th ed.

## 931.01 Phosphorus in Plants

**Micro Method**
**Final Action**

### A. Reagents

(**a**) *Phosphorus std soln.*—0.025 mg P/mL. Dissolve 0.4394 g pure dry $KH_2PO_4$ in $H_2O$ and dil. to 1 L. Dil. 50 mL of this soln to 200 mL.

(**b**) *Ammonium molybdate soln.*—Dissolve 25 g $NH_4$ molybdate in 300 mL $H_2O$. Dil. 75 mL $H_2SO_4$ to 200 mL and add to $NH_4$ molybdate soln.

(**c**) *Hydroquinone soln.*—Dissolve 0.5 g hydroquinone in 100 mL $H_2O$, and add 1 drop $H_2SO_4$ to retard oxidn.

(**d**) *Sodium sulfite soln.*—Dissolve 200 g $Na_2SO_3$ in $H_2O$, dil. to 1 L, and filter. Either keep this soln well stoppered or prep. fresh each time.

### B. Preparation of Solution

To 1 or 2 g sample in small porcelain crucible add 1 mL $Mg(NO_3)_2$ soln (dissolve 950 g P-free $Mg(NO_3)_2.6H_2O$ in $H_2O$ and dil. to 1 L), and place on steam bath. After few min, cautiously add few drops HCl, taking care that gas evolution does not push portions of sample over edge of crucible. Make 2 or 3 further addns of few drops HCl while sample is on bath so that as it approaches dryness it tends to char. If contents become too viscous for further drying on bath, complete drying on hot plate. Cover crucible, transfer to cold furnace, and ignite 6 hr at 500°, or until even gray ash is obtained. (If necessary, cool crucible, dissolve ash in little $H_2O$ or alc.-glycerol, evap. to dryness, and return uncovered to furnace 4–5 hr longer.) Cool, take up with HCl (1+4), and transfer to 100 mL beaker. Add 5 mL HCl and evap. to dryness on steam bath to dehydrate $SiO_2$. Moisten residue with 2 mL HCl, add ca 50 mL $H_2O$, and heat few min on bath. Transfer to 100 mL vol. flask, cool immediately, dil. to vol., mix, and filter, discarding first portion of filtrate.

### C. Determination

To 5 mL aliquot filtrate in 10 mL vol. flask add 1 mL $NH_4$ molybdate soln, rotate flask to mix, and let stand few sec. Add 1 mL hydroquinone soln, again rotate flask, and add 1 mL $Na_2SO_3$ soln. (Last 3 addns may be made with Mohr pipet.) Dil. to vol. with $H_2O$, stopper flask with thumb or forefinger, and shake to mix thoroly. Let stand 30 min, and measure $A$ with spectrophtr set at 650 nm. Report as % P.

Refs.: JAOAC **14,** 216(1931). J. Biol. Chem. **59,** 255(1924).

CAS-7723-14-0 (phosphorus)

## 936.04★ Selenium in Plants

**Gravimetric Method**

**Final Action**
**Surplus 1974**

(Applicable to materials contg >2 ppm Se)

*See* **3.073**, 11th ed.

## 969.06 Selenium in Plants

**Fluorometric Method**

**First Action 1969**
**Final Action 1974**

(*Caution:* See safety notes on photofluorometer, wet oxidation, nitric acid, and perchloric acid.)

### A. Apparatus

(**a**) *Micro-Kjeldahl flasks.*—30 mL Pyrex, ca 170 mm total length with ₮ 12/18 outer joint at mouth.

(**b**) *Air condensers.*—10 × 140 mm Pyrex tubes with ₮ 12/18 inner joint.

(**c**) *Micro-Kjeldahl digestion unit with glass fume duct.*—Fit rack to hold flasks and attached air condensers in nearly upright position during early stages of digestion. Use in fume hood.

(**d**) *Fluorometer.*—Capable of illuminating sample at 369 nm and measuring fluoresced light at 525 nm. Spectrofluorometer set to above wavelengths is also satisfactory.

### B. Reagents

(Use deionized $H_2O$ distd in glass for prepg solns and dilns.)

(**a**) *Nitric acid.*—Redistd in glass.

(**b**) *Hydroxylamine-ethylenediaminetetraacetic acid soln.*—Add ca 20 mL $H_2O$ to 1.9 g EDTA (acid form). Slowly add ca 5*N* $NH_4OH$ with stirring until EDTA just dissolves. Some excess $NH_4OH$ is not harmful. Dissolve 6 g $NH_2OH.HCl$ in 100 mL $H_2O$. Combine solns and dil. to 250 mL with $H_2O$.

(**c**) *Cresol red indicator.*—Dissolve 0.1 g cresol red in 10 mL $H_2O$ and 1 drop 50% NaOH soln. Dil. to 50 mL with $H_2O$.

(**d**) *Selenium std soln.*—0.3 μg Se/mL. Add 10 mL $HNO_3$ to 30.0 mg Se (purity ≥99%) and warm to dissolve. Dil. to 100 mL with $H_2O$, mix well, and transfer exactly 1 mL to micro-Kjeldahl flask. Add 2 mL 70% $HClO_4$ and 1 glass bead. Boil gently to $HClO_4$ fumes and cool. Add 1 mL $H_2O$ and 1 mL HCl (1+4); heat 30 min in boiling $H_2O$ bath. Transfer to 1 L vol. flask and dil. to vol. with ca 1*N* HCl. Store in all-glass container. Soln is stable several months at room temp.

(**e**) *Decalin.*—Eastman Kodak No. 1905 decahydronaphthalene, or equiv.

(**f**) *2,3-Diaminonaphthalene* (*DAN*) *soln.*—Prep. soln in semidarkened room or in room with only yellow light at time of detn. Protect from light and prep. fresh for each set of detns. Add 50 mL ca 0.1 *N* HCl to 0.05 g DAN (No. 13,653-0, Aldrich Chemical Co.). Place in 50° $H_2O$ bath in dark 15 min. Cool to approx. room temp. and ext twice with 10 mL decalin, shaking vigorously each time and discarding decalin. Filter thru paper satd with $H_2O$. For >8 detns, prep. larger amt.

### C. Preparation of Samples

Grind air-dried samples to pass No. 18 or finer sieve. Cut fresh or wet samples finely with scissors or knife, or grind in food chopper to assure representative sample.

Some plants (e.g., *Astragalus bisulcatus, A. racemosus, Stanleya bipinnata,* and *Oonopsis condensata*) contain Se in volatile form that is lost during drying. Analyze these plants without drying. With usual agricultural crops, this is not a problem if drying is performed at 60–70°.

### D. Preparation of Fluorometric Blanks and Standard

(**a**) *Blank.*—Place 1 mL $H_2O$ in micro-Kjeldahl flask. (For samples contg <0.1 ppm, carry 10 mL $HNO_3$ as blank thru entire detn.)

(**b**) *Std.*—Place 1.0 mL Se std soln in micro-Kjeldahl flask.

Add 2 mL 70% $HClO_4$ to each flask and continue as in detn, beginning "Mix contents of flasks . . ."

### E. Determination for Low Level Samples

*For samples containing ≤4 ppm selenium.*—Weigh ≤1 g sample (air-dried wt basis) contg ≤0.4 μg Se into micro-Kjeldahl flask. Add 1 glass bead, previously cleaned with $HNO_3$. Add 10 mL $HNO_3$ and let stand at room temp. ≥4 hr. (Use 5 mL $HNO_3$ for samples <0.5 g.) Affix air condenser and place flask in nearly upright position on micro-Kjeldahl digestion unit. Heat ca 15 min with low flame and then increase heat until $HNO_3$ condenses in lower part of condenser. Heat 10 min longer, turn off burner, and let cool 5 min. Wash down sides of flask with 2 mL 70% $HClO_4$ thru air condenser. Swirl flask and continue refluxing 15 min. Remove condenser and continue heating, drawing off fumes in fume duct, until $HClO_4$ fumes appear and then 15 min longer. Cool, add 1 mL $H_2O$, and again heat to $HClO_4$ fumes and 1–2 min longer. Cool, and add 1 mL $H_2O$.

Mix contents of flasks and add 1 mL HCl (1+4) to each. Place in boiling $H_2O$ bath 30 min. Cool to ca room temp.

To each flask add 5 mL $NH_2OH$-EDTA soln and 2 drops cresol red indicator. Neutze to yellow with ca 5 *N* $NH_4OH$ and add HCl (1+4) to orange-pink. *From this point, perform all operations in semidarkened room or room with yellow light only.* Prep. DAN soln, add 5 mL to each flask, and dil. to neck with ca 0.1 *N* HCl. Mix and place in 50° $H_2O$ bath in dark 25 min.

Remove flasks from $H_2O$ bath and cool to ca room temp. in pan of $H_2O$. Pour solns into 125 mL separators with Teflon stopcocks and contg 10.0 mL decalin. Shake vigorously ≥30 sec, let stand ca 1 min, and drain and discard lower layer. Wash decalin twice by shaking vigorously ≥15 sec with 25 mL ca 0.1 *N* HCl. (VirTis, Rt 208, Gardiner, NY 12525, Extractomatic shaker with 100 mL separators may be substituted. When used, shake ext 5 min and wash 1 min periods.) Transfer decalin layer to 12 mL centrf. tubes and centrf. 2 min at moderate speed. Pour decalin soln into fluorometer tubes, zero fluorometer against decalin, and read all tubes at 525 nm within 5 min. Correct std and unknown readings for blank.

$$\text{ppm Se} = 0.3 \times \text{sample reading}/\text{std reading} \times \text{g sample}$$

### F. Determination for Higher Level Samples

*For samples containing >4 ppm selenium.*—Proceed as above, thru second par. Dil. digest to adequate vol. and take aliquot contg ca 0.3 μg Se for detn. Alternatively, digest sample in 10 vols $HNO_3$ 2 hr on steam bath. Dil. to definite vol., and carry appropriate aliquot thru detn. Latter method is especially applicable when proper sampling requires large sample. Do not dil. decalin soln contg piazselenol, as this introduces errors.

Ref.: JAOAC **52,** 627(1969).

CAS-7782-49-2 (selenium)

## 920.10 Sulfur in Plants
### Sodium Peroxide Method
### Final Action

(*Caution:* See safety notes on sodium peroxide.)

#### *A. Preparation of Solution*

Place 1.5–2.5 g sample in ca 100 mL Ni crucible and add 5 g anhyd. $Na_2CO_3$. Mix thoroly, using Ni or Pt rod, and moisten with ca 2 mL $H_2O$. Add $Na_2O_2$, ca 0.5 g at time, mixing thoroly after each addn, and continue until mixt. becomes nearly dry and quite granular (ca 5 g $Na_2O_2$). Place crucible over S-free flame or elec. hot plate and heat carefully, stirring occasionally, until contents are fused. (If material ignites, detn is worthless.)

After fusion, remove crucible, let cool somewhat, and cover hardened mass with more $Na_2O_2$ to depth of ca 5 mm. Heat gradually and finally with full flame until fusion again takes place, rotating crucible occasionally to bring any particles adhering to sides into contact with oxidizing material. Continue heating 10 min after fusion is complete. Cool somewhat, place warm crucible and contents in 600 mL beaker, and carefully add ca 100 mL $H_2O$. After initial violent action ceases, wash material out of crucible, make slightly acid with HCl (adding small portions at time), transfer to 500 mL vol. flask, cool, dil. to vol., and filter.

#### *B. Determination*

Dil. aliquot of prepd soln to ca 200 mL with $H_2O$ and add HCl until ca 0.5 mL free acid is present. Heat to bp and add 10 mL 10% $BaCl_2$ soln dropwise with constant stirring. Continue boiling ca 5 min, and let stand $\geq$5 hr in warm place. Decant thru ashless paper or ignited and weighed gooch. Add 15–20 mL boiling $H_2O$ to ppt, transfer to filter, and wash with boiling $H_2O$ until filtrate is Cl-free. Dry ppt and filter, ignite, and weigh as $BaSO_4$. Wt ppt $\times$ 0.1374 = S.

Refs.: USDA Bur. Chem. Bull. **105,** p. 151; **116,** p. 92; **137,** p. 30.

CAS-7704-34-9 (sulfur)

## 923.01 Sulfur in Plants
### Magnesium Nitrate Method
### Final Action

#### *A. Preparation of Solution*

Weigh 1 g sample into large porcelain crucible. Add 7.5 mL $Mg(NO_3)_2$ soln (dissolve 950 g P-free $Mg(NO_3)_2.6H_2O$ in $H_2O$ and dil. to 1 L), so that all material comes in contact with soln. (It is important that enough $Mg(NO_3)_2$ soln be added to ensure complete oxidn and fixation of the S present. For larger samples and for samples with high S content, proportionally larger vol. of this soln must be used.) Heat on elec. hot plate (180°) until no further action occurs. Transfer crucible while hot to furnace ($\leq$500°) and let it remain until sample is thoroly oxidized. (No black particles should remain. If necessary, break up sample and return to furnace.) Remove crucible and let cool. Add $H_2O$; then HCl in excess. Bring soln to boil, filter, and wash thoroly. If preferred, transfer soln to 250 mL vol. flask before filtering and dil. to vol. with $H_2O$.

#### *B. Determination*

Dil. entire filtered soln, **923.01A**, to 200 mL, or take 100 mL aliquot of the measured vol., dil. to 200 mL, and proceed as in **920.10B**.

Ref.: JAOAC **6,** 415(1923).

## OTHER CONSTITUENTS

## 931.02 Sugars in Plants
### Preparation of Sample
### Final Action

#### *A. Preparation of Solution*

(**a**) *General method.*—Prep. fresh sample as in **922.02(b)**. Pour alc. soln thru filter paper or extn thimble, catching filtrate in vol. flask. Transfer insol. material to beaker, cover with 80% alcohol, warm on steam bath 1 hr, let cool, and again pour alc. soln thru same filter. If second filtrate is highly colored, repeat extn. Transfer residue to filter, let drain, and dry. Grind residue so that all particles will pass thru 1 mm sieve, transfer to extn thimble, and ext 12 hr in Soxhlet app. with 80% alcohol. Dry residue and save for starch detn. Combine alc. filtrates and dil. to vol. at definite temp. with 80% alcohol.

For dried materials, grind samples finely, and mix well. Weigh sample into beaker, and continue as above, beginning ". . . cover with 80% alcohol, . . ."

(**b**) *Applicable when starch is not to be determined.*—Prep. fresh sample as in **922.02(b)**, but boil on steam bath 1 hr. Decant soln into vol. flask, comminute solids in high-speed blender with 80% alcohol. Boil blended material on steam bath 0.5 hr, cool, transfer to vol. flask, dil. to mark with 80% alcohol at room temp., filter, and take aliquot for analysis.

Grind dry material to pass No. 20 sieve or finer, transfer weighed sample to vol. flask, and add 80% alcohol and enough $CaCO_3$ to neutze any acidity. Boil 1 hr on steam bath, cool, adjust vol. at room temp. with 80% alcohol, filter, and take aliquot for analysis.

#### *B. Clarification with Lead (1)*

Place aliquot alc. ext in beaker on steam bath and evap. off alcohol. Avoid evapn to dryness by adding $H_2O$ if necessary. When odor of alcohol disappears, add ca 100 mL $H_2O$ and heat to 80° to soften gummy ppts and break up insol. masses. Cool to room temp. and proceed as in (**a**) or (**b**):

(**a**) Transfer soln to vol. flask, rinse beaker thoroly with $H_2O$, and add rinsings to flask. Add enough *satd neut. $Pb(OAc)_2$ soln* to produce flocculent ppt, shake thoroly, and let stand 15 min. Test supernate with few drops of the $Pb(OAc)_2$ soln. If more ppt forms, shake and let stand again; if no further ppt forms, dil. to vol. with $H_2O$, mix thoroly, and filter thru dry paper. Add enough solid Na oxalate to filtrate to ppt all the Pb, and refilter thru dry paper. Test filtrate for presence of Pb with little solid Na oxalate.

(**b**) Add twice min. amt of satd neut. $Pb(OAc)_2$ soln required to cause complete pptn, as found by testing portion of supernate with few drops dil. Na oxalate soln. Let mixt. stand only few min; then filter into beaker contg estd excess of Na oxalate crystals. Let Pb ppt drain on filter and wash with cold $H_2O$ until filtrate no longer gives ppt in oxalate soln. Assure excess of oxalate by testing with 1 drop $Pb(OAc)_2$. Filter and wash pptd Pb oxalate, catching filtrate and washings in vol. flask. Dil. to vol. with $H_2O$ and mix.

#### *C. Clarification with Ion-Exchange Resins (2)*

Place aliquot alc. ext, **931.02A**, in beaker and heat on steam bath to evap. alcohol. Avoid evapn to dryness by adding $H_2O$. When odor of alcohol disappears, add ca 15–25 mL $H_2O$ and heat to 80° to soften gummy ppts and break up insol. masses. Cool to room temp. Prep. thin mat of Celite on filter paper in buchner or on fritted glass filter and wash until $H_2O$ comes thru clear. Filter sample thru Celite mat, wash mat with $H_2O$, dil. filtrate and washings to appropriate vol. in vol. flask, and mix well.

Place 50.0 mL aliquot in 250 mL erlenmeyer; add 2 g *Amberlite IR-120(H)* analytical grade cation (replaced by REXYN 101(H) resin, Fisher Scientific Co.) and 3 g *Duolite A-4(OH)* anion ion exchange resins. Let stand 2 hr with occasional swirling. Take 5 mL aliquot deionized soln and det. reducing sugars as glucose as in **959.11B**.

Refs.: (1) JAOAC **14,** 73, 225(1931); **15,** 71(1932).
(2) JAOAC **36,** 402(1953).

## 933.02 Glucose in Plants

**Micro Method**

**Final Action 1965**

*See* **959.11B**.

## 960.06 Fructose in Plants

**Somogyi Micro or Munson-Walker Method**

**First Action 1960**
**Final Action 1961**

### *A. Reagents*

(**a**) *Glucose oxidase preparation.*—Add slowly, stirring constantly, 100 mL $H_2O$ to 5 g glucose oxidase prepn ("DeeO L-750" code 4633000, Miles Laboratories, Inc., 1127 Myrtle St, PO Box 70, Elkhart, IN 46514). Stir ca 1 min and centrf. or filter to obtain clear soln. Add ca 1 mL $CHCl_3$ and refrigerate. Soln is stable ≥1 month.

(**b**) *McIlvaine's citrate-phosphate buffer.*—Dissolve 214.902 g $Na_2HPO_4.12H_2O$ and 42.020 g citric acid in $H_2O$ and dil. to 1 L.

### *B. Determination*

To suitable aliquot add $^1/_4$ its vol. of buffer to give pH ca 5.8. Add 30% as much glucose oxidase prepn as estd glucose content (for 500 mg glucose add 150 mg glucose oxidase, *i.e.*, 3 mL soln), and few drops 30% $H_2O_2$ (omit if Somogyi method is to be used in detn). Let stand overnight at room temp.

Det. fructose by Somogyi micro method, **959.11B**, or by Munson-Walker method, **906.03**, using Table **960.06**. Check equivs in range of interest, using pure fructose as std, and correct as necessary.

Refs.: JAOAC **41,** 307, 681(1958); **42,** 650(1959); **43,** 512(1960); **44,** 267(1961).

## 906.01 Sugars (Reducing) in Plants

**Munson-Walker General Method**

**Final Action**

*See* **906.03**.

**Table 960.06 Abbreviated Munson and Walker Table for Calculating Fructose**

*(From Official and Tentative Methods of Analysis, AOAC, 5th Ed., 1940)*

| Cuprous Oxide, mg | Fructose, mg | Cuprous Oxide, mg | Fructose, mg |
|---|---|---|---|
| 10 | 4.5 | 300 | 148.6 |
| 50 | 23.5 | 350 | 174.9 |
| 100 | 47.7 | 400 | 201.8 |
| 150 | 72.2 | 450 | 229.2 |
| 200 | 97.2 | 490 | 253.9 |
| 250 | 122.7 | — | — |

## 921.03★ Sugars (Reducing) in Plants

**Quisumbing-Thomas Method**

**Final Action**
**Surplus 1970**

*See* **31.048–31.049**, 11th ed.

## 930.07 Sucrose in Plants

**Inversion Methods**

**Final Action**

### *A. Hydrochloric Acid Inversion*

Using aliquot of cleared soln, **931.02B**, proceed as in **925.05**.

### *B. Invertase Inversion*

(**a**) *For plants giving hydrolysis end point within 2 hours.*—Pipet aliquot of cleared soln, **931.02B**, into 400 mL Pyrex beaker and make slightly acid to Me red with HOAc. Add 3 drops 1% soln of *Wallerstein invertase scales*. Let mixt. stand at room temp. 2 hr. Add reagents as in **923.09B**, and det. reducing power. Calc. results as invert sugar. Deduct reducing power of original soln, also expressed as invert sugar, and multiply difference by 0.95.

(**b**) *For plants giving slower hydrolysis end point.*—Place aliquot of soln, **931.02B**, in small vol. flask. Make slightly acid to Me red with HOAc. Add 3 drops 1% soln of *Wallerstein invertase scales* and few drops toluene. Stopper flask and let stand overnight or longer at room temp. Dil. to vol. with $H_2O$ and use aliquot for reducing power as above.

CAS-57-50-1 (sucrose)

## 930.09 Ether Extract of Plants

**Gravimetric Method**

**Final Action**

*See* **920.39B**.

## 930.10 Fiber (Crude) in Plants

**Digestion Method**

**Final Action**

*See* **962.09**.

## 978.04 Nitrogen (Total) (Crude Protein) in Plants

**Kjeldahl Methods**

**First Action 1976**
**Final Action 1978**

### *A. Kjeldahl Method for Nitrate-free Samples*

*See* **955.04**.

### *B. Kjeldahl Method for Nitrate-Containing Samples*

*See* **968.01**.

## 977.02 Nitrogen (Total) (Crude Protein) in Plants

**First Action 1977**

### *A. Automated Method*

*See* **976.05**.

### B. Semiautomated Method

*See* **976.06**.

## 948.02 Starch in Plants

### Titrimetric Method

### Final Action 1965

### A. Reagents

(**a**) *Iodine-potassium iodide soln.*—Grind 7.5 g I and 7.5 g KI with 150 mL $H_2O$, dil. to 250 mL, and filter.

(**b**) *Alcoholic sodium chloride soln.*—Mix 350 mL alcohol, 80 mL $H_2O$, and 50 mL 20% NaCl soln, and dil. to 500 mL with $H_2O$.

(**c**) *Alcoholic sodium hydroxide soln.*—0.25*N*. Mix 350 mL alcohol, 100 mL $H_2O$, and 25 mL 5*N* NaOH, and dil. to 500 mL with $H_2O$.

(**d**) *Dilute hydrochloric acid.*—0.7*N*. Dil. 60 mL HCl to 1 L with $H_2O$.

(**e**) *Somogyi phosphate sugar reagent.*—Dissolve 56 g anhyd. $Na_2HPO_4$ and 80 g Rochelle salt in ca 1 L $H_2O$, and add 200 mL 1.00*N* NaOH. Then slowly add, with stirring, 160 mL 10% $CuSO_4.5H_2O$ soln. Dissolve 360 g anhyd. $Na_2SO_4$ in this soln, transfer to 2 L vol. flask, and add exactly 200 mL 0.1*N* $KIO_3$ soln (3.5667 g/L). Dil. to vol., mix well, let stand several days, and filter thru dry paper into dry flask, discarding first 50 mL filtrate. Store reagent at 20–25°. It is 0.01*N* with respect to $KIO_3$; 5.00 mL is equiv. to 10 mL 0.005*N* $Na_2S_2O_3$.

Det. glucose factor of reagent as follows: Accurately weigh 150 mg NBS glucose SRM into 1 L vol. flask, dissolve in $H_2O$, dil. to vol., and mix well. Transfer 5 mL aliquot to 25 × 200 mm Pyrex test tube, add exactly 5 mL Somogyi reagent, stopper with size 00 crucible, and heat (together with several blanks contg 5 mL $H_2O$ and 5 mL reagent) exactly 15 min in boiling $H_2O$ bath. Titr. as in detn. From difference between blank and std titrns, calc. mg glucose equiv. to 1 mL exactly 0.005*N* $Na_2S_2O_3$. Effective range for detn is 0.05–1.0 mg glucose in 5 mL aliquot.

(**f**) *Sodium thiosulfate std soln.*—0.005 *N*. Dissolve 2.73 g $Na_2S_2O_3.5H_2O$ in $H_2O$ and dil. to 2 L. Stdze daily as follows: Add 1 mL KI soln, (**g**), and 3 mL 1.5 *N* $H_2SO_4$ to 5 mL Somogyi sugar reagent. Let stand 5 min, and titr. with $Na_2S_2O_3$ soln, adding starch indicator, (**h**), just before end point.

(**g**) *Potassium iodide soln.*—2.5%. Stabilize with little $Na_2CO_3$.

(**h**) *Starch indicator.*—Make 1.5 g sol. starch into paste with few mL $H_2O$, and add slowly, with stirring, to 300 mL boiling $H_2O$.

### B. Determination

Select sample as in **922.01**, remove all foreign matter, dry, and grind to pass No. 80 sieve. Accurately weigh 0.1–1.0 g powd sample contg ca 20 mg starch into 25 × 150 mm Pyrex test tube. Add ca 200 mg fine sand and 5 mL $H_2O$, and mix well with stirring rod to wet sample. Heat tube in boiling $H_2O$ bath 15 min to gelatinize starch. Cool to room temp., and place in 22–25° bath. Add 5 mL 60% $HClO_4$ rapidly with const agitation. Grind tissue against lower wall of tube with stirring rod for approx. min at time. Repeat grinding frequently during 30 min; then without delay, transfer quant. to 100 mL vol. flask with $H_2O$. Add 3 mL 5% *uranyl acetate soln* to ppt protein, dil. to vol. with $H_2O$, mix well, and centrf. portion of mixt. Pipet 10 mL clear supernate into 25 × 150 mm test tube. Add ca 100 mg Celite, 5 mL 20% NaCl soln, and 2 mL I-KI reagent, and mix well. Let stand overnight, centrf., and decant.

Wash starch-I ppt by suspending it in 5 mL alc. NaCl soln, centrf., and decant. Add 2 mL alc. NaOH soln to packed ppt. Gently shake and tap tube until ppt is no longer blue. (Do not use stirring rod; allow ample time for complex to decompose.) Wash walls of tube with 5 mL alc. NaCl soln, centrf. liberated starch, and wash with 5 mL alc. NaCl soln as before. Add 2 mL 0.7 *N* HCl to ppt. Stopper tube loosely with size 00 crucible, and heat 2.5 hr in boiling $H_2O$ bath. (Bath should have cover with holes to accommodate tubes; holes not occupied by tubes must be covered.) Cool, and transfer quant. to 25 mL vol. flask. Add drop phenol red, **941.17A**, and neutze with 1 *N* NaOH. Discharge color with *0.1 N oxalic acid,* dil. to vol., and mix well. Transfer 5 mL aliquot to 25 × 200 mL Pyrex test tube, add exactly 5 mL Somogyi reagent, and stopper tube with size 00 crucible. Heat together with several blanks contg 5 mL $H_2O$ and 5 mL Somogyi reagent in vigorously boiling $H_2O$ bath exactly 15 min. Remove tube from bath and cool to 25–30°. Add 1 mL 2.5% KI soln down wall of tube without agitation and then add 3 mL 1.5 *N* $H_2SO_4$ rapidly with agitation. After all $Cu_2O$ dissolves, titr. soln with 0.005 *N* $Na_2S_2O_3$, adding starch indicator, (**h**), just before end point is reached. Treat blank solns similarly.

$$\% \text{ Starch} = [50(\text{mL blank} - \text{mL sample}) \times 0.90/\text{mg sample}] \times (N/0.005) \times G \times 100$$

where 50 = diln factor, 0.90 = factor glucose to starch, $N$ = actual normality $Na_2S_2O_3$ soln, and $G$ = mg glucose equiv. to 1 mL 0.005 *N* $Na_2S_2O_3$.

Refs.: Anal. Chem. **20**, 850(1948). JAOAC **39**, 423(1956).

## 932.01 Lignin in Plants

### Direct Method

### Final Action 1965

### A. Preparation of Sample

Grind sample in mill to pass No. 80 sieve and dry at 105°. Ext weighed sample (5–10 g) 30 hr in Soxhlet app. with alcohol-benzene soln (32 parts alcohol and 68 parts benzene by wt). Dry material in oven to free it from solvs and place in flask of suitable size. Add 150 mL $H_2O$/g sample, and reflux 3 hr. Filter mixt, while still hot, preferably thru weighed fritted glass crucible, and transfer extd material to flask. Add 1% HCl (111 g concd HCl + 3890 mL $H_2O$) in proportion of 150 mL acid soln/g plant material, and reflux 3 hr. Filter mixt. while still hot thru fritted glass crucible previously used, wash with $H_2O$ until acid-free, dry at 105°, and weigh. Calc. % total loss due to successive extn with alcohol-benzene soln, hot $H_2O$, and 1% HCl. (With samples not especially rich in carbohydrates and proteins, extn with hot $H_2O$ may be omitted.)

### B. Apparatus

App., Fig. **932.01**, consists of: (1) 1.5 L bottle, *A*, to which is attached by 2-hole rubber stopper 250 mL dropping funnel, *C*, having lower end of stem bent as illustrated and placed close to bottom of *A*; (2) Drechsel gas-washing bottle, *D*; (3) 3 Pyrex test tubes, 38 × 300 mm diam., *G, G′, G″*, connected in parallel by device, *O*, and immersed in wooden box, *L*, filled with crushed ice, *H*; and (4) bottle contg $H_2O$ for absorption of excess HCl, *K*. *G, G′*, and *G″* are provided with 2-hole rubber stoppers; glass tube with right angle bend extends thru 1 hole nearly to bottom of test tube, and similar tube extending ca 10 mm into test tube passes thru other hole. Rubber connections and stopcocks for regulating flow of gas are provided as indicated in diagram. *A* is filled with ca 500 mL $H_2SO_4$ and *C* with HCl; HCl flowing thru stopcock *B* into *A* generates HCl gas, which is dried by $H_2SO_4$ in *D*, and flows into *G, G′*, and *G″* contg samples and fuming HCl reagent.

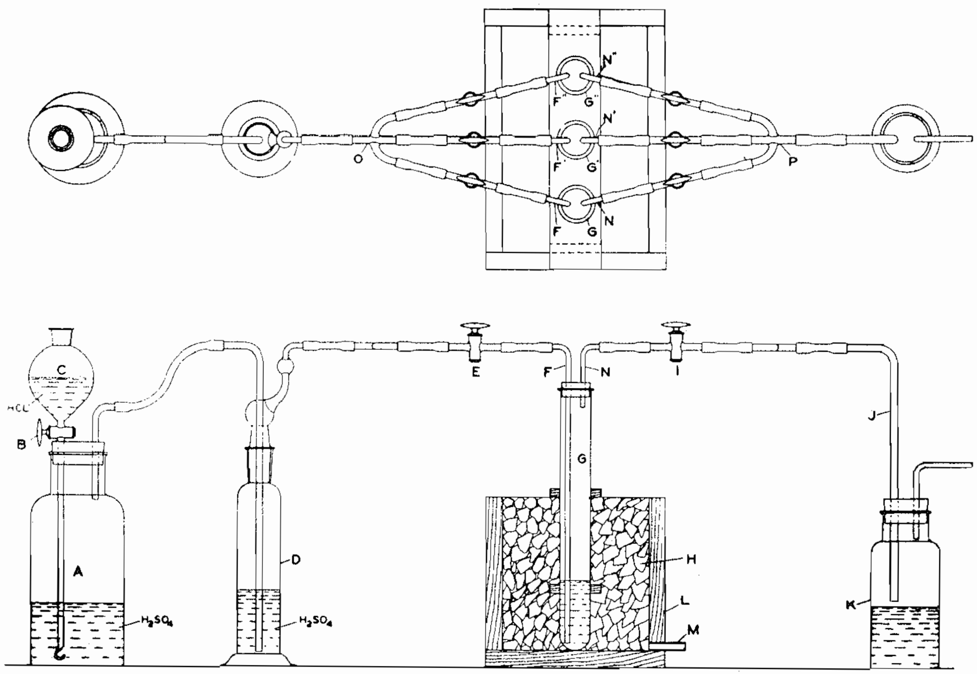


**FIG. 932.01—Apparatus for determining lignin**

### *C. Reagent*

*Fuming hydrochloric acid.*—(*Caution:* See safety notes on fuming acids.) Density 1.212–1.223 at 15°. To 500 g NaCl in 1 L g-s Pyrex distg flask, add cold soln of 250 mL $H_2O$ in 450 mL $H_2SO_4$. Connect side tube of distg flask to glass tube passing thru $H_2SO_4$ wash bottle, and connect outlet tube of $H_2SO_4$ wash bottle to another glass tube, immersed in flask contg 3 L HCl. Surround flask contg HCl with crushed ice. Heat distg flask with small flame and pass HCl gas into acid soln until it attains sp gr of 1.212–1.223 at 15°. Keep reagent refrigerated at ≤0°. If only few detns are to be made, prep. correspondingly smaller amt.

### *D. Determination*

Weigh three 1 g samples of extd and dried sample in weighing bottle and place in 3 large test tubes, *G*, *G′*, and *G″*. Add 20 mL of the reagent to each tube, using this acid to wash down any particles clinging to sides. When all material is wet with reagent, add addnl 30 mL reagent. Add ca 3 drops *capryl alcohol* to minimize foaming. Place test tubes, *G*, *G′*, and *G″*, in wooden box, *L*, and surround with crushed ice. Lubricate tubes *F*, *F′*, and *F″* with drop of glycerol so that they move easily thru holes in rubber stoppers. Lead dry HCl gas from generator into reaction mixts thru tubes *F*, *F′*, and *F″* (*F′* and *F″* are shown in top view), which reach nearly to bottom of tubes *G*, *G′*, and *G″*. Regulate flow of gas thru reaction mixts in *G*, *G′*, and *G″* by stopcocks shown in top view, continuing passage of gas 2 hr. (At first rather slow stream of gas passes in, but during last 15 min, flow is fairly rapid.)

After reaction period, discontinue flow of gas, and disconnect long tubes *F*, *F′*, and *F″* and outlet tubes of test tubes *G*, *G′*, and *G″* from *O* and *P*. Pull tubes *F*, *F′*, and *F″* just above surface of reaction mixt., and close with short pieces of rubber tubing having one end plugged with short piece of glass rod. Similarly close off outlet tubes, *N*, *N′*, and *N″*. Place tubes contg reaction mixt. in cold room or refrigerator (8–10°) 24 hr.

Transfer contents of *G*, *G′*, and *G″* to 1 L erlenmeyers, taking care to remove any material adhering either to inside or outside of tubes *F*, *F′*, and *F″*. Dil. reaction mixts to 500 mL with $H_2O$. Connect flasks to reflux condensers and boil 1 hr. Prep. 3 gooches in usual manner, dry at 105°, and weigh. Ignite one of weighed crucibles, *X*, over Bunsen burner, cool in desiccator, and reweigh. Let contents of flasks cool to room temp. and filter thru weighed gooches. Wash ppts collected in gooches with hot $H_2O$, dry at 105°, and weigh in weighing bottles. Ignite crude lignin in crucible *X* over Bunsen flame and det. wt ash. Place one of other 2 gooches in wide-neck Kjeldahl flask and det. % N in crude lignin as in **955.04**. If methoxyl in lignin is to be detd, collect ppt from one of flasks in dried (105°) fritted glass crucible and proceed as in **956.07C**.

Wt lignin
= wt crude lignin − wt ash − wt crude protein (N × 6.25)

Calc. % lignin in original dry unextd material.

Refs.: JAOAC **15,** 124(1932); **18,** 386(1935); **19,** 107(1936).

CAS-9005-53-2 (lignin)

## **949.04 Lignin in Plants**
### **Indirect Method**
### **Final Action 1965**

(*Caution:* See safety notes on asbestos.)

Ext 1 g sample with alcohol-benzene (1+2) 4 hr in Soxhlet or comparable app. (extn vessel may be either coarse porosity Alundum or paper thimble, closed at top with filter paper or plug of cotton). Wash sample in thimble with suction, using 2 small portions alcohol followed by 2 small portions ether. Heat at 45° in nonsparking oven to drive off ether, and transfer sample to 250 mL wide-mouth erlenmeyer. Add 40 mL *1% soln of pepsin in 0.1 N HCl,* wetting sample well by adding small portion reagent, stirring or shaking thoroly, and finally washing down sides of flask with remaining soln. Incubate at 40° overnight.

Add 20–30 mL hot $H_2O$ and filter, using filter stick. (Filter sticks are made with Pyrex fritted glass disk, 30 mm diam., medium porosity. Thin layer of pre-ashed diat. earth (Hyflo Super-Cel, or similar filter-aid) is sucked onto disk from $H_2O$ suspension. This is usually enough for easy filtration; if not, add extra Super-Cel to material being filtered. Some sticks filter slowly with some samples. It is advisable to obtain more than needed and discard slow-filtering ones. It is convenient to arrange filter sticks in set of 12 attached to vac. manifold by rubber tubing.)

Repeat washing twice and then wash residue into flask by forcing 7–8 mL 5% (w/w) $H_2SO_4$ downward thru filter stick, using air pressure. Wash stick further with the $H_2SO_4$, finally adding enough to bring total vol. to ca 150 mL. Reflux vigorously on hot plate 1 hr, adding $H_2O$ occasionally to maintain original vol. Filter off acid. Wash residue with three 20–30 mL portions hot $H_2O$, two 15–20 mL portions alcohol, and two 15 mL portions ether. Leave vac. on few min to dry residue, and transfer from stick to flask by tapping and brushing. Heat to drive off any residual ether. If disk formed upon drying is difficult to break up into finely divided state (sometimes in case of immature plant samples), disperse residue in ether in flask and then boil off ether on steam bath. Add 20 mL 72% (w/w) $H_2SO_4$ at 20° to residue and hold 2 hr at 20°, stirring occasionally. Add 125 mL $H_2O$, filter, wash once with 20 mL hot $H_2O$, and filter again. Wash residue from filter stick and reflux as before 2 hr, using 150 mL 3% (w/w) $H_2SO_4$. Filter residue onto gooch with asbestos pad and wash with hot $H_2O$ until acid-free. Dry at 105–110° and det. lignin by loss in wt on ignition at 600°.

Ref.: JAOAC **32,** 288(1949).

CAS-9005-53-2 (lignin)

## PIGMENTS

### 940.03 Chlorophyll in Plants

**Photoelectric Colorimetric Method for Total Chlorophyll Only**

**Final Action**

#### *A. Apparatus*

(**a**) *Mortar and pestle.*—Deep glass mortar ca 10 cm id with well-defined lip.

(**b**) *Photoelectric colorimeter.*—Calibrate for chlorophyll, using plant ext as in **940.03C** and light filters with max. *T* near 660 nm. (Combination of Kopp Glass Works filters K2408 and K3965 is suitable.)

(**c**) *Wash bottles.*—Type fitted with rubber bulb, permitting operation with one hand.

(**d**) *High-speed blender.*—Waring Blendor, or equiv.

#### *B. Reagents*

(**a**) *Acetone.*—(*1*) Undild acetone and (*2*) 85% aq. soln by vol. Com. acetone, tech. grade, is satisfactory.

(**b**) *Quartz sand.*—Acid-washed and dried.

#### *C. Determination*

(*Caution:* See safety notes on blenders, toxic solvents, and acetone.)

Select field material carefully to ensure representative sample. Remove representative portion from field sample, and if fresh, cut finely with hand shears and mix as thoroly as possible. Grind dried material in mill and mix thoroly.

Weigh 1–5 g into mortar and add ca 0.1 g $CaCO_3$ or $Na_2CO_3$. Macerate tissue with pestle, add quartz sand, and grind short time; then add 85% acetone, little at time, and continue grinding until tissue is finely ground. Transfer mixt. to funnel, filter with suction, and wash residue with 85% acetone. Return residue to mortar with more 85% acetone and grind again. Filter and wash as before. Repeat procedure until tissue is devoid of any green, and washings are colorless. (It is advisable to grind residue at least once with undild acetone and then to add enough $H_2O$ at end to bring acetone concn to 85%. High-speed blender may be used instead of mortar to macerate and ext tissue (*see* **942.04C**), but each investigator should satisfy himself that device used exts tissue completely.) When extn is complete, transfer filtered ext to vol. flask of appropriate size and dil. to vol.

Measure *T* of soln with photoelec. colorimeter, and read amt of chlorophyll present from curve relating *T* and concn. Express chlorophyll values as mg/g tissue, or in other convenient manner.

Calibrate photoelec. colorimeter as follows: Ext sample of fresh, green leaf material with 85% acetone, filter, wash residue, and dil. ext to vol. as above. Make series of dilns of ext and measure *T* of original and of each of dild solns with instrument in same manner as when chlorophyll prepn is being used as calibration std. Transfer aliquot of original ext to ether and evaluate total chlorophyll spectrophtric as in **942.04C(b)** and **(c)**. From value thus obtained, calc. chlorophyll content of original ext and that of each of dild solns, and construct curve relating concn of chlorophyll with *T* or *A*.

Refs.: Ind. Eng. Chem. Anal. Ed. **12,** 148(1940); **15,** 524 (1943).

CAS-1406-65-1 (chlorophyll)

### 942.04 Chlorophyll in Plants

**Spectrophotometric Method for Total Chlorophyll and the a and b Components**

**Final Action**

#### *A. Apparatus*

Use app. in **940.03A** (except photoelec. colorimeter), plus following:

(**a**) *Scrubbing tubes for washing ether solns.*—Open tubes ca 20 mm diam. to one end of each of which is sealed tube of smaller diam. drawn to fine jet at lower end.

(**b**) *Spectrophotometer.*—Capable of isolating spectral region of ca 3 nm near 660 nm with negligible stray radiation. Tubulated cells with tightly fitting glass stoppers are recommended for work with ether.

#### *B. Reagents*

Those listed in **940.03B** and following:

*Ether.*—Com. grade is satisfactory without further purification.

#### *C. Determination*

(Wash glassware with concd $Na_3PO_4$ soln to remove traces of acid that may decompose chlorophyll.)

(**a**) *Extraction of chlorophyll from tissue.*—Select and prep. sample as in **940.03C**. Disintegrate weighed portion (2–10 g, depending on chlorophyll content) of fresh plant tissue in blender cup that contains ca 0.1 g $CaCO_3$, or by use of mortar as in **940.03C**. After tissue is thoroly disintegrated, filter ext thru buchner fitted with quant. paper. Wash residue with 85% acetone, **940.03B(a)**, and if necessary, use little ether to remove last traces of pigment. If extn is incomplete, return residue and paper to blender container with more 85% acetone and repeat extn. Filter and wash, as before, into flask contg first filtrate. Transfer filtrate to vol. flask of appropriate size and dil. to vol. with 85% acetone.

Pipet aliquot of 25–50 mL into separator contg ca 50 mL ether. Add $H_2O$ carefully until it is apparent that all fat-sol. pigments have entered ether layer. Drain and discard $H_2O$ layer. Place separator contg ether soln in upper rack of support. Add ca 100 mL $H_2O$ to second separator placed in rack below first. Set scrubbing tube in place, and let ether soln run thru it to bottom of lower separator and rise in small droplets thru the $H_2O$. When all soln has left upper separator, rinse it and scrubbing tube with little ether added from medicine dropper. Place scrubbing tube in upper separator and exchange its place in support with separator now contg ether soln. Drain and discard $H_2O$ in upper separator, add similar portion of fresh $H_2O$ to lower separator, and repeat washing process. Continue washing ether soln until all acetone is removed (5–10 washings). Then transfer ether soln to 100 mL vol. flask, dil. to vol., and mix.

(**b**) *Spectrophotometric measurements*.—Add ca teaspoonful (ca 5 mL) anhyd. $Na_2SO_4$ to 60 mL reagent bottle, and fill it with ether soln of pigment. When this soln is optically clear, pipet aliquot into another dry bottle and dil. with enough dry ether to give $A$ value of 0.2–0.8 at wavelength to be used. (Most favorable value is near 0.6 at 660 nm, since such soln yields satisfactory value at 642.5 nm.)

Fill 2 clean g-s absorption cells with dry ether from pipet and polish outside surfaces of each, first with cotton wet with alcohol and then with dry cotton. Place cells in instrument, and det. whether each gives same galvanometer deflection. If not, clean again or select cells that do, and do this daily. Empty one cell, fill it with the dried ether soln, and place in instrument. Adjust entrance and exit slits until spectral region isolated is 3–4 nm at 660.0 nm.

Det. whether instrument is in proper adjustment for wavelength by taking $A$ readings thru soln against solv. at 1 nm intervals from 658 to 665 nm. Highest value should be at 660.0 nm; if not, adjust instrument until it is, or make 660.0 nm readings at wavelength setting that gave highest $A$. With grating instrument, apply same correction at 642.5 nm; however, with prism instrument, correction at 642.5 nm must be obtained from wavelength calibration curve for particular instrument in use. Calibrate instrument for wavelength in this way often enough to ensure that it remains in proper adjustment. Det. $A$ at 660.0 and 642.5 nm (or corrected settings) for each unknown soln.

(**c**) *Calculation of chlorophyll concentration*.—Calc. total chlorophyll and each of $a$ and $b$ components (mg/L) as follows:

(1) Total chlorophyll $= 7.12\,A_{660.0} + 16.8\,A_{642.5}$

(2) Chlorophyll $a$ $= 9.93\,A_{660.0} - 0.777\,A_{642.5}$

(3) Chlorophyll $b$ $= 17.6\,A_{642.5} - 2.81\,A_{660.0}$

### *D. Supplementary Information*

Factors involved in spectrophtric analysis of chlorophyll system have been discussed in detail (Plant Physiol. **17**, 198 (1942)). These authors used Beer's law in form:

$$c = (\log_{10} I_0/I)/a \times t\ [= A/a \times t]$$

where $I_0$ is intensity of light transmitted by solv.-filled cell; $I$ is intensity of light transmitted by soln-filled cell; $c$ is concn of chlorophyll (g/L); $a$ is absorptivity; $t$ is thickness of soln layer in cm; and $A$ is absorbance.

**Table 942.04 Absorption Constants Used in Analysis (after Comar and Zscheile)**

| Wavelength, nm | Absorptivities (for Ether Solns) Chlorophyll a | Chlorophyll b |
|---|---|---|
| 660.0 | 102 | 4.50 |
| 642.5 | 16.3 | 57.5 |
| 600.0 | 9.95 | 9.95 |
| 581.0 | 8.05 | 8.05 |
| 568.0 | 7.11 | 7.11 |
| 613.0 | 15.6 | 8.05 |
| 589.0 | 5.90 | 10.3 |

Since, at given wavelength, observed $A$ value of soln having 2 components represents sum of $A$ values of each of components, following equation holds in case of chlorophylls $a$ and $b$ at given wavelength:

$$A_{\text{observed}} = A_a + A_b \tag{4}$$

If 1 cm cell is used, this equation may be expressed as:

$$A_{\text{observed}} = a_a c_a + a_b c_b \tag{5}$$

Concns of chlorophylls $a$ and $b$ in given ether soln can now be calcd by equation (5) as follows:

(**a**) Det. $A$ for soln at 2 different wavelengths (660.0 and 642.5 nm have been found advantageous for this purpose).

(**b**) From Table **942.04**, select proper absorptivities corresponding to wavelengths used.

(**c**) Substitute observed $A$ value and absorptivities in equation (5) for each of the 2 wavelengths used as illustrated for 660.0 and 642.5 nm in equations (6) and (7). Solve these 2 equations simultaneously for 2 unknowns, the concns of chlorophylls $a$ and $b$.

$$A_{660.0} = 102\ c_a + 4.50\ c_b \tag{6}$$

$$A_{642.5} = 16.3\ c_a + 57.5\ c_b \tag{7}$$

Equations (1), (2), and (3) were derived this way.

Criterion for accuracy of chlorophyll values detd by spectrophtric method is agreement between analytical results detd from measurements at different wavelengths. Measurements at 660.0 and 642.5 nm are convenient for routine analysis (Plant Physiol. **17**, 198 (1942)); however, readings may be made at other wavelengths to check these values. Absorptivities for chlorophylls $a$ and $b$ in ether soln that may be used for this purpose are presented in Table **942.04**.

These values may be used for calcns as follows:

(**a**) Values for total chlorophyll and % composition may be calcd from $A$ at 660.0 and 642.5 nm as described.

(**b**) Check values for total chlorophyll may be calcd from $A$ at intersection points 600.0, 581.0, and 568.0 nm.

(**c**) Check values for % composition may be calcd from $A$ for each of points 613.0 and 589.0 nm in combination with value of total concn obtained from (**a**) or (**b**).

Refs.: Ind. Eng. Chem. Anal. Ed. **14,** 877(1942). Plant Physiol. **17,** 198(1942).

CAS-1406-65-1 (chlorophyll)

## 955.10 Carotenes in Plants

**Final Action**

*See* **941.15**.

## TOBACCO

## 966.02 Moisture in Tobacco
### Gravimetric Method
**First Action 1966**
**Final Action 1968**

### A. Apparatus

(**a**) *Drying oven*.—Forced-draft, regulated to 99.5±0.5°. Suggested dimensions: 19 × 19 × 19″ (48 cm). Approx. oven settings: fresh air intake vent 1/5 open; air control damper 1/4 open; air exhaust vent 1/3 open.

(**b**) *Moisture dish*.—Al, diam. 45–65 mm, depth 20–45 mm, with tight-fitting cover.

### B. Determination

Accurately weigh ca 5 g sample (ground to pass ≤1 mm screen) into weighed moisture dish and place uncovered dish in oven.

Do not exceed 1 sample/10 sq in. (650 sq cm) shelf space, and use only 1 shelf. Dry 3 hr at 99.5±0.5°. Remove from oven, cover, and cool in desiccator to room temp. (ca 30 min). Reweigh to nearest 1 mg and calc. % moisture.

Ref.: JAOAC **49,** 525(1966).

## 963.05 Chlorides in Tobacco
### Potentiometric Method
**First Action 1963**
**Final Action 1964**

### A. Reagents

*Silver nitrate std soln*.—0.1 *N*. Stdze against KCl as in detn.

### B. Apparatus

(**a**) *pH meter*.—Leeds and Northrup, Sumneytown Pike, N Wales, PA 19454, Beckman Instruments, or equiv., equipped with Ag and glass electrodes.

(**b**) *Buret*.—10 mL, graduated in 0.05 or 0.02 mL, preferably reservoir-type.

### C. Determination

Accurately weigh ca 2 g sample, ground to pass No. 40 sieve, into 250 mL electrolytic beaker. Add 100 mL $H_2O$, small amt at first to thoroly wet sample; then remainder. Let stand ≥5 min at room temp., stirring intermittently. Pipet 5 mL $HNO_3$ (1+9) into mixt. and insert clean electrodes. Start mag. stirrer and continue stirring thruout titrn at rate that produces vigorous agitation without spattering. Titr. with std 0.1 *N* $AgNO_3$ soln to potential previously established as equivalence point. Det. equivalence point potential graphically by making several titrns on one or more tobacco samples. Recheck occasionally, and redet. when either electrode is replaced. Record vol. of titrant and calc.:

$$\% \text{ Cl} = \text{mL } AgNO_3 \times \text{normality} \times 3.5453/\text{g sample}$$

Ref.: JAOAC **46,** 415(1963).

## 959.04 Nitrogen in Tobacco
### Kjeldahl Method for Samples Containing Nitrates
**Final Action 1964**

(For nitrate-free samples, omit salicylic acid and thiosulfate treatment.)

### A. Reagents

*See* **920.02A** and the following:

(**a**) *Sodium hydroxide-thiosulfate soln*.—Dissolve 500 g NaOH pellets and 40 g $Na_2S_2O_3.5H_2O$ in $H_2O$ and dil. to 1 L.

(**b**) *Indicators*.—(*1*) Dissolve 1 g Me red in 200 mL alcohol; or (*2*) prep. mixed indicator by dissolving 0.8 g Me red and 0.2 g methylene blue in 500 mL alcohol.

### B. Apparatus

*See* **920.02B**.

### C. Determination

Place weighed sample (1–2 g) in digestion flask. Add vol. $H_2SO_4$ (contg 2 g salicylic acid/40 mL) corresponding to wt sample (35 mL for 1 g, 40 mL for 2 g for $NO_3$-contg samples; 20 and 25 mL, resp., for $NO_3$-free samples). Shake until thoroly mixed; let stand ≥30 min with occasional shaking; then add 5 g $Na_2S_2O_3.5H_2O$. Shake, let stand 5 min, and heat carefully until frothing ceases. Turn off heat, add 0.7 g HgO (or metallic Hg) and 15 g $K_2SO_4$, and boil briskly 1–1.5 hr after soln clears.

Cool, add ca 200 mL $H_2O$, cool to ca room temp., and add few Zn granules. Tilt flask and carefully add 50 mL NaOH-thiosulfate soln without agitation. Immediately connect flask to distn bulb on condenser whose tip is immersed in 50 mL std 0.1 *N* acid in receiving flask. Then rotate digestion flask carefully to mix contents. Heat until ≥150 mL distillate collects, and titr. excess acid with std base, using Me red or mixed indicator. Correct for blank detn on reagents.

Ref.: JAOAC **42,** 302(1959).

CAS-7727-37-9 (nitrogen)

## 966.03 Potassium in Tobacco
### Flame Photometric Method
**First Action 1966**
**Final Action 1968**

### A. Reagents

(**a**) *Potassium std solns*.—(*1*) *Stock soln*.—1000 ppm K. *See* **956.01A(a)**. (*2*) *Working solns*.—Place 0, 5, 10, 15, 20, 25, and 30 mL stock soln in seven 1 L vol. flasks, add 40 mL 3 *N* HCl to each, and dil. to vol. with $H_2O$.

(**b**) *Diatomaceous earth*.—Celite 545, acid-washed.

### B. Apparatus

(**a**) *Flame photometer*.—Natural gas-air fuel, or equiv., adequate for K analysis.

(**b**) *Chromatographic tube*.—20 × 150 mm with coarse fritted disk.

### C. Preparation of Sample Solution

Accurately weigh ca 0.5 g tobacco dust into ca 40 mL weighing dish. Add ca 1 g Celite and mix intimately with spatula. Transfer quant. thru powder funnel into chromatgc tube. Add addnl Celite thru funnel into tube until 2.5 cm layer accumulates on top of sample-Celite mixt. Compact sample and Celite by tapping tip of tube on table top, and insert tip of tube into neck of 1 L vol. flask. Add 40 mL 3 *N* HCl into tube by pipet or dispenser, washing down sides, and let elute into vol. flask. When liq. level reaches top of Celite, add 25 mL $H_2O$ and let elute. Add second 25 mL portion of $H_2O$, let elute by gravity, or force thru rapidly with compressed air. Rinse tip of tube into vol. flask, dil. to vol. with $H_2O$, and mix well.

### D. Determination

Det. % $T$ for sample eluate and K stds as specified in instruction manual of instrument. *See* also **956.01D**.

Prep. calibration curve and det. ppm K of sample from curve.

$$\% \text{ K} = \text{ppm K} \times 0.1/\text{g sample}$$
$$\% \text{ K}_2\text{O} = \text{ppm K} \times 0.1205/\text{g sample}$$

Ref.: JAOAC **49,** 521(1966).

CAS-7440-09-7 (potassium)

## 971.02 Glycerol, Propylene Glycol, and Triethylene Glycol in Cased Cigarette Cut Filler and Ground Tobacco

**Gas Chromatographic Method**

**First Action 1971**
**Final Action 1985**

(*Caution:* See safety notes on pipets and methanol.)

### A. Apparatus

(**a**) *Gas chromatograph.*—With programmed temp. oven and W hot wire detector; F&M Model 720 (current models 5700 series; Hewlett-Packard Co.), or equiv. Conditions: Detector bridge 140 ma; temps (°): injection 265, detector 280, column 90–240 at 15°/min; He 60 mL/min adjusted, if necessary, to facilitate sepns; attenuation 4, adjusted according to sensitivity to yield peaks of sufficient size for accurate measurement (use same attenuation for all stds and samples); chart speed, 12″/hr.

(**b**) *Column.*—42 (105 cm) × $^3/_{16}$″ Cu tubing packed with 5% Carbowax 20M-terephthalic acid (TPA) on 60–80 mesh Chromosorb G AW-DMCS (Hewlett-Packard Co., No. 8501-6223 or Applied Science No. 04388). Prep. packing by placing 30.0 g Chromosorb in 500 mL ₮ r-b flask. Add soln of 1.50 g Carbowax 20M-TPA in 150 mL $CHCl_3$, and slurry. Remove $CHCl_3$ under vac. in rotary evaporator and air dry overnight at room temp. Condition new column 2 hr at 240°; then inject three 30 μL samples tobacco ext before analyzing samples. Recondition columns removed from app. before use.

### B. Reagents

(**a**) *Extracting soln.*—Dil. 20.0 mL 1,3-butylene glycol stock std soln, (**b**), to 2 L with anhyd. MeOH.

(**b**) *1,3-Butylene glycol stock std soln.*—Accurately weigh 20.00 g USP 1,3-butanediol into 100 mL vol. flask and dil. to vol. with anhyd. MeOH.

(**c**) *Glycerol stock std soln.*—Accurately weigh 10.00 g USP glycerol into 100 mL vol. flask and dil. to vol. with extg soln.

(**d**) *Propylene glycol stock std soln.*—Accurately weigh 5.00 g USP propylene glycol into 100 mL vol. flask and dil. to vol. with extg soln.

(**e**) *Triethylene glycol stock std soln.*—Accurately weigh 5.00 g triethylene glycol into 100 mL vol. flask and dil. to vol. with extg soln.

(**f**) *Humectant std solns.*—Into each of four 100 mL vol. flasks, pipet 1.0, 2.0, 3.0, and 4.0 mL, resp., glycerol, propylene glycol, and triethylene glycol stock std solns. Dil. to vol. with extg soln. Each soln contains (in mg/100 mL):

| Soln | Propylene Glycol | Glycerol | Triethylene Glycol |
|---|---|---|---|
| 1 | 50 | 100 | 50 |
| 2 | 100 | 200 | 100 |
| 3 | 150 | 300 | 150 |
| 4 | 200 | 400 | 200 |

### C. Extraction

Place 10.00 g sample in 250 mL ₮ erlenmeyer. Pipet 100 mL extg soln into flask and stopper. Shake mech. 1 hr and let settle few min until supernate is clear. Alternatively, shake mech. 30 min and let stand overnight.

### D. Determination

Prime column by injecting two 30 μL aliquots supernate ext. Then alternately inject 30 μL supernate exts and a humectant std soln until all samples and stds have been run, repeating ext injections, if necessary. (Sequence is $ext_1$, $ext_1$, $std_1$, $ext_1$, $std_2$, $ext_2$, $std_3$, $ext_3$, $std_4$, $ext_4$, $std_1$, $ext_5$, $std_2$, etc. If <4 exts are available, distribute ext injections among those available so that sequence thru $std_4$ is run.) Det. peak hts and calc. ratios of propylene glycol, glycerol, and triethylene glycol to butylene glycol for each std and sample soln. Plot peak ht ratios against polyol concn for std solns and construct std curve for each humectant. Det. concn in mg/100 mL for propylene glycol, glycerol, and triethylene glycol in sample soln from resp. std curves.

$$\% \text{ Humectant} = (\text{mg}/100 \text{ mL}) \times 0.01$$

Ref.: JAOAC **54,** 560(1971).

CAS-56-81-5 (glycerol)
CAS-57-55-6 (propylene glycol)
CAS-112-27-6 (triethylene glycol)

## 960.07 Alkaloids (Total As Nicotine) in Tobacco

**Distillation Method**

**First Action 1960**
**Final Action 1964**

### A. Apparatus

(**a**) *Distillation apparatus.*—500 mL Kjeldahl flask fitted with inlet tube for steam, trap bulb, and condenser; Griffith still (Tobacco Sci. **1**, 130(1957), available from Lab Glass, Inc., PO Box 610, Vineland, NJ 08360); or other suitable steam distn app.

(**b**) *Spectrophotometer.*—Beckman Instruments Model DU, 24, or 25 (replacement model DU-64), or other instrument capable of accurately measuring $A$ in 200–300 nm range, equipped with 1 cm quartz cells.

### B. Reagents

(**a**) *Alkali-salt soln.*—Dissolve 300 g NaOH in 700 mL $H_2O$ and sat. with NaCl.

(**b**) *Silicotungstic acid soln (for gravimetric determination).*—Dissolve 120 g $SiO_2.12WO_3.26H_2O$ in $H_2O$ and dil. to 1 L. (Soln should be clear and free from green color.)

### C. Standardization

(*Caution:* Nicotine is very toxic. Avoid contact with skin and eyes. See safety notes on distillation and vacuum.)

Purify best grade of nicotine com. available by successive vac. distns until center cuts from 2 successive distns have same $a$ at 259 nm (ca 34.3). Accurately weigh ca 0.2 g purified nicotine; dissolve in and dil. to 1 L with ca 0.05 $N$ HCl. Dil. 10 mL aliquot of this soln to 100 mL with ca 0.05 $N$ HCl. Det. $A$ at 259 nm and calc. $a = A/(c \times b)$, where $c$ is concn of nicotine in g/L and $b$ is cell length in cm.

### D. Distillation

Accurately weigh 2–5 g tobacco sample and transfer to distn flask or app. (If final detn of nicotine is gravimetric, use sample contg ≥0.1 g alkaloids; if spectrophtric, use ≥2 g sample.) (If Griffith still is used, use 0.05–0.2 g sample.) Place 25 mL HCl (1+4) in receiver (1 L vol. flask is desirable) and place receiver so that condenser tube dips into acid. (With Griffith still, use 10 mL HCl (1+4) in 250 mL vol. flask.) Add 50 mL alkali-salt soln to distn flask so that sample is rinsed into bottom of flask. (With Griffith still, use 5 mL alkali-salt soln.) If large vol. of liq. is required for proper function of still, add more alkali-salt soln; do not dil. Connect flask to app. immediately and steam distil with as rapid current of steam as can be condensed efficiently. Effluent condensate should not be above room temp. Apply heat to distn flask from burner, mantle, or other heat source to keep vol. in flask approx. const. Collect ca 900 mL condensate (or distil addnl 100 mL after condensate shows no nicotine by silicotungstic acid test). (With Griffith still, collect 225 mL.) Dil. distillate to vol.

### *E. Determination*

(**a**) *Spectrophotometric.*—Dil. aliquots of distillate (if necessary) with 0.05 *N* HCl so that *A* at 259 nm is 0.5–0.8 and read *A* at 236, 259, and 282 nm. Calc. corrected $A'_{259} = 1.059 \times [\text{observed } A_{259} - {}^1/_2\,(A_{236} + A_{282})]$ after correcting all observed *A* values to original distillate vol. basis. Concn, *c*, of alkaloids as nicotine in g/L is given by $c = A'_{259}/(a \times b)$, where *a* is absorptivity at 259 nm, and *b* is cell length in cm.

$$\%\ \text{alkaloid (as nicotine)} = c \times \text{vol. distillate (L)} \times 100/\text{g sample}$$

(**b**) *Gravimetric.*—Det. alkaloids in distillate as in **920.35B**, but double amt of silicotungstic acid specified, i.e., 2 mL/each 10 mg alkaloids expected.

Ref.: JAOAC **43,** 524(1960).

CAS-55-11-5 (nicotine)

## 960.08 Alkaloids (Total As Nicotine) in Tobacco

### Cundiff-Markunas Method

**First Action 1960**
**Final Action 1964**

(Total alkaloids (as nicotine), tertiary alkaloids (as nicotine), and secondary alkaloids (as nornicotine))

### *A. Reagents*

(**a**) *Benzene-chloroform soln.*—Mix equal parts by vol. of benzene and $CHCl_3$ and sat. with $H_2O$.

(**b**) *Sodium hydroxide soln.*—36%. Dissolve 500 g NaOH in $H_2O$ and dil. to 1 L.

(**c**) *Dilute acetic acid.*—5%. Dil. 50 mL HOAc to 1 L with $H_2O$.

(**d**) *Crystal violet indicator.*—Dissolve 0.5 g crystal violet in 100 mL HOAc.

(**e**) *Perchloric acid std soln.*—0.025 *N*. Add 4.7 mL 72% $HClO_4$ to freshly opened 5 lb bottle HOAc and mix. (*Caution:* See safety notes on acetic acid, acetic anhydride, and perchloric acid.) Stdze as follows: Accurately weigh 0.1 g KH phthalate (NIST) into 125 mL erlenmeyer, add 50 mL HOAc, and heat to dissolve. Cool, add 2 drops indicator, and titr. to blue-green end point. Perform blank titrn on 50 mL HOAc and 2 drops indicator soln, and correct vol. of titrant.

$$N = \text{wt KH phthalate} \times 4.896/\text{mL } HClO_4 \text{ soln}$$

### *B. Determination*

Accurately weigh 2.5 g finely ground tobacco into 250 mL erlenmeyer. Add 15 mL 5% HOAc and swirl until tobacco is thoroly wetted. Pipet 100 mL benzene-$CHCl_3$ soln into flask, and then 10 mL 36% NaOH soln. Stopper flask tightly and shake 20 min, using wrist-action shaker. Add 4.5–5 g (2 teaspoonfuls) Filter-Cel, mix, and filter most of benzene layer thru Whatman No. 2 paper into second flask. If filtrate has any turbidity, add 2–2.5 g (1 teaspoonful) addnl Filter-Cel and refilter thru Whatman No. 2 paper. Filtrate must be clear.

Pipet 25 mL aliquots of filtrate into each of two 125 mL erlenmeyers. Pass stream of air over surface of soln in first flask 5 min, add 2 drops indicator, and titr. to green end point with 0.025 *N* $HClO_4$. Add 1.0 mL $Ac_2O$ to second flask and let stand ≥15 min. Add 25 mL HOAc and 2 drops indicator, and titr. to blue-green end point with 0.025 *N* $HClO_4$. Take first appearance of blue-green thruout soln as end point. For each series of analyses perform blank titrns and correct respective vols of titrant.

Calc. % alkaloids as follows: % total alkaloids (as nicotine) $= V_1 \times N \times 32.45$/wt sample; % tertiary alkaloids (as nicotine) $= (2V_2 - V_1) \times N \times 32.45$/wt sample; % secondary alkaloids (as nornicotine) $= 2(V_1 - V_2) \times 29.64$/wt sample; where $V_1$ = vol. titrant for nonacetylated aliquot; $V_2$ = vol. titrant for acetylated aliquot; and *N* = normality $HClO_4$.

Ref.: JAOAC **43,** 524(1960).

CAS-55-11-5 (nicotine)
CAS-494-97-3 (nornicotine)

## 979.01 Nicotine on Cambridge Filter Pads

### Gas Chromatographic Method

**First Action 1979**
**Final Action 1984**

### *A. Apparatus and Reagents*

(**a**) *Gas chromatograph.*—With flame ionization detector, heated injection port, and thermostated column oven. Following conditions have been found satisfactory: Column, 1.8 m (6′) × ${}^1/_8$″ stainless steel; packing, 2% KOH and 10% Carbowax 20M (based on final packing wt) on 45–60 mesh calcined diat. earth (such as Chromosorb W, or equiv.), resieved before use to mesh range to remove fines and lumps; temps (°): column 165, detector and injection port 200–250; carrier gas flow, ca 40 mL/min. Adjust H and air flows for max. sensitivity and stability. Under these conditions, column should have ht equiv. to theoretical plate (HETP) <1 mm and resolution of >2, calcd with nicotine and anethole.

(**b**) *Measuring system.*—Measure peak areas with electronic integrator or other system with resolution of ≥1 count/mv-sec.

(**c**) *Mechanical shaker.*—Capable of extg ≥99% nicotine. Burrell Wrist-Action shaker has been found satisfactory.

(**d**) *Extracting soln.*—2-Propanol contg 1 mg anethole/mL as internal std for nicotine. If $H_2O$ is also to be detd, add 20 mg EtOH/mL 2-propanol as addnl internal std.

(**e**) *Nicotine std solns.*—(*1*) *Stock soln.*—Weigh 2.500 g nicotine, **960.07C**, or equiv. amt of nicotine salt. Transfer quant. into 100 mL vol. flask, and dil. to vol. with extg soln. (*2*) *Working std solns.*—Pipet 1, 2, 3, 4, and 5 mL stock soln into five 100 mL vol. flasks, and dil. to vol. with extg soln (0.25, 0.50, 0.75, 1.00, and 1.25 mg nicotine/mL).(*Caution: See* precaution in **960.07C**.)

### *B. Extraction*

Place Cambridge filter material in flask or serum bottle accommodated by shaker used, add 10.00 mL extg soln, stopper, and shake until ≥99% of nicotine is extd (usually ca 15 min).

### C. Standardization

Prime column with aliquots of 1.25 mg/mL std soln. Let baseline stabilize, inject 1 μL each std soln in succession, and repeat sequence 3 times. Det. area ratio (nicotine:anethole) for each injection, and calc. slope and intercept of response curve, preferably by method of least squares (*See Definition of Terms and Explanatory Notes*). Correlation coefficient should be ≥0.99 and intercept ≤0.05 mg/mL.

### D. Determination

Prime column with aliquots of ext, **979.01B**. Let baseline stabilize, and inject 1 μL of each sample soln. Calc. nicotine concn in soln ($C$, mg/mL) = m$x$ + b, where m = slope of stdzn curve, b = intercept, and $x$ = area ratio of nicotine to anethole.

Nicotine yield/cigarette = ($C$ × 10.00)/(No. cigarettes/pad)

Ref.: JAOAC **62,** 229(1979).

CAS-55-11-5 (nicotine)

## 968.02 Menthol in Cigarette Filler
### Colorimetric Method
#### First Action 1968
#### Final Action 1970

### A. Apparatus and Reagents

(**a**) *Distillation apparatus*.—*See* Fig. **968.02**.

(**b**) *Spectrophotometer*.—With matched cells; capable of measuring $A$ at 550 nm.

(**c**) *Menthol std. soln*.—1 mg/mL. Accurately weigh 100 mg USP *l*-menthol into 100 mL vol. flask, add alcohol to dissolve, and dil. to vol. with alcohol.

(**d**) *DMAB color reagent*.—Dissolve 0.5 g *p*-dimethylaminobenzaldehyde (Sigma Chemical Co.) in 100 mL $H_2SO_4$ (1.6+1).

### B. Preparation of Calibration Curve

Prep. dil. stds by pipeting aliquots contg 0, 3, 4, 6, 8, and 10 mg menthol into 100 mL vol. flasks and dilg to vol. with alcohol (1+1). Pipet 1 mL each dil. std into 10 mL test tube, add 5 mL color reagent, mix, and place in boiling $H_2O$ bath *exactly* 2 min. Cool in tap $H_2O$, and within 15 min det. $A$ at 550 nm against 0 std. Prep. calibration curve by plotting $A$ against menthol concn (mg/100 mL).

### C. Determination

Accurately weigh 2.00–2.15 g cigaret filler and transfer to distn flask, **A**. Add 80 mL $H_2O$ and few boiling stones, connect flask to condenser with tube, **B**, attach adapter, **C**, to condenser, and immerse tip in 20 mL alcohol in 100 mL vol. receiving flask.

Gently heat distn flask until distn begins; then increase heat and lower receiving flask, **D**, so tip of adapter is no longer immersed. Distil until 20 mL distillate collects. Disconnect condenser from tube, and wash down condenser with alcohol. Remove receiving flask, dil. distillate to ca 70 mL with alcohol, and add $H_2O$ almost to vol. Mix, add alcohol to vol., and mix again.

Pipet 1 mL distillate into 10 mL test tube, add 5 mL color reagent, mix, and place in boiling $H_2O$ bath *exactly* 2 min. Cool in tap $H_2O$, and within 15 min det. $A$ at 550 nm, using "color" soln from nonmentholated tobacco carried thru detn as blank. (If nonmentholated sample corresponding to mentholated sample is not available, use reagent blank.) Use nonmentholated tobacco blank within 15 min after color development step. Fresh nonmentholated tobacco blank soln may be required during multiple sample runs. Det. mg menthol from calibration curve.

% Menthol = mg menthol/(g original sample × 10)

Ref.: JAOAC **51,** 650(1968).

CAS-1490-04-6 (menthol)

## 968.03 Menthol in Cigarette Filler
### Gas Chromatographic Method
#### Final Action 1970

### A. Apparatus and Reagents

(**a**) *Gas chromatograph*.—Equipped with flame ionization detector and thermostated injection port and column oven. Use following conditions for analysis: Column, 1.5 m (5′) × $^1/_8$″ od stainless steel packed with 10% (w/w) silicone oil DC-550 on 60–80 mesh Chromosorb W; temps (°): column 150, detector 150, injection port 175; N carrier gas flow ca 35 mL/min. Adjust H and air flows for max. sensitivity and reasonable stability.

(**b**) *Mechanical shaker*.—Wrist action.

(**c**) *Menthol-anethole std soln*.—0.250 mg menthol and 0.50 mg anethole/mL. Weigh exactly 0.5000 g tech. grade anethole and wash into 1 L vol. flask with 200 mL alcohol. Transfer 0.2500 g USP *l*-menthol to the vol. flask with enough alcohol to bring to vol. Store soln in dark g-s bottle. Do not use >6 weeks.

(**d**) *Extracting soln*.—0.50 mg anethole/mL. Dissolve 1.000 g anethole in alcohol in 2 L vol. flask, dil. to vol. with alcohol, and store in dark.

### B. Determination of Ratio Factor

Weigh ca 3 g nonmentholated control filler, contg all usual humectants but no menthol or anethole, into 125 mL rubber-stoppered flask. Pipet 50 mL std menthol-anethole soln into flask, stopper, and shake 1 hr on mech. shaker. Let solids settle 15 min and chromatograph 2 μL aliquot of supernate. Repeat twice more to obtain total of 3 replicates of std chromatogram. For quant. results, inject both std and unknown samples by inserting 2″ (5 cm) needle to hilt, injecting 2 μL

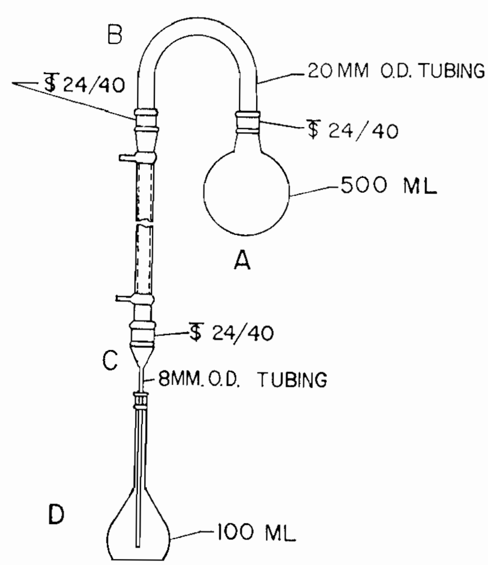


**FIG. 968.02—Distillation apparatus; *see* 968.02C for explanation of symbols**

rapidly, and withdrawing needle at once. (Menthol elutes in ca 3 min, anethole in ca 5 min.) After ca 10 min, all other compds are eluted and new injection can be made.

Draw baselines under menthol and anethole peaks and measure peak hts in mm. Using mean peak ht of menthol and anethole from 3 std chromatograms, calc. std ratio factor of menthol to anethole as follows:

Std ratio factor = peak ht for menthol (0.25 mg/mL)/ peak ht for anethole (0.50 mg/mL)

### *C. Determination*

Accurately weigh 8–8.5 g mentholated cigarette filler and place in 250 mL rubber-stoppered erlenmeyer. Pipet 100 mL extg soln into flask, stopper, and mech. shake 2 hr. Let solids settle 15 min and chromatograph 2 μL aliquot of supernate. Draw baselines under menthol and anethole peaks and measure peak hts in mm. Calc. ratio factor of unknown menthol as follows:

Ratio factor for unknown = peak ht for unknown menthol/ peak ht for anethole (0.50 mg/mL)

% Menthol = (unknown ratio factor × 0.25 × 10)/ (std ratio factor × g sample)

Ref.: JAOAC **51,** 650(1968).

CAS-1490-04-6 (menthol)

# 4. Animal Feed

**Joel M. Padmore, *Associate Chapter Editor***
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### 965.16 Sampling of Animal Feed

**Procedure**

Use slotted single or double tube, or slotted tube and rod, all with pointed ends.

Take ≥500 g sample, 1 kg preferred, as follows: Lay bag horizontally and remove core diagonally from end to end. Det. number of cores as follows: From lots of 1–10 bags, sample all bags; from lot of ≥11, sample 10 bags. Take 1 core from each bag sampled, except that for lots of 1–4 bags take enough diagonal cores from each bag to total ≥5 cores. For bulk feeds draw ≥10 cores from different regions; in sampling small containers (≤10 lb) 1 package is enough. Reduce composite sample to amt required, preferably by riffling, or by mixing thoroly on clean oil-cloth or paper and quartering. Place sample in air-tight container.

A sample from less than these numbers of bags may be declared an official sample if guarantor agrees. For samples that cannot be representatively taken with probe described, use other sampling means.

### 950.02 Animal Feed

**Preparation of Sample**
**Final Action**

Grind sample to pass sieve with circular openings 1 mm ($^1/_{25}$") diam. and mix thoroly. If sample cannot be ground, reduce to as fine condition as possible. Do not grind molasses feeds.

Refs.: JAOAC **33,** 424(1950); **41,** 223(1958); **48,** 658(1965); **51,** 467(1968).

### 934.01 Moisture in Animal Feed

**Drying in Vacuo at 95–100°**
**Final Action**

Dry amt sample contg ca 2 g dry material to const wt at 95–100° under pressure ≤100 mm Hg (ca 5 hr). For feeds with high molasses content, use temp. ≤70° and pressure ≤50 mm Hg. Use covered Al dish ≥50 mm diam. and ≤40 mm deep. Report loss in wt as moisture.

Ref.: JAOAC **17,** 68(1934); **51,** 467(1968); **60,** 322(1977).

### 925.04 Moisture in Animal Feed

**By Distillation with Toluene**
**Final Action**

***A. Apparatus***

Connect 250 mL flask of Pyrex or other resistant glass by means of Bidwell-Sterling moisture receiver to 500 mm Liebig condenser. Calibrate receiver, 5 mL capacity, by distg known amts $H_2O$ into graduated column, and estg column of $H_2O$ to 0.01 mL. Clean tube and condenser with chromic acid cleaning mixt., rinse thoroly with $H_2O$, then alcohol, and dry in oven to prevent undue amt $H_2O$ from adhering to inner surfaces during detn.

***B. Determination***

If sample is likely to bump, add dry sand to cover bottom of flask. Add enough toluene to cover sample completely (ca 75 mL). Weigh and introduce enough sample into toluene to give 2–5 mL $H_2O$ and connect app. Fill receiving tube with toluene, pouring it thru top of condenser. Bring to boil and distil slowly, ca 2 drops/sec, until most of the $H_2O$ passes over; then increase rate of distn to ca 4 drops/sec.

When all $H_2O$ is apparently over, wash down condenser by pouring toluene in at top, continuing distn short time to see whether any more $H_2O$ distils over; if it does, repeat washing-down process. If any $H_2O$ remains in condenser, remove by brushing down with tube brush attached to Cu wire and satd with toluene, washing down condenser at same time. (Entire process is usually completed within 1 hr.) Let receiving tube come to room temp. If any drops adhere to sides of tube, force them down, using Cu wire with end wrapped with rubber band. Read vol. $H_2O$ and calc. to %.

Refs.: JAOAC **8,** 295(1925); **9,** 30(1926).

### 920.36★ Moisture in Animal Feed

**Drying without Heat over Sulfuric Acid**
**Final Action**
**Surplus 1974**

See **7.006–7.007**, 12th ed.

### 930.15 Moisture in Animal Feed

**Drying at 135°**
**Final Action**

(Not to be used when fat detn is to be made on same sample)

Regulate air oven to 135±2°. Using low, covered Al dishes, **934.01**, weigh ca 2 g sample into each dish and shake until contents are evenly distributed. With covers removed, place dishes and covers in oven as quickly as possible and dry samples 2 hr. Place covers on dishes and transfer to desiccator to cool. Weigh, and calc. loss in wt as $H_2O$.

Refs.: JAOAC **13,** 173(1930); **14,** 152(1931); **17,** 178(1934); **18,** 80(1935).

### 953.07 Moisture in Animal Feed

**In Highly Acid Milk By-products**
**Final Action**

Add ca 2 g ZnO, freshly ignited or oven dried, to flat-bottom dish ≥5 cm diam. and weigh. Add ca 1 g sample and weigh quickly. Add ca 5 mL $H_2O$ and distribute sample evenly on bottom of dish. Heat on steam bath, exposing max. surface of dish bottom to live steam until apparently dry. Heat at 98–100° in air oven 3 hr or to const wt. Cool in desiccator; then weigh quickly. Det. wt residue. Dil. with twice its vol. $CO_2$-free $H_2O$. Add 2 mL phthln, and titr. with 0.1N NaOH to first persistent pink. Calc. as % lactic acid by wt. (1 mL 0.1N NaOH = 0.0090 g lactic acid.). To compensate for $H_2O$ formed when acid is neutzd by ZnO, add 0.1 g to residue wt for each g acid (as lactic) in weighed sample. Report % residue (corrected) as total solids.

Refs.: JAOAC **36,** 213(1953); **37,** 253(1954).

## 942.05 Ash of Animal Feed

**Final Action**

Weigh 2 g sample into porcelain crucible and place in temp. controlled furnace preheated to 600°. Hold at this temp. 2 hr. Transfer crucible directly to desiccator, cool, and weigh immediately, reporting % ash to first decimal place.

Refs.: JAOAC **25,** 857(1942); **26,** 220(1943).

## 935.11★ Protein in Animal Feed

**Qualitative Tests**

**Final Action**

**Surplus 1965**

***A. Biuret Test***

*See* **22.012–22.013**, 10th ed.

***B. Millon Test***

*See* **22.014–22.015**, 10th ed.

***C. Glyoxylic Acid Test (Hopkins-Cole)***

*See* **22.016–22.017**, 10th ed.

***D. Adamkiewicz Test***

*See* **22.018**, 10th ed.

***E. Xanthoproteic Test***

*See* **22.019**, 10th ed.

## 954.01 Protein (Crude) in Animal Feed

**Kjeldahl Method**

**Final Action**

Det. N as in **955.04**. Multiply result by 6.25, or in case of wheat grains by 5.70.

Refs.: JAOAC **37,** 241(1954); **38,** 56(1955).

## 988.05 Protein (Crude) in Animal Feed

**$CuSO_4/TiO_2$ Mixed Catalyst Kjeldahl Method**

**First Action 1988**

(*Caution:* See safety notes on sulfuric acid and sodium hydroxide.)

***A. Principle***

Sample is digested in $H_2SO_4$, using $CuSO_4/TiO_2$ as catalysts, converting N to $NH_3$ which is distd and titrd.

***B. Reagents***

(**a**) *Sodium hydroxide soln.*—Dissolve ca 450 g NaOH pellets or flakes (low N) in $H_2O$, cool, and dil. to 1 L; or use soln with sp. gr. ≥1.36.

(**b**) *Boiling stones.*—Alundum, 8–14 mesh (No. 1590-D18; Thomas Scientific Co.).

(**c**) *Methyl red indicator.*—Dissolve 1 g Me red (Na salt) in 100 mL MeOH.

(**d**) *Hydrochloric or sulfuric acid std soln.*—0.5N. Prep. as in **936.15** or **890.01**.

(**e**) *Sodium hydroxide std soln.*—0.1N. Prep. as in **936.16**.

After stdzg both acid and base by methods suggested in (**d**) and (**e**), also check one against the other. In addn, check entire method by analyzing NIST Std Ref. material No. 194, $NH_4H_2PO_4$, certified 12.15% N, and a high purity lysine·HCl.

***C. Apparatus***

(**a**) *Digestion.*—Kjeldahl flasks with capacity of 500–800 mL.

(**b**) *Distillation.*—Digestion flask (e.g., Corning Glass No. 2020) connected to distn trap by rubber stopper. Distn trap is connected to condenser with low-S tubing. Outlet of condenser tube should be <4 mm diam.

***D. Determination***

Weigh 0.250–1.000 g sample into digestion flask. Add 16.7 g $K_2SO_4$, 0.01 g anhyd. $CuSO_4$, 0.6 g $TiO_2$, 0.3 g pumice, 0.5–1.0 g Alundum granules, and 20 mL $H_2SO_4$. (Add addnl 1.0 mL $H_2SO_4$ for each 0.1 g fat or 0.2 g other org. matter if sample wt is >1 g.)

Include at least 1 sample of high purity lysine·HCl in each day's run as check of correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.

To digest sample, first adjust heat to bring 250 mL $H_2O$ at 25° to rolling boil in 5 min. Add a few boiling chips to prevent superheating. Then heat samples at this 5-min boil rate until dense white fumes clear bulb of flask (ca 10 min), swirl gently, and continue heating addnl 40 min. (Note: Reagent proportions, heat input, and digestion time are critical factors—do not change.) Cool, cautiously add about 250 mL $H_2O$, and cool to room temp. (Note: Add $H_2O$ as soon as possible to reduce amt of caking. If excessive bumping occurs during distn, increase diln $H_2O$ from 250 mL to ca 300 mL.)

Prep. titrn beaker by adding appropriate vol. of acid std soln to amt of $H_2O$ such that condenser tip will be sufficiently immersed to trap all $NH_3$ evolved. Add 3–4 drops of indicator soln (**c**).

Add addnl 0.5–1.0 g Alundum granules to cooled digestion flask. Optionally, 2–3 drops of tributyl citrate may also be added to reduce foaming. Slowly down side of flask, add sufficient NaOH soln (**a**) such that mixt. will be strongly alk. Immediately connect flask to distn app., mix completely, and distill at ca 7.5-min boil rate until ≥150 mL distillate is collected in titrn beaker.

Titr. excess std acid in distillate with NaOH std soln (**e**). Correct for blank detn on reagents. Calc. % nitrogen:

$$\%N = \{[(N_{acid})(mL_{acid}) - (mL_{bk})(N_{NaOH}) - (mL_{NaOH})(N_{NaOH})] \times 1400.67\}/\text{mg sample}$$

where $mL_{NaOH}$ = mL std base needed to titr. sample; $mL_{acid}$ = mL std acid used for that sample; $mL_{bk}$ = mL std base needed to titr. 1 mL std acid minus mL std base needed to titr. reagent blank carried thru method and distd into 1 mL std acid; $N_{acid}$ = normality of std acid; $N_{NaOH}$ = normality of std base. Calc. % crude protein, defined as 6.25 × % nitrogen, or 5.7 × % nitrogen for wheat grains.

Ref.: JAOAC **70,** 907(1987).

## 968.06 Protein (Crude) in Animal Feed

**Dumas Method**

**First Action 1968**
**Final Action 1969**

### A. Principle

N, freed by pyrolysis and subsequent combustions, is swept by $CO_2$ carrier into nitrometer. $CO_2$ is absorbed in KOH and vol. residual N is measured and converted to equiv. protein by numerical factor.

### B. Apparatus and Reagents

(**a**) *Nitrogen analyzer and accessories.*—Consists of combustion and collection and measuring systems. Suitable instrument, Model 29A, with following accessories and reagents is available from Oak Brook Instruments Div. of Perkin Elmer Corp., 2000 York Rd, Oak Brook, IL 60521 (Perkin Elmer's current model of nitrogen analyzer is PE2410N nitrogen analyzer): Al combustion boats, No. 29–412; Vycor combustion tubes, No. 29–328; CuO-Pt catalyst (CuO wire form with 2.5% Pt reforming catalyst), No. 29–160; reduced Cu wire, No. 29–120; $Co_3O_4$, No. 29–170; CuO powder, fines, No. 29–140; 45% KOH, No. 29–110.

(**b**) *Balance.*—Accurate to 0.01 mg.

(**c**) *Barometer.*—Hg type, readable to 0.1 mm.

### C. Preparation of Samples

Grind to pass No. 30 sieve. Store in capped bottles.

### D. Determination

Operate instrument in accordance with instructions of manufacturer. (Following directions apply to Coleman Model 29A Nitrogen Analyzer. Consult Operating Directions D-360B, Coleman Cat. No. 29–904, for addnl details.)

After combustion furnaces have come to thermal equilibrium, turn combustion cycle control to START and let proceed normally thru cycle. Observe indicated temp. on pyrometer of both upper and lower combustion furnaces at end of combustion portion of cycle. Furnace temps should be 850–900°. If not, adjust.

Prep. combustion tube by inserting stainless steel screen in lower end of combustion tube (end farthest from trademark). In upper end, place enough glass wool to form 6 mm plug when packed. With 11 mm glass rod, drive glass wool down to stainless steel plug. Holding tube vertically, pour CuO-Pt catalyst directly from dispenser bottle into combustion tube until it reaches upper end of trademark. Tap or vibrate tube on bench until reagent settles to approx. center of trademark.

Weigh and record wt of empty Al combustion boat. Place sample in boat. Weigh and record wt of sample and combustion boat. Difference between wts is sample wt. Use following sample wts (mg) as guides to suitable sample sizes: bermuda grass 150–300; rice bran, wheat shorts, dehydrated alfalfa 150–250; range feed 100–200; cottonseed meal 75–150; edible soy protein 50–150. Weigh sample to nearest 0.01 mg. To avoid wt changes, record wt within 1 min after sample and boat are placed on balance. If this is impossible, weigh sample inside weighing bottle, such as Kimble No. 15165 or 15166.

Turn combustion tube to horizontal, and carefully insert loaded sample boat into open end of tube. Slide or push boat, without spilling contents, until it reaches trademark. Raise open end until tube forms 60–70° angle to horizontal. Tap or vibrate combustion tube on bench top while rotating tube between thumb and forefinger. Raise open end of tube and add vol. $Co_3O_4$ and vol. CuO fines equal to vol. sample. For convenient means of adding above reagents to samples, place vol. CuO fines and vol. $Co_3O_4$, each equal to vol. sample, in addnl combustion boat; add contents of boat, but not boat itself, to combustion tube; and rotate partially filled combustion tube between thumb and forefinger while varying angle of tube 20–45° from horizontal. Continue rotating, tapping, and vibrating until sample is dispelled from boat and is thoroly mixed with oxidizing agents. Raise open end until tube forms 60–70° angle to horizontal; add CuO-Pt catalyst ca 12 mm above sample boat. Tap or vibrate gently to eliminate voids. Add CuO-Pt catalyst to within 20 mm of top of tube, again tapping or vibrating gently to eliminate voids.

Install prepd combustion tube in N analyzer. Adjust 45% KOH soln meniscus to calibrating mark in nitrometer with digital readout meter. Record counter reading, $R_1$. (Counter reading should preferably lie between 500 and 1000 μL at this point. Vent control may be used to assist in arriving at this counter setting, if necessary.) Record syringe temp., $t_1$, indicated on special scale thermometer. Add 2 min more to combustion portion of cycle by turning auxiliary timer to setting 3. (Once this is done, addnl 2 min will be automatically programmed into each subsequent cycle.) Turn combustion cycle control to START. Let analyzer proceed thru its cycle. After cycle is complete and combustion cycle control has entered STAND-BY section, readjust KOH meniscus to calibration mark with digital readout counter. Record new counter reading, $R_2$, and syringe temp., $t_2$. Det. blank for instrument under same conditions as actual analysis except omit sample.

### E. Calculations

(**a**) Record observed N vol., $V_o = R_2 - R_1$, where $V_o$ = observed N vol. (μL), $R_1$ = initial counter reading, and $R_2$ = final counter reading.

(**b**) Det. corrected N vol. (in μL), $V_c = V_o - (V_b + V_t)$, where $V_b$ = vol. blank (μL), $V_t$ = vol. correction for temp. (μL) = $C_f(t_2 - t_1)$. $C_f$ is obtained from Table **968.06A** (based on final counter reading); $t_2$ and $t_1$ are in °K.

(**c**) Det. corrected barometric pressure, $P_c = P_o - (P_b + P_v)$, where $P_o$ = observed barometric pressure (mm Hg), $P_b$ = barometric temp. correction (from Table **968.06B**), and $P_v$ = pressure correction for vapor pressure of KOH soln (from Table **968.06C**).

**Table 968.06A Volume Correction for Temperature Correction Factor ($C_f$) (μL/°K)[a]**

| Final Counter Reading, μL | $C_f$ (Nitrometers with Check Value) |
|---|---|
| 0 | 12 |
| 5000 | 29 |
| 10000 | 45 |
| 15000 | 62 |
| 20000 | 79 |
| 25000 | 95 |
| 30000 | 112 |
| 35000 | 129 |
| 40000 | 145 |
| 45000 | 162 |
| 50000 | 179 |

[a] Vol. correction, $V_t = C_f(t_2 - t_1)$

**Table 968.06B Barometric Temperature Correction ($P_b$)**

| | $P_o$ (mm Hg) | |
|---|---|---|
| Temperature, °C | 700–749 | 750–780 |
| 10 | 1.2 | 1.3 |
| 15 | 1.8 | 1.9 |
| 20 | 2.3 | 2.5 |
| 25 | 2.9 | 3.1 |
| 30 | 3.5 | 3.7 |
| 35 | 4.1 | 4.3 |

**Table 968.06C Pressure Correction ($P_v$) for Vapor Pressure of KOH (for Practical Purposes, Temp. of KOH is Same as Syringe)**

| Temperature, °K | $P_v$, mm Hg |
|---|---|
| 288 | 4.1 |
| 293 | 5.7 |
| 298 | 7.4 |
| 303 | 9.6 |
| 308 | 12.5 |
| 313 | 16.5 |

(*Note:* Empirical approximation of $(P_b + P_v) = 11.0$ will be satisfactorily accurate for $P_o$ between 740 and 780 mm Hg and syringe temp. between 298 and 305°K.)

(**d**) Calc. % N = $(P_c \times V_c \times 0.0449)/(T \times W)$, where $T$ = final syringe temp. in °K and $W$ = sample wt in mg.

*Example:*

$P_o$ = 750.1 mm Hg at 25°C; $W$ = 148.91 mg

| | *Start* | *Finish* |
|---|---|---|
| Counter readings, blank | 500 µL | 524 µL |
| Counter readings, sample | 524 | 6955 |

$t_1 = 302.7°K$, $t_2 = 303.0°K$, $V_o = 6955 - 524 = 6431$ µL

$$V_c = 6431 - [24 + C_f(t_2 - t_1)] = 6431 - (24 + 35 \times 0.3) = 6396 \text{ µL}$$

$$P_c = 750.1 - (3.1 \times 9.6) = 737.4$$

$$\% \text{ N} = (737.4 \times 6396 \times 0.04493)/(303.0 \times 148.91) = 4.69\%$$

(**e**) Calc. % protein = % N × 6.25, or % N × 5.70 in case of wheat grains.

Ref.: JAOAC **51,** 766(1968).

## 976.05 Protein (Crude) in Animal Feed

### Automated Kjeldahl Method

**First Action 1976**
**Final Action 1977**

### A. Principle

Automation of macro Kjeldahl method is in 6 steps: sample and reagent addn, initial and final digestion, cooling and diln, NaOH addn, steam distn and titrn, and automatic pumping of flask contents to waste. Chemistry is carried out in macro Kjeldahl flasks equipped with side arms which are rotated at 3 min intervals thru each successive step.

### B. Apparatus

(**a**) *Kjeldahl (protein/nitrogen) analyzer.*—Kjel-Foss Automatic, Model 16210 (Foss Food Technology Corp.), or equiv.

(**b**) *Weighing papers.*—120 × 120 mm N-free tissues, Foss Food Technology Corp., or equiv.

### C. Reagents

(**a**) *Kjel-tabs.*—Contg 5 g $K_2SO_4$ and 0.25 g HgO (Foss Food Technology Corp.).

(**b**) *Kjeldahl (protein/nitrogen) analyzer reagents.*—Prep. following according to manufacturer's instructions: (*1*) *Sulfuric acid.*—96–98%. (*2*) *Hydrogen peroxide.*—30–35%. (*3*) *Ammonium sulfate std solns.*—(*a*) *Std soln I.*—Dissolve 30.000 ± 0.030 g $(NH_4)_2SO_4$ in $H_2O$ and dil. to 1 L with $H_2O$. (*b*) *Std soln II.*—Dissolve 0.750 ± 0.001 g $(NH_4)_2SO_4$ in $H_2O$ and dil. to 1 L with $H_2O$. (*4*) *Mixed indicator soln.*—Dissolve 1.000 g Me red and 0.250 g methylene blue in alcohol and dil. to 1 L with alcohol. Dil. 10 mL this soln to 1 L with $H_2O$. (*5*) *Sodium hydroxide-sodium thiosulfate soln.*—40% NaOH-8% $Na_2S_2O_3.5H_2O$. (*6*) *Dilute sulfuric acid soln.*—0.6%. Dil. 30 mL 96–98% $H_2SO_4$ to 5 L with $H_2O$.

### D. Determination

(*Caution:* See safety notes on wet oxidation, sulfuric acid, mercury, and peroxides.)

Place 3 Kjel-tabs in special flask (500 mL of design compatible to Foss instrument) in position 1. Shift dispenser arm over flask and depress $H_2SO_4$ lever, initiating simultaneous addn of 10 mL 30–35% $H_2O_2$ and 12–15 mL 96–98% $H_2SO_4$ (depending on fat content of sample). To flask, add accurately weighed sample (ca 1.0 g if <45% protein, and ca 0.5 g if >45% protein) wrapped in weighing paper and close lid. Flask automatically rotates to position 2 where sample digests 3 min, and then to position 3 for 3 min addnl digestion. In position 4, flask is cooled by centrifugal blower, lid opens automatically, and 140 mL $H_2O$ is added automatically. Flask rotates to position 5, where NaOH-$Na_2S_2O_3$ soln is automatically introduced in excess. Released $NH_3$ is steam distd quant. into 200 mL tall-form titrn beaker contg 50 mL mixed indicator soln, and is simultaneously titrd automatically with dil. $H_2SO_4$ soln delivered by photometrically regulated syringe. Final position of syringe is measured by potentiometer, output of which feeds electronic circuitry for conversion to visual display and/or printout in % N or % protein with appropriate conversion factors. In position 6, flask is emptied. Calibrate instrument initially each day with aliquots of $(NH_4)_2SO_4$ std solns and check periodically as stated in operating manual.

Ref.: JAOAC **59,** 141(1976).

## 976.06 Protein (Crude) in Animal Feed

### Semiautomated Method

**First Action 1976**
**Final Action 1977**

### A. Principle

Samples are digested in 250 mL calibrated tubes, using block digestor. *A* of $NH_3$-salicylate complex is read in flowcell at 660 nm, or $NH_3$, is distd into std acid and back-titrd with std alkali.

### B. Apparatus

(**a**) *Block digestor.*—Model BD-20 (Technicon Instruments Corp.) or Model DS-20 (Tecator, Inc., 2875C Towerview Rd, Herndon, VA 22071). Capable of maintaining 410° and digesting 20 samples at a time in 250 mL calibrated volumetric tubes constricted at top. Block must be equipped with removable shields to enclose exposed area of tubes completely at or above ht of constriction.

(**b**) *Automatic analyzer.*—AutoAnalyzer with following modules (Technicon Instruments Corp.), or equiv.: Sampler II or IV with 40/hr (2:1) cam (higher ratio cams result in carry-over and poorer peak sepn); proportioning pump III; $NH_3$ anal. cartridge No. 116-D531-01 (or construct equiv. manifold from flow diagram); AAII single channel colorimeter with 15 × 1.5–2.0 mm id tubular flowcell, matched 660 nm interference filters, and voltage stabilizer; and recorder of appropriate span. (*See* Fig. **976.06**.)

### C. Reagents

(**a**) *Phosphate-tartrate buffer soln.*—pH 14.0. Dissolve 50 g NaK tartrate and 26.8 g $Na_2HPO_4.7H_2O$ in 600 mL $H_2O$. Add 54 g NaOH and dissolve. Add 1 mL Brij-35 (Technicon Instruments Corp.), dil. to 1 L with $H_2O$, and mix.

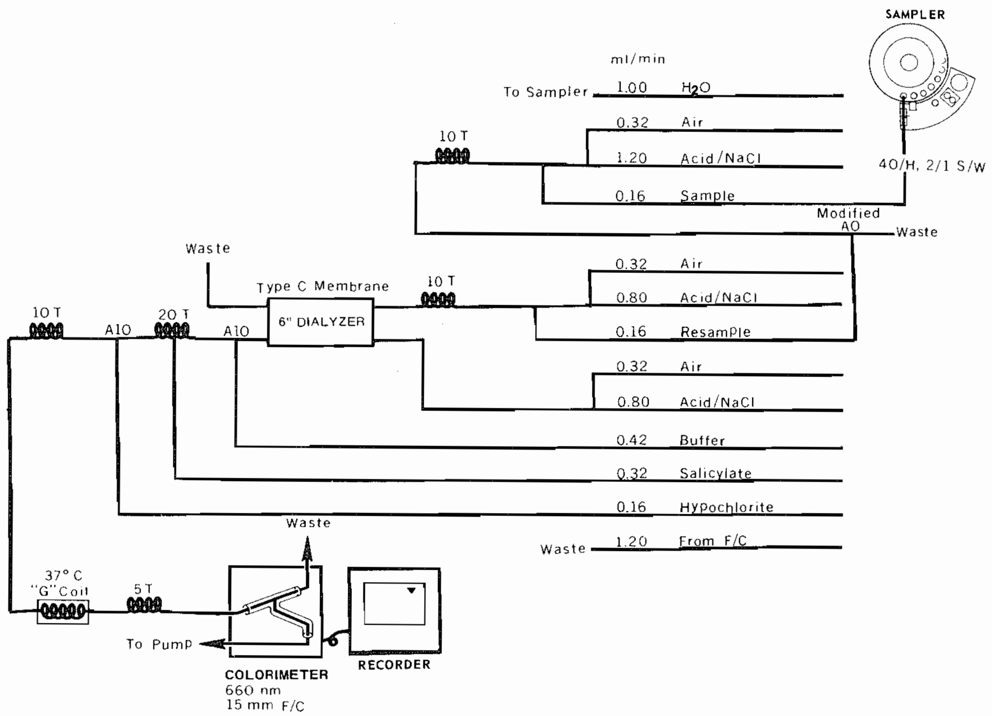


**FIG. 976.06—Flow diagram for semiautomated analysis for crude protein**

(**b**) *Sodium chloride-sulfuric acid soln.*—Dissolve 200 g NaCl in $H_2O$ in 2 L vol. flask. Add 15 mL $H_2SO_4$ and 2 mL Brij-35. Dil. to vol. with $H_2O$ and mix.

(**c**) *Sodium hypochlorite soln.*—Dil. 6 mL com. bleach soln contg 5.25% available Cl (Clorox, or equiv.) to 100 mL with $H_2O$ and mix. Prep. fresh daily.

(**d**) *Sodium nitroprusside-sodium salicylate soln.*— Dissolve 150 g $NaC_7H_5O_3$ and 0.3 g $Na_2Fe(CN)_5.NO.2H_2O$ in 600 mL $H_2O$. Add 1 mL Brij-35, dil. to 1 L with $H_2O$, and mix.

(**e**) *Nitrogen std solns.*—Prep. 6 stds by accurately weighing (±10 mg) 59, 118, 177, 236, 295, and 354 mg $(NH_4)_2SO_4$ primary std (Fisher Scientific Co. No. A-938, or equiv.; dry 2 hr at 105° before use and assume theoretical value of 21.20% N after drying) into individual 250 mL digestion tubes. Proceed as in **976.06G**, beginning "Add 9 g $K_2SO_4$, 0.42 g HgO, and 15 mL $H_2SO_4$ . . ." Stds may be stored and reused until exhausted.

(**f**) *Sodium hydroxide-potassium sulfide soln.*—Dissolve 400 g NaOH in $H_2O$. While still warm, dissolve 30 g $K_2S$ in soln, and dil. to 1 L.

### D. Analytical System

If manifold is to be constructed, use clear std pump tubes for all air and soln flows. All fittings, coils, and glass transmission lines are AAII type and size. Use glass transmission tubing for all connections after pump to colorimeter. Construct modified AO fitting on sample diln loop using AO fitting, N13 stainless steel nipple connector, and 1/2" length of 0.035" id Tygon tubing. Insert N13 nipple approx. halfway into 0.035" Tygon tubing. Insert tubing into side arm of AO fitting far enough so resample line will not pump any air. Space pump tubes equally across pump rollers. Cut 0.16 mL/min resample pump tube ≤1" at entrance before connecting to side arm of AO fitting. In operation, add buffer and hypochlorite solns thru metal side arms of A10 type fittings; add salicylate soln, (**d**), thru metal insert to 20T coil. Air, reagents, and sample are combined immediately after pump thru injection fittings.

### E. Start-Up

Start automatic system and place all lines except salicylate line in resp. solns. After ≥5 min, place salicylate line in resp. soln and let system equilibrate. If ppt forms after addn of salicylate, pH is too low. Immediately stop proportioning pump and flush coils with $H_2O$, using syringe. Before restarting system, check concns of NaCl-$H_2SO_4$ soln and phosphate-tartrate buffer soln.

Pump lowest concn N std soln continuously thru system ≥5 min and adjust baseline control on colorimeter to read 10% full scale. Pump highest concn N std soln continuously thru system until no drift exists (usually ≥10 min) and adjust "std. cal." control to read 85% full scale. Recorder tracings must be stable and show <0.3 division noise. If noisy conditions exist, replace dialyzer membrane. When recorder tracing indicates stable condition, immediately start sampling.

### F. Shut-Down

Place reagent lines in $H_2O$, removing salicylate line first. Let system wash out ≥20 min.

### G. Colorimetric Determination

(*Caution:* See safety notes on mercury.)

Weigh samples (*See* Table **976.06**) into dry digestion tubes. Add 9 g $K_2SO_4$, 0.42 g HgO, and 15 mL $H_2SO_4$ to each tube.

**Table 976.06 Sample Weight**

| Protein, % | Sample, g |
|---|---|
| 6–24 | 1.5±0.1 |
| 25–40 | 1.0±0.1 |
| 41–50 | 0.8±0.1 |
| 51–60 | 0.7±0.1 |
| 61–90 | 0.5±0.01 |
| >90 | Weigh sample equiv. to 50 mg N |

(Calibrated metal scoops may be used for solids.) Insert tubes into digestor block preheated to 410°, place shields around tubes, and digest 45 min.

After digestion, remove rack of tubes from block, place in hood, and let cool 8–10 min. (Time depends upon air flow around tubes.) Direct rapid spray of $H_2O$ (kitchen sink dish rinsing sprayer works well) to bottom of each tube to dissolve acid digest completely. If ppt forms, place tube in ultrasonic bath to aid in redissolving salt. Let cool, dil. to vol., and mix thoroly. Transfer portion of each sample soln to AutoAnalyzer beaker.

Place stds in tray in increasing order of concn, followed by group of samples. Analyze lowest concn std in duplicate, discarding first peak. Precede and follow each group of samples with std ref. curve to correct for possible drift. Analyze stds and samples at rate of 40/hr, 2/1 sample-to-wash ratio. Prep. std curve by averaging peak hts of first and second set of stds. Plot av. peak ht stds against N concn contained in each 250 mL tube.

$$\% \text{ Protein} = [(\text{mg N}/250 \text{ mL from graph}) \times 6.25 \times 100]/\text{mg sample}$$

### H. Titrimetric Determination

Digest as in **976.06G**. Cool 5 min and add only enough $H_2O$ to dissolve salts (70–75 mL). Cool and attach digestion tube to distn head according to manufacturer's directions. Place receiver flask contg 25 mL std acid, **936.15A** or **890.01A**, and 5–7 drops Me red indicator on platform. Condenser tip must be below surface of std acid soln. Add 50 mL NaOH-$K_2S$ soln to tube and steam distil vigorously until 125 mL distillate collects. Titr. excess acid with std 0.1*N* NaOH soln, **936.16**. Correct for reagent blank.

$$\%\text{N} = [(\text{mL std acid} \times \text{normality acid}) - (\text{mL std NaOH} \times \text{normality NaOH})] \times 1.4007/\text{g sample}$$

$$\% \text{ crude protein} = \% \text{ N} \times 6.25$$

Refs.: JAOAC **59,** 134(1976); **62,** 290(1979).

## 984.13 Protein (Crude) in Animal Feed
### Copper Catalyst Kjeldahl Method
### First Action 1984

(*Caution:* See safety notes on sulfuric acid and sodium hydroxide.)

### A. Principle

Sample is digested in $H_2SO_4$, using $CuSO_4$ as catalyst, converting N to $NH_3$ which is distd and titrd.

### B. Reagents

(**a**) *Sodium hydroxide.*—Pellets, flakes, or soln witn sp. gr. ≥1.36, low N. Dissolve ca 450 g NaOH in $H_2O$, cool, dil. to 1 L.

(**b**) *Alundum.*—Boiling stones, 8–14 mesh (Thomas Scientific Co., No. 1590-D18).

(**c**) *Methyl red indicator.*—Dissolve 1 g Me red (Na salt) in 100 mL MeOH.

(**d**) *Hydrochloric or sulfuric acid std soln.*—0.5N. Prep. as in **936.15** or **890.01**.

(**e**) *Sodium hydroxide std soln.*—0.1N. Prep. as in **936.16**.

After stdzg both acid and base by methods suggested in (**d**) and (**e**), also check one against the other. In addn, check entire method by analyzing NIST Std Ref. Material No. 194, $NH_4H_2PO_4$, certified 12.15% N, and high purity lysine.HCl.

### C. Apparatus

(**a**) *Digestion.*—Use Kjeldahl flasks with capacity of 500–800 mL.

(**b**) *Distillation.*—Use digestion flask (Corning Glass Works, or equiv.) connected to distn trap by rubber stopper. Distn trap is connected to condenser with low-S tubing. Outlet of condenser tube should be <4 mm diam.

### D. Determination

Weigh 0.250–1.000 g sample into digestion flask. Add 15 g $K_2SO_4$, 0.04 g anhyd. $CuSO_4$, 0.5–1.0 g alundum granules, and 20 mL $H_2SO_4$. (Add addnl 1.0 mL $H_2SO_4$ for each 0.1 g fat or 0.2 g other org. matter if sample wt is >1 g.)

Include at least one sample of high purity lysine.HCl in each day's run as check of correctness of digestion parameters. If recovery is not complete, make appropriate adjustments.

Heat flask at 5-min boil rate (burner preheated and adjusted to bring 250 mL $H_2O$ at 25° to rolling boil in 5 min) until dense white fumes clear bulb of flask, swirl gently, continue heating addnl 90 min. (Note: Reagent proportions, heat input, and digestion time are critical factors—do not change.) Cool, cautiously add 250 mL $H_2O$, and cool to room temp. (Note: If bumping occurs during distn, vol. of $H_2O$ may be increased to ca 275 mL.)

Prep. titrn beaker by adding accurately measured appropriate vol. std acid soln to amt of $H_2O$ such that condenser tip will be sufficiently immersed. Add 3–4 drops indicator soln (**c**).

Add 2–3 drops of tributyl citrate to digestion flask to reduce foaming; add another 0.5–1.0 g alundum granules. Slowly down side of flask, add sufficient NaOH soln (**a**) such that mixt. will be strongly alk. Immediately connect flask to distn app., mix completely, and distil at ca 7.5-min boil rate until ≥150 mL distillate is collected in titrn beaker.

Titr. excess std acid in distillate with std NaOH soln. Correct for blank detn on reagents. Calc. % N:

$$\% \text{ N} = [(N_{acid})(mL_{acid}) - (mL_{bk})(N_{NaOH}) - (mL_{NaOH})(N_{NaOH})][1400.67]/\text{mg sample}$$

where $mL_{NaOH}$ = mL std base needed to titr sample; $mL_{acid}$ = mL std acid used for that sample; $mL_{bk}$ = mL std base needed to titr. 1 mL std acid minus mL std base needed to titr. reagent blank carried thru method and distd into 1 mL std acid; $N_{acid}$ = normality of std acid; $N_{base}$ = normality of std base. Calc. % crude protein, defined as 6.25 × % nitrogen, or 5.7 × % nitrogen for wheat grains.

Ref.: JAOAC **67,** 869(1984).

## 989.03 Fiber (Acid Detergent) and Protein (Crude) in Animal Feed and Forages
### Near-Infrared Reflectance Spectroscopic Method
### First Action 1989

(Generally applicable to detn of acid-detergent fiber and crude protein in any forage or feed sample)

Method Performance:

Crude protein:

$$s_R = 0.15; RSD_R = 0.42\%$$

Acid-detergent fiber:

$$s_R = 0.34; RSD_R = 1.14\%$$

Successful use of method is based on obtaining suitable calibration for instrument by selecting learning set of samples and performing calibration described in *Determination* (**b**). Four rules to be followed in calibration of instrument are stated in USDA Agriculture Handbook No. 643, p. 45 ("Near Infrared Reflectance Spectroscopy (NIRS): Analysis of Forage Quality." *U.S. Dep. Agric. Handb. 643*, U.S. Government Printing Office, Washington, DC, 96 pp.). In brief, rules are as follows:

(*1*) Be certain calibration samples adequately represent population to be analyzed.

(*2*) Conduct accurate laboratory analyses on calibration samples. This step cannot be overemphasized.

(*3*) Select appropriate data processing technique to extract pertinent information from spectra.

(*4*) Select correct wavelengths.

It may be necessary to periodically update a calibration. Analyst must monitor results of method to ascertain when this is necessary. Test sample is usually run each day and its analytical value is detd periodically by laboratory analysis method. Accuracy of any chemometric procedure is limited only by validity of laboratory method used to measure desired quality parameter for all samples and selection of appropriate learning set.

### A. Principle

Random portions of prepd sample are loaded into sample holder of NIR spectrometer. Instrument is part of system that has been calibrated using representative samples from population to be tested. Equations selected from calibration statistics, which have been validated, are used to calc. acid-detergent fiber and crude protein content of feed and forage samples.

### B. Apparatus

(**a**) *Wavelength-scanning instrument*.—Model 6100 or 6350 grating monochromator (Pacific Scientific Corp., Gardner/Neotec Instrument Div., Silver Spring, MD 20910), or equiv. Monochromator is described in detail in (*1*) Landa, I. Rev. Sci. Instrum. **50,** 34–40(1979); and (*2*) Landa, I., & Norris, K.H. Appl. Spectrosc. **23,** 105–107(1979).

(**b**) *Computer*.—PDP 11 Series computer equipped with 64 K bytes of main memory; dual RX02 double-density floppy disks; RL01 5-megabyte or RL02 10-megabyte hard disks. PDP system software RT-11 V5.0 or later (Digital Equipment Corp., Nashua, NH 03061), or equiv.

(**c**) *USDA public software*.—Software is described in detail in USDA Agriculture Handbook No. 643. Software consists of 14 programs written in FORTRAN IV to collect, store, and process NIRS data. Repository for software: U.S. Department of Agriculture, Richard B. Russell Agricultural Research Center, Plant Structure and Composition Research Unit, PO Box 5677, Athens, GA 30613. Commercial software is available from several vendors.

(**d**) *Mill*.—Tecator cyclone sample mill with 1 mm screen (Fisher Scientific Co.), or equiv. Periodically change grinding ring to ensure consistency of particle size over time.

(**e**) *NIRS sample holder*.—Nylon, 2.5 cm diam., 1 cm thick, with IR transmittance quartz window. Sample capacity 0.75–1.75 g. Sample is held in place with sep. back made of rubber or foam core (Pacific Scientific Corp., Gardner/Neotec Instrument Div.), or equiv.

(**f**) *Sample storage container*.—For maintaining const moisture concn in samples. For best results, use Poly Kraft Bags-Mil-B-121 Type II, Grade A, Class I. Place sample in bag and heat-seal (EDCO Supply Corp., Brooklyn, NY 11232, or equiv.).

### C. Instrument Operation

(**a**) *Start-up*.—For best results, run instrument continuously. If instrument is cold, warm-up time should be >15 min and may require 1 h.

(**b**) *Monochromator diagnostic tests*.—(*1*) *Instrument noise*.—Scan ceramic ref. to itself. Collect 25 repetitions of 64 scans. Express deviations from zero as av. deviation (bias), and as root mean sq (RMS), expressed as log $(1/R)/10^6$, where R = reflectance. Bias indicates any systematic change in log (1/R) level of scans taken over time. Bias values that are all pos. or all neg. indicate problem with instrument. RMS value can range from low of 10 to high of 50 without affecting analysis. Monochromators should have av. noise level below 30 RMS over 100 scans.

(*2*) *Wavelength accuracy*.—Use clear polystyrene petri dish to measure wavelength repeatability and accuracy. Place petri dish in light beam and then pull out sample drawer to expose ceramic std. Ref. this scan to measurements without petri dish. Locate and compare major styrene peaks with known locations at 1680.3, 2164.9, and 2304.2 nm. Repeatability std dev. should be <0.05 nm, and accuracy from known location should be <0.5 nm. Large pos. values for wavelength accuracy and repeatability usually indicate mech. problems in monochromator.

(**c**) *Maintenance*.—Whenever dust accumulates, use vacuum, brush, or soft tissue to clean ceramic std, all parts of drawer assembly, and windows above and below detector. Instrument operation is described in detail in USDA Agriculture Handbook No. 643 and in Shenk, J.S., Westerhaus, M.O., & Hoover, M.R. Proc. Am. Soc. Agric. Eng. (1978), p. 242.

### D. Determination

(**a**) *Preparation of sample*.—Grind samples for NIRS analysis in cyclone mill through 1 mm screen. Clean mill between samples to minimize cross-contamination. Prior to grinding, dry samples contg >25% moisture in 60° air oven for 24 h. Mix milled samples well, and place random portion in sample holder. Continue to add random portions until NIRS sample holder is $^2/_3$ full. Press back into holder until it is tight and level. As check, invert holder and make certain sample is firmly pressed against window. If any abnormality is apparent, remove back and repeat procedure. Consistency in sample handling and prepn is crucial to successful use of NIRS technique.

(**b**) *Calibration*.—To calibrate system for acid-detergent fiber and crude protein detns, randomly select samples that are representative of population to be analyzed, using either finite or infinite population. Finite population has defined boundaries set by analyst which limit population; infinite population has no such boundaries. Select sufficient number of samples to represent range of acid-detergent fiber and crude protein concns and all other variables that may affect chem. and physical composition of feed or forage (stage of growth, species, preservation method, etc.). In practical terms, min. of 50 samples should be considered.

Collect reflectance (R) measurements (log 1/R) of calibration samples with program SCAN, *Apparatus* (**c**), at 2.0 nm intervals from 1100 to 2500 nm. Develop multiterm calibration equations by multiple linear regression of reflectance measurements to acid-detergent fiber and crude protein concns using program CAL (**c**). Before regression statistics are evaluated, examine differences (residuals) between NIRS data and ref. method data for samples with large *t*-values. Large pos. or neg. *t*-value indicates that residual is 2.5 times std error of difference between NIRS detn and ref. method detn, and that laboratory values from ref. method were inaccurate or did not represent samples at time scan was taken (i.e., subsampling error). Reanalyze these samples by ref. method.

In addn, evaluate output for samples that have large H-values. Large H-statistic (>3) indicates that NIRS spectra used in calibration for that sample differ substantially from NIRS spectra of other samples. Calcn of H was mathematically derived from covariance matrix according to formula: H =

$X(s^1x)^{-1}x^1$ [Landa. *Apparatus* (**a**)]. High value on diagonal of H matrix indicates sample that is dissimilar to calibration set at wavelengths used in equation. Rescan such samples. If 2 scans agree and sample belongs in population, then retain sample. If scans disagree, then first scan was mistake and should be discarded.

Examine std error of calibration (SEC) to det. fit of calibration samples: the lower the SEC, the better the fit. Select equation with SEC about 2 times laboratory repeatability std dev. for acid-detergent fiber and crude protein ref. method. Examine coeff. of detn ($R^2$) to det. proportion of variation in ref. method values among samples explained by NIRS regression equation. Low $R^2$ values often indicate that laboratory data from ref. method are imprecise. If laboratory repeatability error from ref. method is $^1/_4$ of std dev., select equation with $R^2 \geq 0.75$.

Examine $F$-statistic of regression coeffs. High $F$-values indicate that regression coeff. is significantly different from zero; small $F$-values indicate that coeff. contributes little to equation except to fit random errors. Probability that observed $F$-value was obtained solely through chance does not follow std $F$ tables because $F$ is selected as max. of all wavelength combinations considered. As number of choices increases, large $F$-values are needed to signify coeff. fitting more than just random errors. Reject equations with $F$-values $<10$.

(**c**) *Validation*.—Conduct NIRS analysis (program PRE) with equations selected from calibration statistics on population of unknown samples. Examine NIRS data for samples with larger H-values. Large H-value ($>3.0$) for a few samples indicates that their NIR spectra are different from spectra of calibration population. Large $t$-value (2.5 times SED) for a few samples indicates that laboratory values from ref. method were inaccurate or did not represent samples at time scan was taken. If many validation samples have large $t$- and H-values, over-fitting has occurred, and equation is specific to samples in calibration set. Next, use validation statistics from program STAT to examine std error of analysis (SEA) by NIRS of chem. composition of validation samples. SEA is true indication of performance of equation on unknown samples. Select equation with lowest bias and SEA. Unlike SEC, which must decrease with each addnl term, SEA only decreases with addnl terms until over-fitting becomes important and forces it to increase. Best equation for routine NIRS analysis is based on both superior calibration and validation statistics.

Ref.: JAOAC **71**, 1162(1988).

## 941.04 Urea and Ammoniacal Nitrogen in Animal Feed

### Urease Method

### Final Action

#### A. Reagents

(**a**) *Defoaming soln*.—Dow Corning Corp. Antifoam B Emulsion.

(**b**) *Urease soln*.—Prep. fresh soln by dissolving stdzd urease in $H_2O$ so that each 10 mL neutzd soln will convert N of $\geq 0.1$ g pure urea.

*Standardization*.—To det. alky of com. urease prepn dissolve 0.1 g in 50 mL $H_2O$ and titr. with 0.1$N$ HCl, using Me red, **984.13B(c)**. Add same vol. 0.1$N$ HCl to each 0.1 g urease in prepg urease soln. To det. enzyme activity, prep. ca 50 mL neutzd 1% soln. Add different amts of soln to 0.1 g samples pure urea and follow with enzyme digestion and distn as in detn. Calc. activity of urease prepn from amt of this urease soln that completely converted urea, as detd by complete recovery of N by distn.

(**c**) *Calcium chloride soln*.—Dissolve 25 g $CaCl_2$ in 100 mL $H_2O$.

#### B. Determination

Place 2 g sample in Kjeldahl flask with ca 250 mL $H_2O$. Add 10 mL urease soln, stopper tightly, and let stand 1 hr at room temp. or 20 min at 40°. Cool to room temp. if necessary. Use addnl urease soln if feed contains $>5\%$ urea (ca 12% protein equiv.). Rinse stopper and neck with few mL $H_2O$. Add $\geq 2$ g MgO (heavy type), 5 mL $CaCl_2$ soln, and 3 mL defoaming soln, and connect flask with condenser by Kjeldahl connecting bulb. Distil 100 mL into measured vol. std acid, **936.15** or **890.01**, and titr. with std alkali, **936.16**, using Me red, **984.13B(c)**.

Refs.: JAOAC **24**, 867(1941); **25**, 874(1942); **27**, 494(1944).

## 967.07 Urea in Animal Feed

### Colorimetric Method

### First Action 1967
### Final Action 1970

(Applicable to animal feeds and their ingredients)

#### A. Apparatus

*Spectrophotometer*.—Instrument with max. band width 2.4 nm at 420 nm, with 1 cm cells.

#### B. Reagents

(**a**) *p-Dimethylaminobenzaldehyde* (*DMAB*) *soln*.—Dissolve 16.00 g (Eastman Kodak Co. No. 95 only) in 1 L alcohol and add 100 mL HCl. Stable 1 month. Prep. new std curve with each new batch of reagent.

(**b**) *Zinc acetate soln*.—Dissolve 22.0 g $Zn(OAc)_2.2H_2O$ in $H_2O$, add 3 mL HOAc, and dil. to 100 mL.

(**c**) *Potassium ferrocyanide soln*.—Dissolve 10.6 g $K_4Fe(CN)_6.3H_2O$ in $H_2O$ and dil. to 100 mL.

(**d**) *Vegetable charcoal*.—Darco G-60.

(**e**) *Phosphate buffer soln*.—pH 7.0. Dissolve 3.403 g anhyd. $KH_2PO_4$ and 4.355 g anhyd. $K_2HPO_4$ sep. in ca 100 mL portions freshly distd $H_2O$. Combine solns and dil. to 1 L with $H_2O$.

(**f**) *Urea std solns*.—(*1*) *Stock soln*.—5 mg/mL. Dissolve 5.000 ± 0.001 g reagent grade urea in $H_2O$ and dil. to 1 L with $H_2O$. (*2*) *Working solns*.—0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8, and 2.0 mg urea/5 mL. Pipet 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20 mL stock soln into 250 mL vol. flasks and dil. to vol. with phosphate buffer. (*3*) *Reference soln*.—Use std soln contg 1.0 mg urea/5 mL as ref. std. Store at $<24°$. Stable 1 week.

#### C. Preparation of Standard Curve

Pipet 5 mL aliquots of working std solns into 20 × 150 mm (25 mL) test tubes and add 5 mL DMAB soln to each. Prep. reagent blank of 5 mL buffer soln and 5 mL DMAB soln. Shake tubes thoroly and let stand 10 min in $H_2O$ bath at 25°. Read $A$ in 1 cm cell at 420 nm with reagent blank at zero $A$. Plot $A$ against concn urea. Plot should be straight line; if not, repeat, using new lot of DMAB.

#### D. Determination

Weigh 1.00 g ground sample into 500 mL vol. flask. Add 1 g charcoal, ca 250 mL $H_2O$, 5 mL $Zn(OAc)_2$ soln, and 5 mL $K_4Fe(CN)_6$ soln. Shake mech. 30 min and dil. to vol. with $H_2O$. Let stand until ppt settles. Decant thru Whatman No. 40 paper and collect clear filtrate. Pipet 5 mL filtrate into test tube, add 5 mL DMAB soln, and shake thoroly. Include reference std (5 mL soln (**f**)(*3*) and 5 mL DMAB soln) and reagent blank with each group of samples. Let stand 10 min in $H_2O$ bath at 25°. Read $A$ at 420 nm against reagent blank.

% Urea = $(1.0 \times A_{sample} \times 100)/(A_{std} \times$ mg sample in aliquot)

Ref.: JAOAC **50,** 56(1967).

CAS-57-13-6 (urea)

## 920.37★ Nitrogen (Albuminoid) in Animal Feed

**Final Action**
**Surplus 1965**

*See* **22.020–22.021**, 10th ed.

## 920.38★ Nitrogen (Amido) in Animal Feed

**Final Action**
**Surplus 1965**

% Amido N = % Total N − % albuminoid N

## 968.07 Nitrogen (Nitrate and Nitrite) in Animal Feed

**Colorimetric Method**
**First Action 1968**
**Final Action 1970**

### *A. Principle*

Nitrate and nitrite are extd with Cd and Ba chloride soln. Bulk of sol. proteins are pptd in alk. soln and clarified soln is passed thru metallic Cd column, reducing nitrate to nitrite. Nitrite is measured colorimetrically.

(*Caution:* Cd salts are toxic.)

### *B. Reagents and Apparatus*

(**a**) *Nitrate nitrogen std solns.*—(*1*) *Stock soln.*—12 μg nitrate N/mL. Dissolve 0.867 g $KNO_3$ in 1 L $H_2O$. Dil. 25 mL to 250 mL with $H_2O$. (*2*) *Working solns.*—0.6, 1.2, 1.8, 2.4, 3.0 μg N/mL. Dil. 5, 10, 15, 20, and 25 mL stock soln to 100 mL with $H_2O$.

(**b**) *Extracting soln.*—Dissolve and dil. 50 g $CdCl_2$ and 50 g $BaCl_2$ to 1 L with $H_2O$. Adjust to pH 1 with HCl.

(**c**) *Ammonium chloride buffer soln.*—pH 9.6. Dissolve 50 g $NH_4Cl$ in 500 mL $H_2O$ and adjust pH with $NH_4OH$. Dil. to 1 L with $H_2O$.

(**d**) *Sodium hydroxide soln.*—2.5*N*. Dissolve 50 g NaOH in 500 mL $H_2O$.

(**e**) *Sulfanilamide soln.*—0.5%. Dissolve 1.25 g sulfanilamide in 250 mL HCl (1+1). Soln is stable 1–2 months.

(**f**) *Coupling reagent.*—Dissolve 0.5 g *N*-(1-naphthyl)ethylenediamine.HCl in 100 mL $H_2O$. Store in g-s dark bottle in refrigerator. Soln is stable several weeks.

(**g**) *Salt soln.*—Dissolve 100 g NaCl in 500 mL $H_2O$. Add 50 mL buffer soln, (**c**), and dil. to 1 L with $H_2O$.

(**h**) *Reduction tube.*—25 mL buret or equiv. id chromatgc tube with stopcock and reservoir (Kontes Glass Co. Cat. No. K-420280 or Lurex Scientific Cat. No. JC-1506).

### *C. Preparation of Columns*

Prep. supply of metallic Cd by placing Zn rods into 500 mL 20% $CdSO_4$ soln. After reaction for 3 hr, discard soln and scrape moss-like Cd growth from Zn rods. Place Cd in high-speed blender, add 500 mL $H_2O$, and blend 2 sec. Wash fine metal particles with $H_2O$ onto sieves, collecting only 20–40 mesh size. Fill reduction tube with $H_2O$ and add 2 cm plug of glass wool. Press any trapped air from glass wool as it is pushed to bottom of column with glass rod. Add Cd to depth of 10 cm, using min. of very gentle tapping. Wash column with 25 mL 0.10*N* HCl, two 25 mL portions $H_2O$, and finally 25 mL buffer, (**c**), dild 1 + 9. Keep column covered with salt soln, (**g**), when not in use.

Normally columns can be used repeatedly if kept under salt soln between analyses. When succession of highly proteinaceous or other sol. org. contg samples are treated, flow rate may decrease gradually. Repeating 25 mL 0.10*N* HCl treatment may restore original flow rate; if not, prep. new column. Reproducible flow rate is important. Actual rate can be 3–5 mL/min but once established, it must be identical (±0.1 mL) for samples and stds.

### *D. Preparation of Standard Curve*

Prep. std curve of 3, 6, 9, 12, and 15 μg nitrate-nitrite N by pipeting 5.0 mL aliquots of working std solns into 30 mL beakers. Add 5 mL buffer soln, (**c**), and 15 mL $H_2O$, mix well, and transfer quant. to reduction column, using min. $H_2O$. Adjust flow rate thru column to 3–5 mL/min. Just as reservoir empties, add 15 mL salt soln, (**g**). Collect eluate, including salt wash, in 50 mL vol. flask (total vol. of eluate should be ca 40 mL). Add 5 mL sulfanilamide soln, (**e**), mix, and let stand 3 min. Add 2 mL coupling reagent, (**f**), mix, dil. to vol. with $H_2O$, mix, and let stand 20 min for max. color development. Color is stable ≥2 hr. Det. $A$ in 1 cm cells at 540 nm against reagent blank. Plot $A$ against μg nitrate-nitrite N.

### *E. Extraction*

(**a**) *Low level nitrate samples (grains, meals, supplements, etc.).*—Wash 5.0 g finely ground sample into 250 mL vol. flask. Add 100 mL extg soln, (**b**), and 100 mL $H_2O$, and mix. Let stand 1 hr with occasional swirling. Add 20 mL 2.5*N* NaOH, dil. to vol. with $H_2O$, mix, and filter immediately thru rapid paper. Pipet 10 mL buffer soln, (**c**), into 100 mL vol. flask, dil. to vol. with clear filtrate, and mix.

(**b**) *Dry, high level nitrate products (dried plants, hays, meals, etc.).*—Weigh 5.0 g finely ground sample into 500 mL vol. flask. Add 100 mL extg soln, (**b**), and 300 mL $H_2O$, and mix. Let stand 1 hr with occasional swirling, add 40 mL 2.5*N* NaOH, dil. to vol. with $H_2O$, mix, and filter immediately thru rapid paper. Pipet 10 mL buffer soln, (**c**), into 100 mL vol. flask, dil. to vol. with clear filtrate, and mix.

(**c**) *Grasses, silages, and other wet materials.*— Weigh 100 g sample into 1 gal. capacity high-speed blender. Add 100 mL extg soln, (**b**), and 800 mL $H_2O$, including vol. $H_2O$ contributed by sample as detd in **934.01** or **925.04B**. Homogenize 1 min, pour into 2 L beaker, and let stand 1 hr. Add 100 mL buffer soln, (**c**) (total vol. 1 L), mix well, and filter thru Whatman No. 42 paper, collecting portion of clear filtrate.

### *F. Determination*

(**a**) *Nitrate plus nitrite nitrogen.*—Pipet 25 mL buffered sample exts, **968.07E(a)** or (**b**), or 5 mL ext, (**c**), into reduction column and treat as in **968.07D**, beginning, "Adjust flow rate thru column . . ." Rinse column with 30 mL $H_2O$ between samples to remove NaCl. Use portion of buffered sample exts with equiv. diln and pH as ref. soln in detg $A$ at 540 nm. Also det. nitrate-nitrite in reagents and correct for this blank value. Calc. total nitrate-nitrite N from std curve.

(**b**) *Nitrite nitrogen.*—Pipet aliquot clear sample filtrate (contg <15 μg nitrite) into 50 mL vol. flask and dil. with $H_2O$ to ca 40 mL. Mix well, add 5 mL sulfanilamide soln, (**e**), mix, and let stand 3 min. Add 2 mL coupling reagent, (**f**), and dil.

to vol. with $H_2O$. Mix well and let stand 20 min for max. color development. Measure $A$ in 1 cm cells against sample ext with equiv. diln at 540 nm. Correct for nitrite reagent blank.

(**c**) *Nitrate nitrogen.*—Calc. by difference between (**a**) and (**b**) above.

### G. Calculation

ppm $NO_2$-N and/or $NO_3$-N
$= \mu g$ $NO_3$-N found × diln factor/g sample

Diln factors for exts: **968.07E(a)**, 11.1; (**b**), 22.2; (**c**), 200.

Ref.: JAOAC **51**, 763(1968).

## 971.09 Pepsin Digestibility of Animal Protein Feeds

**Filtration Method**

**First Action 1971**
**Final Action 1973**

### A. Principle

Defatted sample is digested 16 hr with warm soln of pepsin under const agitation. Insol. residue is isolated by filtering, washed, dried, and weighed to det. % residue. Residue is examined microscopically and analyzed for protein. Filtration method is applicable to all animal proteins. Methods are not applicable to vegetable proteins or mixed feeds because of presence of complex carbohydrates and other compds not digested by pepsin.

### B. Apparatus

(**a**) *Agitator.—See* Fig. **971.09**. Continuous, slow speed (15 rpm), end-over-end type, to operate inside incubator at 45 ± 2° and carry 8 oz screw-cap prescription bottles, or equiv. Agitator and bottles available from D. E. Sims, 716 Forrest Ave, Quincy, IL 62301. Stirring or reciprocating (shaking) type agitator cannot be used because solid particles collect on sides of bottle and do not contact pepsin soln. If heat from agitator motor raises incubator temp. to >45°, mount motor outside incubator by drilling hole thru side of incubator and connecting motor to agitator with extension shaft and coupling (available from agitator supplier). (*Caution:* See safety notes on electrical equipment.)

(**b**) *Settling rack.*—Wood or metal to hold digestion bottles at 45° angle. May be made from 2 boards nailed horizontally into "V" cut into vertical end pieces. Also available from agitator supplier, (**a**).

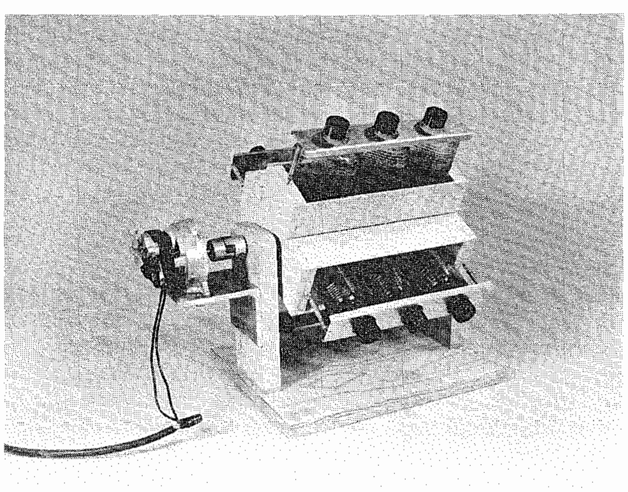

**FIG. 971.09—Agitator**

(**c**) *Filtering device.*—Modified California buchner, **962.09C(d)**, available from Labconco Corp., 8811 Prospect Ave, Kansas City, MO 64132, No. 55100. (If edge of screen is rough, smooth with small-tip soldering iron.) Use with retainer sleeve, 2 × 2.75" od stainless steel tube, available from agitator supplier, (**a**).

(**d**) *Glass fiber filter.*—7 cm, Whatman, Inc., No. 934-AH, or equiv.

(**e**) *Moisture dishes.*—Al, 78 mm od × 20 mm, with outside cover and vertical sides (Curtin Matheson Scientific, Inc., No. 19370-30, or equiv.).

### C. Reagent

*Pepsin soln.*—0.2% pepsin (activity 1:10,000) in 0.075*N* HCl; do *not* use pepsin NF or pepsin of activity other than 1:10,000. *Prep. just before use* by dilg 6.1 mL HCl to 1 L and heating to 42–45°. Add pepsin and stir gently until dissolved. Do not heat pepsin soln on hot plate or overheat.

### D. Preparation of Sample

Sieve sample, **965.16**, thru No. 20 sieve. Grind portion retained on sieve to pass No. 20 sieve. Combine both portions and blend by stirring and shaking in pt (500 mL) jar. Thoro blending is essential. Because of high fat content of many animal products, grinding without sieving may cause sticking in mill, loss of moisture or fat, or poorly blended sample.

### E. Extraction

(*Caution:* See safety notes on distillation, flammable solvents, and diethyl ether.)

Prep. extn thimble from 11 cm Whatman No. 2 paper, or equiv., as follows: Fold paper in half; straighten paper and refold at right angles to first fold; turn paper over and repeat process with folds at 45° to original fold; while holding creased paper in one hand, place short test tube (6–8 mm smaller in diam. than extractor sample holder or cup in which thimble is to be used) at its center; fold along natural crease lines to form 4-pointed star around tube; and wrap points in same direction around tube to complete thimble.

Weigh 1.000 g ground sample (0.500 g of poultry byproducts or hydrolyzed feathers because of gummy nature and amt of residue) into thimble and ext 1 hr with ether at condensation rate of 3–4 drops/sec. (If Soxhlet is used, top of thimble should extend above siphon tube to avoid loss of solid particles. If paper contg sample is totally submerged in siphon cup, sample must be completely wrapped in paper.) Observe ether ext to det. that no solid particles were carried into solv. For approx. fat content detn, evap. ether, and dry and weigh residue. Remove paper from sample container or cup and let dry at room temp. Unfold, and quant. brush defatted sample into digestion bottle, avoiding contamination by brush bristles or filter paper fibers. Use of powder funnel is helpful to avoid loss.

### F. Pepsin Digestion

To defatted sample in agitator bottle add 150 mL freshly prepd pepsin soln prewarmed to 42–45°. Be sure sample is completely wetted by pepsin soln. Stopper bottle, clamp in agitator, and incubate with const agitation 16 hr at 45°.

### G. Treatment of Residue

Dry individual sheets of glass fiber filter, (**d**), 30 min at 110° in moisture dishes with cover open. Cool in desiccator 30 min with cover closed, and weigh ($W_1$).

Remove bottles from agitator. Place in 45° angle settling rack and loosen caps. Let residue settle ≥15 min. Place weighed filter in California buchner, (**c**), apply suction, and moisten with $H_2O$. Place retainer sleeve on filter and press down gently. Rinse particles of residue on cap onto filter with small amt $H_2O$. Carry bottle from rack to filter at same angle as settled and slowly pour contents thru filter as continuous small stream, avoiding all unnecessary agitation. Liq. passes thru paper as rapidly as poured, with residue spreading over surface of filter but not covering it completely until all or practically all of liq. has passed thru. If filtration rate becomes slow, it may be accelerated by adding acetone washes described below, but only if no significant amt of digestion mixt. remains on funnel when acetone is added. (Filtration (passage of aq. mixt. thru filter) should be complete within 1 min with most proteins.) After supernate has passed thru filter, quant. transfer residue onto filter as follows:

Add 15 mL acetone to bottle. Hold thumb over bottle neck and shake vigorously. Release pressure, replace thumb over bottle neck, and shake bottle in inverted position over filter. Remove thumb, letting acetone and residue discharge onto filter. Repeat rinse with second 15 mL portion acetone, shaking and releasing pressure as above. Inspect bottle, and rinse further with acetone, using policeman, if necessary. If >3 mm liq. remains on paper when acetone washes are started, it may be necessary to use three 15 mL acetone washes instead of 2 to increase filtration rate.

After all liq. passes thru funnel, wash residue and inside surface of retainer sleeve with 2 small portions acetone from wash bottle or hypodermic syringe, and suck dry. Remove retainer sleeve from funnel. Transfer filter to original moisture dish. Scrape or brush any residue particles or filter clinging to retainer sleeve or funnel onto filter in moisture dish. Dry in oven, cool, and weigh as before ($W_2$). Calc. % indigestible residue = $(W_2 - W_1) \times 100$/g sample.

Det. indigestible protein by transferring filter contg residue directly to Kjeldahl flask. Proceed as in **954.01**. (*Caution:* Violent reaction may take place when NaOH is mixed with dild digestion mixt., caused by large excess $H_2SO_4$ due to small amt org. material from residue and none from glass filter. Avoid by thoroly mixing and cooling digestion mixt. before addn of NaOH or by using 20 mL $H_2SO_4$ in Kjeldahl digestion instead of 25 mL.) Make blank detn on 1 sheet of glass filter and subtract from each sample detn, if necessary. Calc. % protein based on original sample wt. Result represents % indigestible protein in *sample*. Convert to % crude protein content of sample not digested, "protein indigestible" = % indigestible protein in sample × 100/% total crude protein in sample.

Refs.: J. Agric. Food Chem. **3,** 159(1955). JAOAC **40,** 606(1957); **41,** 233(1958); **42,** 231(1959); **43,** 320 (1960); **54,** 669(1971); **55,** 702(1972).

## 920.39 Fat (Crude) or Ether Extract in Animal Feed

**Final Action**

Use method **920.39A** or **920.39C** for mixed feeds other than (*1*) baked and/or expanded, (*2*) dried milk products, or (*3*) contg urea.

### *A. Indirect Method*

Det. moisture as in **934.01** or **920.36★**; then ext dried substance as in **920.39C**, and dry again. Report loss in wt as ether ext.

***Direct Method***

### *B. Reagent*

*Anhydrous ether.*—Wash com. ether with 2 or 3 portions $H_2O$, add solid NaOH or KOH, and let stand until most of $H_2O$ is abstracted from the ether. Decant into dry bottle, add small pieces of carefully cleaned metallic Na, and let stand until H evolution ceases. Keep ether, thus dehydrated, over metallic Na in loosely stoppered bottles. (*Caution:* See safety notes on sodium metal and diethyl ether.)

### *C. Determination*

(Large amts $H_2O$-sol. components such as carbohydrates, urea, lactic acid, glycerol, and others may interfere with extn of fat; if present, ext 2 g sample on small paper in funnel with five 20 mL portions $H_2O$ prior to drying for ether extn. *Caution:* See safety notes on monitoring equipment, distillation, and diethyl ether.)

Ext ca 2 g sample, dried as in **934.01** or **920.36★**, with anhyd. ether. Use thimble with porosity permitting rapid passage of ether. Extn period may vary from 4 hr at condensation rate of 5–6 drops/sec to 16 hr at 2–3 drops/sec. Dry ext 30 min at 100°, cool, and weigh.

Refs.: JAOAC **64,** 351(1981); **65,** 289(1982).

## 954.02 Fat (Crude) or Ether Extract in Pet Food

**Gravimetric Method**

**Final Action 1977**

(To be used only on products which have been baked and/or expanded, and on intermediate moisture pet foods. Not applicable to canned, fresh, or frozen pet food. Such products should be dried at 70–110°, then ground, and drying completed by **934.01** or **920.36★** followed by **920.39A** or **920.39C**.)

(*Caution:* See safety notes on distillation, diethyl ether, and petroleum ether.)

Place ca 2 g, accurately weighed, ground, well mixed sample in Mojonnier fat-extn tube, add 2 mL alcohol to prevent lumping on addn of acid, and shake to moisten all particles. Add 10 mL HCl (25+11), mix well, and set tube 30–40 min in $H_2O$ bath at 70–80°, shaking frequently. Cool to room temp. and add alcohol until liq. level rises into constricted portion of Mojonnier tube.

Add 25 mL ether, stopper with glass, Neoprene, or good quality rubber stopper thoroly cleaned with alcohol, and shake vigorously 1 min. Carefully release pressure so that no solv. is lost. Wash adhering solv. and fat from stopper back into extn tube with few mL redistd pet ether (bp <60°). Add 25 mL redistd pet ether, stopper, and shake vigorously 1 min. Let stand until upper liq. is practically clear or centrf. 20 min at ca 600 rpm. Pour as much of ether-fat soln as possible thru filter consisting of cotton pledget packed just firmly enough in funnel stem to let ether pass freely into 150 mL beaker contg several glass beads. Rinse lip of tube with few mL pet ether. Re-ext liq. remaining in tube twice, each time with only 15 mL of each ether, shaking 1 min after addn of each ether. Pour clear ether soln thru filter into same beaker as before, and wash tip of tube, stopper, funnel, and end of funnel stem with few mL of mixt. of 2 ethers (1+1). Evap. slowly on steam bath under gentle stream of air or N. Continue heating on steam bath 15 min after solv. has evapd; then cool to room temp.

Redissolve dried fat residue in four 10 mL portions Et ether, filtering each portion thru small pledget of cotton into 100 mL beaker, contg few glass beads, that has been predried 30 min at 100°, cooled to room temp. in desiccator, and weighed immediately. Use fifth 10 mL portion ether for rinsing cotton and funnel. Evap. ether on steam bath, dry 90 min at 100°, cool to room temp. in desiccator, and weigh immediately. Correct this wt by blank detn on reagents used.

Refs.: JAOAC **37,** 250(1954); **38,** 225(1955); **59,** 1218(1976); **60,** 322(1977); **65,** 456(1982).

## 932.02 Fat (Crude) or Ether Extract in Dried Milk Products

**Final Action**

Proceed as in **932.06A(b)** and **932.06B**, using 8.5 mL $H_2O$ and 1.5 mL $NH_4OH$.

Refs.: JAOAC **15,** 524(1932); **17,** 190(1934); **18,** 351(1935); **28,** 80(1945).

## 948.04 Fat (Crude) or Acetone Extract in Fish Meal

*See* **948.16** and **969.24**.

## 962.09 Fiber (Crude) in Animal Feed

**Ceramic Fiber Filter Method**

**First Action 1962**
**Final Action 1971**
**Revised First Action 1982**

***AOCS-AOAC Method***

### *A. Principle*

Crude fiber is loss on ignition of dried residue remaining after digestion of sample with 1.25% $H_2SO_4$ and 1.25% NaOH solns under specific conditions. Method is applicable to grains, meals, flours, feeds, and fiber-bearing material from which fat can be extd to leave workable residue.

### *B. Reagents*

(**a**) *Sulfuric acid soln.*—0.255 ± 0.005*N*. 1.25 g $H_2SO_4$/100 mL. Concn must be checked by titrn.

(**b**) *Sodium hydroxide soln.*—0.313 ± 0.005*N*. 1.25 g NaOH/100 mL, free, or nearly so, from $Na_2CO_3$. Concn must be checked by titrn.

(**c**) *Prepared ceramic fiber.*—Place 60 g ceramic fiber (Cerafiber, 8 lb/cu ft, E. J. Bartell Co., 700 Powell Ave, S.W., Renton, WA 98055) in blender, add 800 mL $H_2O$, and blend 1 min at low speed.

Det. blank by treating ca 2 g (dry wt) of prepd ceramic fiber with acid and alkali as in detn. Correct crude fiber results for any blank, which should be negligible (ca 2 mg).

(**d**) *Alcohol.*—95% or reagent alcohol, MeOH, or isopropanol.

(**e**) *Antifoam.*—Dow Corning Corp. Antifoam A compd dild 1+4 with mineral spirits or pet ether, or $H_2O$-dild Antifoam B Emulsion (1+4). Do not use Antifoam Spray.

(**f**) *Bumping chips or granules.*—Broken Alundum crucibles or equiv. granules (RR Alundum 90 mesh, Norton Co., 1 New Bond St, Worcester, MA 01606) are satisfactory.

### *C. Apparatus*

(**a**) *Digestion apparatus.*—With condenser to fit 600 mL beaker, and hot plate adjustable to temp. that will bring 200 mL $H_2O$ at 25° to rolling boil in 15±2 min. (Available from Labconco Corp., 8811 Prospect Ave, Kansas City, MO 64132.)

(**b**) *Ashing dishes.*—Silica, Vitreosil 70 × 16 mm; or porcelain, Coors Ceramics Co., 600 9th St, Golden, CO 80401, No. 60230, or equiv.

(**c**) *Desiccator.*—With efficient desiccant such as 4–8 mesh Drierite ($CaCl_2$ is not satisfactory).

(**d**) *Filtering device.*—With No. 200 type 304 or 316 stainless steel screen (C-E Tyler, Inc., 3200 Bessemer City Rd, Hwy 274, PO Box 8900, Gastonia, NC 28053), easily washed free of digested residue. Either Oklahoma State filter screen (*see* Fig. **962.09A**; available from Labconco Corp.) or modified California plastic buchner. (*See* Fig. **962.09B**; consists of 2 piece polypropylene plastic funnel manufactured by Nalge Co., 75 Panorama Creek Dr, PO Box 20365, Rochester, NY 14602, Cat. No. 4280-0700, 70 mm (without No. 200 screen), or equiv. (also available from Labconco Corp.). Seal screen to filtering surface of funnel, using small-tip soldering iron.)

(**e**) *Suction filter.*—To accommodate filtering devices. Attach suction flask to trap in line with aspirator or other source of vac. with valve to break vac.

(**f**) *Liquid preheater.*—For preheating $H_2O$, 1.25% $H_2SO_4$, and 1.25% NaOH solns to bp of $H_2O$. Convenient system, shown in Fig. **962.09C**, consists of sheet Cu tank with 3 coils of $^3/_8$″ (10 mm) od Cu tubing, 12.5′ (3.8 m) long. Solder inlets and outlets where tubing passes thru tank walls. Connect to reflux condenser and fill with $H_2O$. Keep $H_2O$ boiling with two 750 watt thermostatically controlled hot plates. Use Tygon for inlet leads to reservoirs of $H_2O$, acid, and alkali; use gum rubber tubing for outlets. Capacity of preheater is adequate for 60 analyses in 8 hr.

### *D. Preparation of Sample*

Reduce sample (riffle is suitable) to 100 g and place portion in sealed container for $H_2O$ detn. Immediately det. $H_2O$. Grind remainder to uniform fineness. (Weber mill (Sargent-Welch Scientific Co. S-60870) with screen 0.033–0.040″ (No. 18 or 20), Micro mill (Mikropul, Div. of Hosokawa Micron International, Inc., 10 Chatham Rd, Summit, NJ 07901) with screen $^1/_{25}$–$^1/_{16}$″ (No. 18–No. 12), and Wiley mill with 1 mm (No. 18) screen give comparable fineness.) Since most materials lose moisture during grinding, det. $H_2O$ on ground sample at same time sample is taken for crude fiber detn.

### *E. Determination*

Ext 2 g ground material with ether or pet ether (initial boiling temp., 35–38°; dry-flask end point, 52–60°; ≥95% distg

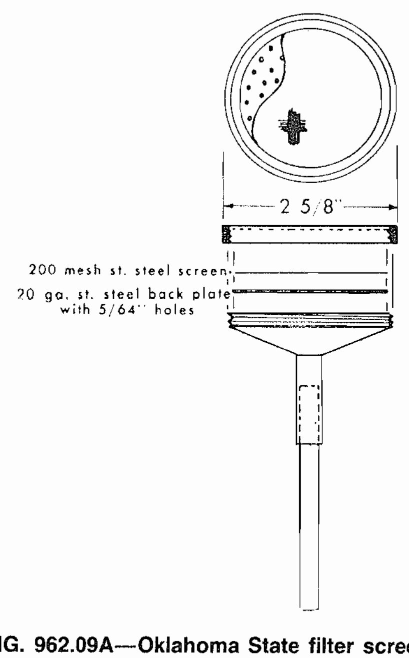


**FIG. 962.09A—Oklahoma State filter screen**

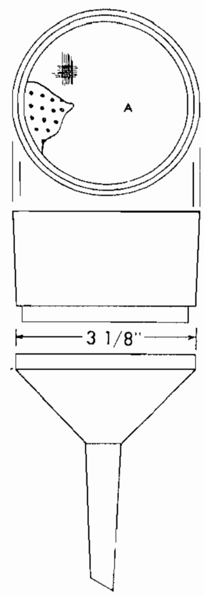


**FIG. 962.09B—Modified California State buchner funnel, 2-piece polypropylene plastic, covered with 200-mesh screen, A, heat-sealed to edge of filtering surface**

<54°, and ≤60% distg <40°; sp gr at 60°F, 0.630–0.660; evapn residue ≤0.002% by wt). If fat is <1%, extn may be omitted. Transfer to 600 mL beaker, avoiding fiber contamination from paper or brush. Add ca 1.5–2.0 g dry wt of prepd ceramic fiber, 200 mL boiling 1.25% $H_2SO_4$, and 1 drop dild antifoam. (Excess antifoam may give high results; use only if necessary to control foaming.) Bumping chips or granules may also be added. Place beaker on digestion app. with preadjusted hot plate and boil exactly 30 min, rotating beaker periodically to keep solids from adhering to sides. Remove beaker, and filter as in (**a**) or (**b**).

(**a**) *Using Oklahoma filter screen.*—Turn on suction and insert screen (precoated with ceramic fiber if extremely fine materials are analyzed) into beaker, keeping face of screen just under surface of liq. until all liq. is removed. Without breaking suction or raising filter, add 50–75 mL boiling $H_2O$. After

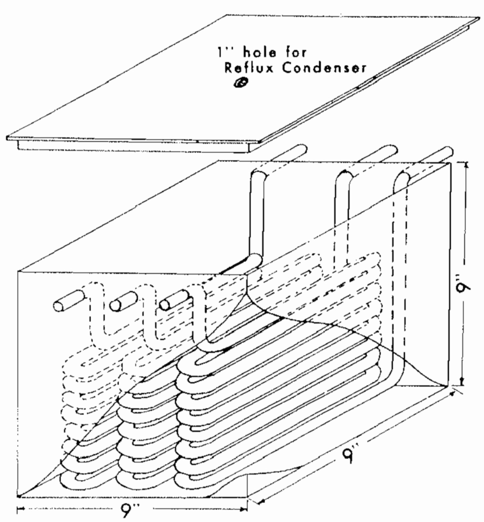


**FIG. 962.09C—Continuous heater for distilled water, 1.25% alkali, and 1.25% acid**

wash is removed, repeat with three 50 mL washings. (Work rapidly to keep mat from becoming dry.) Remove filter from beaker and drain all $H_2O$ from line by raising above trap level. Return mat and residue to beaker by breaking suction and blowing back. Add 200 mL boiling 1.25% NaOH and boil exactly 30 min. Remove beaker, and filter as above. Without breaking suction, wash with 25 mL boiling 1.25% $H_2SO_4$ and three 50 mL portions boiling $H_2O$. Drain free of excess $H_2O$ by raising filter. Lower filter into beaker and wash with 25 mL alcohol. Drain line, break suction, and remove mat by blowing back thru filter screen into ashing dish. Proceed as in (**c**).

(**b**) *Using California buchner.*—Filter contents of beaker thru buchner (precoated with ceramic fiber if extremely fine materials are being analyzed), rinse beaker with 50–75 mL boiling $H_2O$, and wash thru buchner. Repeat with three 50 mL portions $H_2O$, and suck dry. Remove mat and residue by snapping bottom of buchner against top while covering stem with thumb or forefinger and replace in beaker. Add 200 mL boiling 1.25% NaOH and boil exactly 30 min. Remove beaker, and filter as above. Wash with 25 mL boiling 1.25% $H_2SO_4$, three 50 mL portions $H_2O$, and 25 mL alcohol. Remove mat and residue; transfer to ashing dish.

(**c**) *Treatment of residue.*—Dry mat and residue 2 hr at 130±2°. Cool in desiccator and weigh. Ignite 30 min at 600±15°. Cool in desiccator and reweigh.

% Crude fiber in ground sample = $C$
= (Loss in wt on ignition − loss in wt of ceramic fiber blank) × 100/wt sample

% Crude fiber on desired moisture basis
= $C$ × (100 − % moisture desired)/(100 − % moisture in ground sample)

Report results to 0.1%.

Refs.: JAOAC **42,** 222(1959); **43,** 335(1960); **44,** 567(1961); **45,** 578(1962); **65,** 265(1982).

## 978.10 Fiber (Crude) in Animal Feed

**Fritted Glass Crucible Method**

**First Action 1978**
**Final Action 1979**

### *A. Principle*

Principle is same as in **962.09A**, except sample is exposed to min. vac. needed to regulate filtration, and heating of sample solns prevents gelling or pptn of possible satd solns.

### *B. Apparatus and Reagents*

*See* reagents **962.09B(a)**, (**b**), and (**f**); app. **962.09C(a)**, (**c**), (**d**), and (**f**), and in addn:

(**a**) *Filtration apparatus.*—System to permit application of min. vac. necessary for filtration and washing of each sample within 3–5 min. Each unit consists of reservoir manifold connected to (*1*) $H_2O$ aspirator thru 120° stopcock, (*2*) atm. thru second stopcock with metering device, and (*3*) receptacle contg cone-shaped hard rubber gasket which provides vac. seal with crucible. Vac. gage attached to manifold indicates vac. applied to crucible. Crucible can be heated before and during filtration by flow of hot $H_2O$ in surrounding jacket. (For photograph of app., *see* JAOAC **56,** 1353(1973). Filtration unit is available as Model 601 or 602 (replacement models for Model 150) from Analytical Bio-Chemistry Laboratories, Inc., 7200 ABC Ln, Columbia, MO 65205).

(**b**) *Crucible.*—Fritted glass, 50 mL, coarse porosity. Clean as follows: Brush, and flow hot tap $H_2O$ into crucible to remove as much ash as possible. Submerge crucible in base soln, (**c**)*(2)*, $\geq$5 min, remove, and rinse with hot tap $H_2O$. Submerge in HCl (1+1), (**c**) *(1)*, $\geq$5 min, remove, and rinse thoroly with hot tap $H_2O$ followed by distd $H_2O$. After 3–4 uses, back wash by inverting crucible on hard rubber gasket in filtration app., and flowing near-boiling $H_2O$ thru crucible under partial vac.

(**c**) *Cleaning solns.—(1) Acid soln.*—HCl (1+1). *(2) Base soln.*—Dissolve 5 g $Na_2H_2EDTA$, 50 g $Na_2HPO_4$ (tech. grade), and 200 g KOH in $H_2O$, and dil. to 1 L. Storage in sep. wide mouth containers holding 2–3 L soln into which crucibles can be placed is convenient.

### C. Determination

(*Caution:* See safety notes on distillation and petroleum ether.)

Ext 2 g ground material with ether or pet ether (initial boiling temp., 35–38°; dry-flask end point, 52–60°; $\geq$95% distg $<$54°, and $\leq$60% distg $<$40°; sp gr at 60°F, 0.630–0.660; evapn residue $\leq$0.002% by wt). If fat is $<$1%, extn may be omitted. Transfer to 600 mL reflux beaker, avoiding fiber contamination from paper or brush. Add 0.25–0.5 g bumping granules, followed by 200 mL near-boiling 1.25% $H_2SO_4$ soln in small stream directly on sample to aid in complete wetting of sample. Place beakers on digestion app. at 5 min intervals and boil exactly 30 min, rotating beakers periodically to keep solids from adhering to sides. Near end of refluxing place California buchner, **962.09C(d)**, previously fitted with No. 9 rubber stopper to provide vac. seal, into filtration app., and adjust vac. to ca 25 mm Hg (735 mm pressure). At end of refluxing, flow near-boiling $H_2O$ thru funnel to warm it; then decant liq. thru funnel, washing solids into funnel with min. of near-boiling $H_2O$. Filter to dryness, using 25 mm vac., and wash residue with four 40–50 mL portions near-boiling $H_2O$, filtering after each washing. Do not add wash to funnel under vac.; lift funnel from app. when adding wash.

Wash residue from funnel into reflux beaker with near-boiling 1.25% NaOH soln. Place beakers on reflux app. at 5 min intervals and reflux 30 min. Near end of refluxing, turn on filtration app., place crucible, (**b**), in app., and adjust vac. to ca 25 mm. Flow near-boiling $H_2O$ thru crucible to warm it. (Keep near-boiling $H_2O$ flowing thru jacket during filtration and washing.) At end of refluxing, decant liq. thru crucible and wash solids into crucible with min. of near-boiling $H_2O$. Increase vac. as needed to maintain filtration rate. Wash residue once with 25–30 mL near-boiling 1.25% $H_2SO_4$ soln, and then with two 25–30 mL portions near-boiling $H_2O$, filtering after each washing. (Filtering and washing takes ca 3–5 min/sample.) Do not add wash to crucible under vac.

Dry crucible with residue 2 hr at 130±2° or overnight at 110°, cool in desiccator, and weigh. Ash 2 hr at 550±10°, cool in desiccator, and weigh. Do not remove crucibles from furnace until temp. is $\leq$250°, as fritted disk may be damaged if cooled too rapidly.

% Crude fiber = Loss in wt on ignition × 100/wt sample

Ref.: JAOAC **61,** 154(1978).

## 973.18 Fiber (Acid Detergent) and Lignin in Animal Feed

**First Action 1973**
**Final Action 1977**

(*Caution:* See safety notes on asbestos.)

### A. Reagents

(**a**) *Sulfuric acid.*—72% by wt. Stdze reagent grade $H_2SO_4$ to sp gr 1.634 at 20° or 24.00*N*: Add 1200 g $H_2SO_4$ to 440 mL $H_2O$ in 1 L MCA vol. flask with cooling. Stdze to 1634 g/L at 20° by removing soln and adding $H_2O$ or $H_2SO_4$ as required.

(**b**) *Acid-detergent soln.*—Add 20 g cetyl trimethylammonium bromide (tech. grade) to 1 L 1.00*N* $H_2SO_4$, previously stdzd. Agitate to aid soln.

(**c**) *Asbestos.*—Place 100 g asbestos in 3 L flask contg 850 mL $H_2O$. Add 1.4 L $H_2SO_4$ (tech. grade), mix, and let cool 2 hr at room temp. Filter on large buchner and wash with $H_2O$. Resuspend mat in $H_2O$ and pour into bag sewn from rectangle of fiberglass window screening, 14 × 18 mesh (bag should be $\geq$45 cm wide × 30 cm deep). Wash by immersion and agitation in partly filled sink to remove fine particles. Ash recovered asbestos 16 hr in 800° furnace. Store in dry form until use. Used asbestos may be rewashed, reashed, and reused. Com. prepd acid-washed asbestos is unsatisfactory unless treated with 72% $H_2SO_4$ and ashed at 800°.

### B. Apparatus

(**a**) *Refluxing apparatus.*—Any conventional app. suitable for crude fiber detns. Berzelius beakers (600 mL) and condensers made from 500 mL r-b flasks are also satisfactory.

(**b**) *Fritted glass crucibles.*—Use coarse porosity, 40–50 mL Pyrex crucible. Wash new crucibles and ash at 500°. Remove while still hot and place in 100° forced-draft oven $\geq$1 hr. Cool 15 min in desiccator over $P_2O_5$ or $Mg(ClO_4)_2$ and weigh in same order samples are to be weighed. Check balance 0 after each weighing if crucibles are still warm. Hold length of time from oven to balance pan as const as possible and always weigh crucibles in same order.

### C. Determination of Acid-Detergent Fiber

Weigh 1 g air-dried sample ground to pass 1 mm screen, or approx. equiv. amt wet material, into refluxing container. Add 100 mL acid-detergent soln at room temp.

Heat to boiling in 5–10 min; reduce heat to avoid foaming as boiling begins. Reflux 60 min from onset of boiling, adjusting boiling to slow, even level. Remove container, swirl, and filter thru weighed ($W_1$) fritted glass crucible, using min. suction. Increase vac. only as needed. Shut off vac. Break up filtered mat with rod and fill crucible ⅔ full with hot (90–100°) $H_2O$. Stir and let soak 15–30 sec. Dry with vac. and repeat $H_2O$ washing, rinsing sides of crucible. Wash twice similarly with acetone.

Repeat acetone washings until no more color is removed, breaking up all lumps so that solv. wets all particles of fiber. Remove residual acetone with vac. Dry 3 hr or overnight in 100° forced-draft oven and weigh ($W_2$). Calc. % acid-detergent fiber = 100 $(W_2 - W_1)/S$, where $S$ = g sample × g oven-dried matter/g air-dried or wet matter, detd on sep. sample.

### D. Determination of Lignin

To crucible contg fiber, **973.18C**, add 1 g asbestos. Place crucible in 50 mL beaker for support or arrange crucibles in shallow enamel pan. Cover contents of crucible with cooled (15°) 72% $H_2SO_4$ and stir with glass rod to smooth paste, breaking all lumps. Fill crucible about half-way with acid and stir. Leave glass rod in crucible; refill with 72% $H_2SO_4$ and stir hourly as acid drains, keeping crucible at 20–23° (cool if necessary). After 3 hr, filter as completely as possible with vac., and wash with hot $H_2O$ until acid-free to pH paper. Rinse sides of crucible and remove stirring rod. Dry crucible in 100° forced-draft oven, cool in desiccator over $P_2O_5$ or $Mg(ClO_4)_2$, and weigh ($W_3$). Ignite crucible in 500° furnace 2 hr or until C-free. Place crucible while still hot into 100° forced-draft oven 1 hr. Transfer to desiccator, cool, and weigh ($W_4$).

Det. asbestos blank by weighing 1 g asbestos into tared crucible. Proceed as above, beginning "Cover contents of crucible . . ." Record any loss in wt on ashing ($W_5$). Discontinue detn of blank if asbestos blank is <0.0020 g/g asbestos. Calc. % acid-insol. lignin = $(W_3 - W_4 - W_5)/S$.

Refs.: JAOAC **46,** 829(1963); **56,** 781(1973).

CAS-9005-53-2 (lignin)

## 974.06 Sugars (Total) in Animal Feed

### Modified Fehling Solution Method

**First Action 1974**
**Final Action 1975**

#### *A. Reagents*

(**a**) *Soxhlet modification of Fehling soln.*—Prep. as in **923.09A(a)** and (**b**).

(**b**) *Invert sugar std soln.*—1.0%. Prep. as in **923.09A(c)**, but do not neutze. Dil. to 0.5% just before use for analysis of most products.

(**c**) *Lactose std soln.*—1.0%. Dissolve 5.000 g lactose in $H_2O$ and dil. to 500 mL. Prep. daily.

#### *B. Apparatus*

(**a**) *Lamp.*—Fluorescent desk lamp or 150 watt reflector spot lamp, to illuminate boiling soln.

(**b**) *Heater.*—Glas-Col mantle, 250 mL, placed over mag. stirrer. Adjust heat so that 50 mL $H_2O$ contg stirring bar will boil in 3 min. Mag. stirring hot plate is also satisfactory.

#### *C. Preparation of Sample and Inversion*

(**a**) *Feeds containing molasses.*—Weigh appropriate size sample, prepd as in **950.02** but not ground, to provide final soln ca 0.5% invert sugar but ≥5 g, into 250 mL P flask (Corning Glass Works No. 5840, or equiv.). Add 150 mL $H_2O$, swirl to wet and mix, and heat just to bp. Let stand to cool, dil. to vol., mix, and let stand to settle coarse particles. Transfer 50 mL supernate to 100 mL vol. flask and add 2.5 mL HCl (sp gr 1.18 at 20/4°). Let stand overnight at ≥25°, dil. to vol., and mix. (If aliquot to be used in detn is >25 mL, it is necessary to neutze inverted soln.)

(**b**) *Feeds containing milk products.*—Weigh appropriate size sample to provide final soln ca 1% lactose into 250 mL vol. flask. Thoroly moisten sample with $H_2O$, swirl to dissolve lactose, dil. to vol., mix, and let stand to settle coarse particles. Proceed as in **974.06E(b)**.

#### *D. Standardization*

Fill 50 mL buret, with offset tip, with std sugar soln (invert sugar for use with **974.06E(a)** and lactose with **974.06E(b)**). Proceed as in **968.28D**, par. 2, except use same type flask as used in **974.06E**, do not add $H_2O$, and start stirring after addn of indicator.

#### *E. Determination*

(**a**) *Difference method.*—Add reagents and stirring bar to 250 mL extn flask (Corning Glass Works No. 5160, or equiv.) or to erlenmeyer, as in **974.06D**. Transfer aliquot inverted soln, (**a**), to flask so that >1 but <5 mL std soln will be required to reach end point, place on preheated mantle or hot plate, heat to bp, boil 2 min, add ca 1 mL indicator, and begin stirring. Complete detn by titrg with std sugar soln to same end point used in stdzn. Color change is not so sharp as in stdzn, but under suitable light it is definite, discernible, and repeatable.

(**b**) *Alternative method.*—Fill buret with sample soln, (**b**), or inverted sample soln, (**a**). As in **974.06D**, place reagents in flask, place on heater, add sample soln to within 2 mL of final titrn (detd by trial), bring to bp, boil 2 min, and complete titrn as in (**a**).

#### *F. Calculations*

$$\% \text{ Total sugar (as invert or lactose)} = [(F - M) \times I \times 100]/[V \times (W/250) \times D)]$$

where $F$ = mL std sugar required to reduce mixed Soxhlet reagent in stdzn; $M$ = mL std soln required to complete detn (omit in alternative method); $I$ = concn std soln; $V$ = mL sample soln in aliquot used; $W$ = g sample; and $D$ = diln factor.

Report total sugars, expressed as invert or as lactose.

Ref.: JAOAC **57,** 382(1974).

## 925.05 Sucrose in Animal Feed

**Final Action**

Place 10 g sample in 250 mL vol. flask. If material is acid, neutze by adding 1–3 g $CaCO_3$. Add 125 mL 50% alcohol by vol., mix thoroly, and boil on steam bath or by partially immersing flask in $H_2O$ bath 1 hr at 83–87°, using small funnel in neck of flask to condense vapor. Cool and let mixt. stand several hr, preferably overnight. Dil. to vol. with neut. 95% alcohol, mix thoroly, let settle or centrf. 15 min at 1500 rpm, and decant closely. Pipet 200 mL supernate into beaker and evap. on steam bath to 20–30 mL. Do not evap. to dryness. Little alcohol in residue does no harm.

Transfer to 100 mL vol. flask and rinse beaker thoroly with $H_2O$, adding rinsings to flask. Add enough *satd neut.* $Pb(OAc)_2$ *soln* (ca 2 mL) to produce flocculent ppt, shake thoroly, and let stand 15 min. Dil. to vol. with $H_2O$, mix thoroly, and filter thru dry paper. Add enough anhyd. $Na_2CO_3$ or K oxalate to filtrate to ppt all Pb, again filter thru dry paper, and test filtrate with little anhyd. $Na_2CO_3$ or K oxalate to make sure that all Pb has been removed.

Place 50 mL prepd soln in 100 mL vol. flask, add piece of litmus paper, neutze with HCl, add 5 mL HCl, and let inversion proceed at room temp. as in **925.48(c)**. When inversion is complete, transfer soln to beaker, neutze with $Na_2CO_3$, return soln to 100 mL flask, dil. to vol. with $H_2O$, filter if necessary, and det. reducing sugars in 50 mL soln (representing 2 g sample) as in **906.03B**. Calc. results as invert sugar.

% Sucrose = [% total sugar after inversion − % reducing sugars before inversion (both calcd as invert sugar)] × 0.95

Because insol. material of grain or cattle food occupies some space in flask as originally made up, correct by multiplying all results by factor 0.97, as results of large number of detns on various materials show av. vol. of 10 g material to be 7.5 mL.

Refs.: USDA Bur. Chem. Circ. **71.** JAOAC **41,** 276(1958); **42,** 39(1959).

CAS-57-50-1 (sucrose)

## 920.40★ Starch in Animal Feed

**Final Action**
**Surplus**

#### *A. Direct Acid Hydrolysis*

*See* **7.080**, 13th ed.

#### *B. Diastase Method with Subsequent Acid Hydrolysis*

*See* **7.067,** 12th ed.

#### *C. Extraction with Subsequent Enzyme Hydrolysis*

*See* **14.075–14.080**, 13th ed.

#### *D. In Presence of Interfering Polysaccharides*

*See* **22.048**, 10th ed.

***E. In Condensed or Dried Milk Products—Qualitative Test***

*See* **22.049**, 10th ed.

## 920.41★ Pentosans in Animal Feed

**Final Action**
**Surplus 1965**

*See* **22.050–22.051**, 10th ed.

## 920.42★ Galactan in Animal Feed

**Final Action**
**Surplus 1965**

*See* **22.052**, 10th ed.

## 920.43★ Acidity (Water-Soluble) of Animal Feed

**Final Action**
**Surplus 1965**

*See* **22.053**, 10th ed.

## 925.12★ Mineral Salts in Animal Feed

**Final Action**
**Surplus 1974**

***A. Ferrous Salts***

*See* **7.074**, 12th ed.

***B. Copper Salts***

*See* **7.075**, 12th ed.

***C. Potassium Iodide***

*See* **7.076**, 12th ed.

## 968.08 Minerals in Animal Feed

**Atomic Absorption Spectrophotometric Method**
**First Action 1968**
**Final Action 1969**

(*Caution:* See safety notes on AAS.)

***A. Apparatus***

*Atomic absorption spectrophotometer.—See* **965.09A**.

***B. Operating Parameters***

*See* Table **965.09**, except use fuel-rich air-$C_2H_2$ flame for Ca and Mg, and ranges of operation for μg element/mL soln are: Ca 5–20, Cu 2–20, Fe 5–20, Mg 0.5–2.5, Mn 5–20, and Zn 1–5.

***C. Reagents***

(*See* introduction to **965.09B**. Com. prepd std solns may be used.)

(**a**) *Calcium std solns.*—Prep. as in **965.09B(a)**.

(**b**) *Copper, iron, magnesium, manganese, and zinc std solns.*—Prep. stock solns as in **965.09B(b)**, (**c**), (**e**), (**f**), and (**g**), and dil. aliquots with 0.1–0.5*N* HCl to make ≥4 std solns of each element within range of detn.

***D. Preparation of Sample Solution***

(**a**) *Dry ashing* (*not applicable to mineral-mix feeds*).—Ash 2–10 g sample in well-glazed porcelain dish. Start in cold furnace, bring to 550°, and hold 4 hr. Cool, add 10 mL 3*N* HCl, cover with watch glass, and boil gently 10 min. Cool, filter into 100 mL vol. flask, and dil. to vol. with $H_2O$. Subsequent dilns with 0.1–0.5*N* HCl may be necessary to bring sample solns into anal. range, except for Ca. Final Ca diln must contain enough La soln, **965.09B(d)**, to provide 1% La concn after diln to vol. with $H_2O$.

(**b**) *Wet digestion.*—Proceed as in **935.13A(a)**, adding 25 mL $HNO_3$ for each 2.5 g sample and dilg to 100 mL with $H_2O$. Digestion can be made at low heat on hot plate, using 600 mL beaker covered with watch glass. Subsequent dilns with 0.1–0.5*N* HCl may be necessary to bring sample solns into anal. range, as in (**a**).

***E. Determination and Calculation***

*See* **965.09D–E**.

Refs.: JAOAC **51,** 776(1968); **54,** 666(1971); **59,** 937(1976); **60,** 465(1977).

CAS-7440-70-2 (calcium)
CAS-7440-50-8 (copper)
CAS-7439-89-6 (iron)
CAS-7439-96-5 (manganese)
CAS-7440-66-6 (zinc)

## 927.02 Calcium in Animal Feed

**Dry Ash Method**
**Final Action**

(Applicable to mineral feeds only)

Weigh 2 g finely ground sample into $SiO_2$ or porcelain dish and ignite in furnace to C-free ash, but avoid fusing. Boil residue in 40 mL HCl (1+3) and few drops $HNO_3$. Transfer to 250 mL vol. flask, cool, dil. to vol., and mix thoroly. Pipet 25 mL clear liq. into beaker, dil. to ca 100 mL, and add 2 drops Me red, **984.13B(c)**. Add $NH_4OH$ (1+1) dropwise to pH 5.6, as shown by intermediate brownish-orange. If overstepped, add HCl (1+3) with dropper to orange. Add 2 more drops HCl (1+3). Color should now be pink (pH 2.5–3.0), not orange. Dil. to ca 150 mL, bring to boil, and slowly add, with const stirring, 10 mL hot satd (4.2%) soln of $(NH_4)_2C_2O_4$. If red changes to orange or yellow, add HCl (1+3) dropwise until color again changes to pink. Let stand overnight for ppt to settle. Filter supernate thru quant. paper, gooch, or fritted glass filter (fine Pyrex is preferable), and wash ppt thoroly with $NH_4OH$ (1+50). Place paper or crucible with ppt in original beaker, and add mixt. of 125 mL $H_2O$ and 5 mL $H_2SO_4$. Heat to ≥70° and titr. with 0.1*N* $KMnO_4$, **940.35**, to first slight pink. Presence of paper may cause color to fade in few sec. Correct for blank and calc. % Ca.

Refs.: JAOAC **10,** 177(1927); **19,** 93, 574(1936); **28,** 80(1945).

CAS-7440-70-2 (calcium)

## 935.13 Calcium in Animal Feed

**Wet Ash Method**
**Final Action**

***A. Preparation of Solution***

(*Caution:* See safety notes on nitric acid and perchloric acid.)

(**a**) Weigh 2.5 g sample into 500 or 800 mL Kjeldahl flask. Add 20–30 mL $HNO_3$ and boil gently 30–45 min to oxidize all easily oxidizable matter. Cool soln somewhat and add 10 mL 70–72% $HClO_4$. Boil very gently, adjusting flame as necessary, until soln is colorless or nearly so and dense white fumes appear. Use particular care not to boil to dryness (Danger!) at any time. Cool slightly, add 50 mL $H_2O$, and boil to drive out any remaining $NO_2$ fumes. Cool, dil., filter into 250 mL vol. flask, dil. to vol., and mix thoroly.

(**b**) Weigh 2.5 g finely ground sample into $SiO_2$ or porcelain dish and ignite as in **942.05**. Add 40 mL HCl (1+3) and few drops $HNO_3$ to residue, boil, transfer to 250 mL vol. flask, cool, dil. to vol., and mix thoroly.

### B. Determination

Pipet suitable aliquot of clear soln, **935.13A(a)** or (**b**), into beaker, dil. to 100 mL, and add 2 drops Me red, **984.13B(c)**. Continue as in **927.02**, beginning "Add $NH_4OH$ (1+1) dropwise . . ." except use 0.05*N* $KMnO_4$ for titrn.

(100 mL is suitable aliquot of sample soln for grain feeds; for mineral feeds, 25 mL aliquot may be taken and titrd with 0.1*N* $KMnO_4$. For suitable precision, size of sample, aliquot, and concn of $KMnO_4$ must be so adjusted that ≥20 mL std $KMnO_4$ soln is used.)

Refs.: Ind. Eng. Chem. Anal. Ed. **7,** 116, 167(1935). JAOAC **30,** 606(1947); **31,** 98(1948); **32,** 650(1949); **33,** 162(1950); **34,** 563(1951).

CAS-7440-70-2 (calcium)

## 943.01 Chlorine (Soluble) in Animal Feed

**Titrimetric Method**

**Final Action**

### A. Reagents

(**a**) *Potassium chloride std soln.*—0.001 g Cl/mL. Recrystallize reagent KCl 3 times from $H_2O$, dry at 110°, and heat at ca 500° to const wt. Dissolve 2.1028 g in $H_2O$ and dil. to 1 L.

(**b**) *Silver nitrate soln.*—Dissolve 5 g $AgNO_3$ in 1 L $H_2O$ and adjust soln so that 1 mL = 1 mL std KCl soln.

(**c**) *Potassium thiocyanate soln.*—Dissolve 2.5 g KSCN in 1 L $H_2O$ and adjust so that 1 mL = 1 mL std $AgNO_3$ soln. Stdze as in **942.36C**.

(**d**) *Ferric sulfate soln.*—Dissolve 60 g $Fe_2(SO_4)_3{\cdot}nH_2O$ in $H_2O$ and dil. to 1 L.

(**e**) *Ferric sulfate indicator.*—To filtered 25% soln of $Fe_2(SO_4)_3{\cdot}nH_2O$, add equal vol. $HNO_3$.

### B. Determination

Transfer 3 g sample to 300 mL erlenmeyer. Add 50 mL $Fe_2(SO_4)_3$ soln (accurately measured), swirling flask to prevent caking of sample and to facilitate soln of Cl. Add 100 mL (also accurately measured) $NH_4OH$ (1+19). Swirl flask just enough to ensure soln of Cl and thoro mixing of soln. (Very little swirling is necessary. If soln is agitated by vigorous vertical shaking, filtration will be difficult.) Let mixt. settle 10 min. Filter thru dry 11 cm Whatman No. 41 paper, or equiv. Use 50 mL aliquots ($^1/_3$ of total) on samples low in Cl (0–2% Cl) and 25 mL aliquots ($^1/_6$ of total) on samples high in Cl (>2%). For mineral and other feeds contg ≥10% Cl, weigh 1 g and use 15 mL ($^1/_{10}$ of total).

If approx. % Cl in sample is not known, take 10 mL aliquot for trial titrn. To this add 10 mL $HNO_3$ and 10 mL $Fe_2(SO_4)_3$ indicator. Dil. to ca 50 mL. Add 0.5 mL KSCN soln and immediately add, with stirring, enough $AgNO_3$ soln to entirely eliminate any reddish color. From this titrn calc. vol. $AgNO_3$ soln necessary to ppt all Cl in aliquot to be used, adding excess equal to ca 10% total vol. necessary, altho somewhat greater excess will not affect results. Use min. total of 10 mL.

To sample aliquot in 250 mL beaker add 10 mL $HNO_3$ and 10 mL $Fe_2(SO_4)_3$ indicator (or 20 mL soln contg equal vols of these solns). Add, with stirring, calcd vol. $AgNO_3$ soln. Heat to boiling and cool to room temp., stirring enough to coagulate ppt. (Cooling may be hastened by immersion of beakers in cold $H_2O$.) Titr. excess $AgNO_3$ with KSCN. End point is indicated by first appearance of reddish tint that persists 15 sec. For accurate work, use ref. soln contg all ingredients except KSCN. End point is first change in color.

Refs.: JAOAC **26,** 87(1943); **28,** 80(1945).

CAS-7782-50-5 (chlorine)

## 969.10 Chlorine (Soluble) in Animal Feed

**Potentiometric Method**

**First Action 1969**
**Final Action 1970**

### A. Apparatus

*Potentiometer.*—With Ag-AgCl reference electrode and Ag-indicating electrode (Fisher Scientific Co. No. 9–313–216 and 13–639–122, or equiv.).

### B. Standardization

Weigh 125 mg dry NaCl into 400 mL beaker. Add 200 mL $H_2O$ and 1 mL $HNO_3$.

Null potentiometer and titr. NaCl soln with 0.1*N* $AgNO_3$ soln. Plot mL $AgNO_3$ soln against mv or scale readings. Add titrant in small enough increments so that voltage end point is obvious. Use same end point for samples.

### C. Determination

(**a**) *Samples containing less than 5% sodium chloride.*—Weigh 5.844 g sample into 400 mL beaker. Add ca 200 mL $H_2O$ and 1 mL $HNO_3$. Swirl mixt. gently and let stand 10 min for complete soln of chlorides. Titr., while stirring, to same voltage end point as in stdzn.

$$\% \text{ NaCl} = \text{mL } 0.1N \text{ AgNO}_3/10$$

(**b**) *Samples containing more than 5% sodium chloride.*—Weigh 5.844 g sample into 200 mL vol. flask. Add ca 190 mL $H_2O$ and 1 mL $HNO_3$, dil. to vol. with $H_2O$, mix, and let stand 10 min. Transfer aliquot contg equiv. of ca 125 mg NaCl to 400 mL beaker, dil. to ca 200 mL, add 1 mL $HNO_3$, and titr. as in (**a**).

$$\% \text{ NaCl} = \text{diln factor} \times \text{mL } 0.1N \text{ AgNO}_3/10$$

Ref.: JAOAC **52,** 607(1969).

CAS-7782-50-5 (chlorine)

## 952.02 Cobalt in Animal Feed

**Colorimetric Method**

**Final Action**

### A. Reagents

(**a**) *Cobalt std soln.*—0.05 mg Co/mL. Dissolve 0.2385 g $CoSO_4.7H_2O$ (do not dry; use as received) in $H_2O$ and dil. to 1 L. Dil., if necessary, to suitable concn to prep. std curve.

(**b**) *Nitroso-R salt soln.*—Dissolve 1 g $C_{10}H_4OH.NO(SO_3Na)_2$ in $H_2O$ and dil. to 500 mL.

(**c**) *Spekker acid.*—Mix 150 mL 85% $H_3PO_4$ and 150 mL $H_2SO_4$, and dil. to 1 L with $H_2O$.

(**d**) *Sodium acetate soln.*—Dissolve 500 g $NaOAc.3H_2O$ in $H_2O$ and dil. to 1 L with $H_2O$.

### B. Preparation of Standard Curve

To 1, 2, etc., up to 11 mL portions std Co soln in 100 mL vol. flasks add 2 mL Spekker acid, 10 mL nitroso-R salt soln, and 10 mL NaOAc soln. Prep. blank by using 2 mL Spekker acid and 10 mL NaOAc soln, but omitting nitroso-R salt soln. Bring solns to bp on hot plate. Add 5 mL $HNO_3$ and boil $\geq 1$, but $\leq 2$ min. Cool, and dil. to 100 mL.

### C. Determination

(*Caution:* See safety notes on nitric acid and hydrogen sulfide.)

Ash 2 g sample 2 hr at 600°, transfer to 200 mL vol. flask with 20 mL HCl and 50 mL $H_2O$, boil 5 min, cool, and dil. to vol. Let soln settle. Pipet suitable aliquot into small flask. For samples contg 0.01–0.2% Co use equiv. of 0.25 g sample. For other samples, take aliquot contg $\leq 0.5$ mg Co. Soln no longer appears to follow Beer's law above this amt.

Pass brisk current of *$H_2S$* thru soln 10 min. Filter directly into 100 mL vol. flask thru Whatman No. 40 paper. Wash with ca 50 mL *1% $H_2SO_4$ satd with $H_2S$*. Add 2 small glass beads and boil off $H_2S$. (Flasks must be given individual attention, as violent bumping may occur.) Shake flasks often. Add 5 mL $HNO_3$ and boil until nitrous fumes no longer appear. (Take care, as vol. of soln will be low and bumping and spattering may occur. At first indication of this, remove immediately from hot plate.) Small amt $HNO_3$ remaining will not affect result. Cool, add 2 drops phthln, and adjust to first faint pink with ca 30% NaOH soln. Immediately add 2 mL Spekker acid followed by 10 mL nitroso-R salt soln and 10 mL NaOAc soln. Bring to vigorous boil, carefully add 5 mL $HNO_3$, and boil $\geq 1$ but $\leq 2$ min. Cool, and dil. to vol.

Compare color with std Co solns in photoelec. colorimeter, using green or No. 54 filter, or in spectrophtr at 540 nm. Read color within 2 hr. Report % Co to third decimal place.

Ref.: JAOAC **35,** 559(1952).

CAS-7440-48-4 (cobalt)

## 947.03 Copper in Animal Feed

**Colorimetric Method**

**Final Action**

### A. Preparation of Standard Curve

Dissolve 1.9645 g $CuSO_4.5H_2O$ in $H_2O$ and dil. to 500 mL. (1 mL=1 mg Cu.) Use from 1 to 10 mL of this soln to prep. set of stds in 100 mL Pyrex g-s vol. flasks. Add 4 mL HCl, dil. to 50 mL, add 5 mL *tetraethylenepentamine*, dil. to vol. with $H_2O$, stopper, and mix thoroly. Prep. blank, using all reagents except Cu. Filter blank and stds before reading color as in **947.03B**.

### B. Determination

Prep. sample soln as in **952.02C**, using 8 g sample. Pipet 50 mL aliquot into 100 mL Pyrex g-s vol. flask, add 5 mL tetraethylenepentamine, dil. to vol. with $H_2O$, and mix thoroly. Filter, and compare colors within 30 min in photoelec. colorimeter (red or No. 66 filter) or read in spectrophtr at 620 nm. Report % Cu to third decimal place.

Refs.: Anal. Chem. **19,** 325(1947). JAOAC **37,** 246(1954); **38,** 222(1955).

CAS-7440-50-8 (copper)

## 975.08 Fluorine in Animal Feed

**First Action 1975**
**Final Action 1976**

***Colorimetric Method***

### A. Determination

*See* **944.08**, especially **944.08E**.

***Ion Selective Electrode Method***

### B. Apparatus

(**a**) *Electrodes.*—Fluoride ion selective electrode (Model 9409, Orion Research Inc., or equiv.) and single junction calomel ref. electrode, plastic sleeve-type (Model 90-01, Orion Research Inc., or equiv.).

(**b**) *Magnetic stirrer.*—With 4 cm ($1^1/_2$") Teflon-coated stirring bar. Use mat to insulate sample from motor heat.

(**c**) *pH meter.*—Corning digital Model 112 (Corning Scientific Instruments, 63 North St, Medfield, MA 02052, or equiv.).

### C. Reagents

(Deionized $H_2O$ may be used.)

(**a**) *Sodium acetate soln.*—*3M*. Dissolve 408 g $NaOAc.3H_2O$ with $H_2O$ in 1 L vol. flask. When soln warms to room temp., dil. to vol. with $H_2O$. Adjust to pH 7.0 with few drops HOAc.

(**b**) *Sodium citrate soln.*—1.32*M*. Dissolve 222 g Na citrate.$2H_2O$ with ca 250 mL $H_2O$ in 1 L vol. flask. Add 28 mL $HClO_4$, dil. to vol., and mix.

(**c**) *Fluoride std solns.*—(*1*) *Stock soln.*—500 ppm. Accurately weigh 1.105 g NaF (reagent grade, dried 4 hr at 100°) into 1 L vol. flask. Dissolve and dil. to vol. with $H_2O$, and mix. Store in plastic bottle. (*2*) *Intermediate soln I.*—100 ppm. Pipet 20 mL stock soln into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix. (*3*) *Intermediate soln II.*—10 ppm. Pipet 2 mL stock soln into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix. (*4*) *Working solns.*—Pipet 3, 5, and 10 mL intermediate soln II and 5 and 10 mL intermediate soln I into five 100 mL vol. flasks to prep. 0.3, 0.5, 1.0, 5.0, and 10 ppm F working solns, resp. To each add 10.0 mL 1*N* HCl, 25.0 mL $NaOAc.3H_2O$ soln, (**a**), and 25.0 mL Na citrate soln, (**b**). Dil. to vol. with $H_2O$ and mix.

### D. Preparation of Sample

Accurately weigh well mixed sample contg ca 400 μg F into 200 mL vol. flask. Pipet in 20 mL 1*N* HCl and stir 20 min at high speed on mag. stirrer. Add 50.0 mL NaOAc soln, (**a**), and 50.0 mL Na citrate soln, (**b**), to dissolved sample. Dil. to vol. with $H_2O$ and mix.

### E. Determination

Connect F and ref. electrodes to pH meter, place electrodes in low concn F soln, and warm up pH meter. Pour 50–70 mL std and corresponding sample solns into sep. 100 mL beakers. Place electrodes in each soln and while stirring with mag. stirrer at const rate, read mv of std and unknown solns. Rinse and blot off electrodes and stirring bar between solns. Construct std curve on 3 cycle semilogarithmic paper. Read ppm F of sample soln from std curve.

$$\% \text{ F} = \text{ppm F} \times \text{mL sample soln} \times 10^{-6} \times 100/\text{g sample}$$

Ref.: JAOAC **58,** 477(1975).

CAS-7782-41-4 (fluorine)

## 934.02★ Iodine in Mineral Mixed Feeds

**Knapheide-Lamb Method**

**Final Action**

**Surplus 1965**

*See* **22.084–22.086**, 10th ed.

## 935.14 Iodine in Mineral Mixed Feeds

**Elmslie-Caldwell Method**

**Final Action**

(Not applicable to iodized mineral feeds contg little or no org. matter. *Caution:* See safety notes on bromine.)

Place sample contg 3–4 mg I in 200–300 mL Ni dish. Add ca 5 g $Na_2CO_3$, 5 mL NaOH soln (1+1), and 10 mL alcohol, taking care that entire sample is moist. Heat on steam bath to remove alcohol. Then dry at ca 100° to prevent spattering upon subsequent heating (30 min is usually enough).

Place dish and contents in furnace heated to 500° and keep at that temp. 15 min. (Ignition of sample at 500° appears to be necessary only to carbonize any sol. org. matter that would be oxidized by Br-$H_2O$ if not so treated. Temp. >500° may be used if necessary.) Cool, add 25 mL $H_2O$, cover dish with watch glass, and boil gently 10 min. Filter thru 18 cm paper and wash with boiling $H_2O$, catching filtrate and washings in 600 mL beaker (soln should total ca 300 mL). Neutze to Me orange with 85% $H_3PO_4$ and add 1 mL excess.

Add excess Br-$H_2O$ and boil soln gently until colorless, and then 5 min longer. Add few crystals *salicylic acid* and cool soln to ca 20°. Add 1 mL 85% $H_3PO_4$ and ca 0.5 g KI, and titr. I with 0.005*N* $Na_2S_2O_3$, adding starch soln when liberated I color is nearly gone. 1 mL 0.005*N* $Na_2S_2O_3$ = 0.1058 mg I.

Refs.: JAOAC **18,** 338(1935); **21,** 596(1938); **23,** 688(1940); **33,** 83(1950).

CAS-7553-56-2 (iodine)

## 917.04 Manganese (Acid-Soluble) in Animal Feed

**Colorimetric Method**

**Final Action**

### A. Reagent

*Potassium permanganate std soln.*—500 ppm Mn. Prep. and stdze as in **940.35**, except use 1.4383 g $KMnO_4$ and 0.12 g Na oxalate. Transfer aliquot contg 20 mg Mn to beaker. Add 100 mL $H_2O$, 15 mL $H_3PO_4$, and 0.3 g $KIO_4$, and heat to bp. Cool, and dil. to 1 L. Protect from light. Dil. this soln contg 20 ppm Mn with $H_2O$ (previously boiled with 0.3 g $KIO_4$/L) to make convenient working stds in range of concns to be compared.

### B. Determination

(*Caution:* See safety notes on nitric acid and sulfuric acid.)

Ash weighed sample, 5–15 g, at dull red heat (ca 600°) in porcelain dish. Cool, and add 5 mL $H_2SO_4$ and 5 mL $HNO_3$ to ash in dish or to ash transferred to beaker with 20–30 mL $H_2O$. Evap. to white fumes. If C is not completely destroyed, add addnl portions $HNO_3$, boiling after each addn. Cool slightly, transfer to 50 or 100 mL vol. flask, and add vol. dil. $H_3PO_4$ soln (8+92) equal to 1/2 vol. of flask (25 or 50 mL). Cool, dil. to vol., mix, and filter or let stand until clear.

If 50 mL flask was used, pipet 25 mL clear soln into beaker or 50 or 100 mL vol. flask and add 15 mL $H_2O$. If 100 mL flask was used, pipet 50 mL into beaker or 100 mL flask and add 30 mL $H_2O$. Heat nearly to bp, and with stirring or swirling add 0.3 g $KIO_4$ for each 15 mg Mn present. Keep 30–60 min at 90–100°, or until color development is complete. Cool, dil. to measured vol. of 50 or 100 mL, and mix. Compare with std $KMnO_4$ soln in photoelec. colorimeter or in spectrophtr at 530 nm. Calc. ppm Mn.

Refs.: J. Am. Chem. Soc. **39,** 2366(1917). G. Frederick Smith Chemical Co. Pub. 209, 5th ed. (1950). JAOAC **22,** 78, 673(1939); **24,** 865(1941); **25,** 892(1942).

CAS-7439-96-5 (manganese)

## 964.06 Phosphorus in Animal Feed

**Alkalimetric Ammonium Molybdophosphate Method**

**Final Action**

### A. Reagents

(**a**) *Molybdate soln.*—Dissolve 100 g $MoO_3$ in mixt. of 144 mL $NH_4OH$ and 271 mL $H_2O$. Cool, and slowly pour soln, stirring constantly, into cool mixt. of 489 mL $HNO_3$ and 1148 mL $H_2O$. Keep final mixt. in warm place several days or until portion heated to 40° deposits no yellow ppt. Decant soln from any sediment and keep in g-s vessels.

(**b**) *Acidified molybdate soln.*—To 100 mL molybdate soln, (**a**), add 5 mL $HNO_3$. Filter immediately before use.

(**c**) *Sodium hydroxide std soln.*—Dil. 324.03 mL 1*N* alkali, carbonate-free, **936.16**, to 1 L. (100 mL of this soln should neutze 32.40 mL 1*N* acid; 1 mL = 1 mg or 1% $P_2O_5$ on basis of 0.1 g sample.) (Since burets in const use may become so corroded as to increase their capacity, test them at least annually.)

(**d**) *Std acid soln.*—Prep. soln of HCl or of $HNO_3$, corresponding to concn of (**c**) or to 1/2 this concn, and stdze by titrn against (**c**), using phthln.

### B. Determination

Prep. sample soln as in **935.13A(a)**. Pipet, into beaker or flask, aliquot corresponding to 0.4 g sample for $P_2O_5$ content of sample <5%; 0.2 g for 5–20%; 0.1 g for >20%. Add 5–10 mL $HNO_3$, depending on method of soln (or equiv. in $NH_4NO_3$); then add $NH_4OH$ until ppt that forms dissolves only slowly on vigorous stirring, dil. to 75–100 mL, and adjust to 25–30°. If sample does not give ppt with $NH_4OH$ as test of neutzn, make soln slightly alk. to litmus paper with $NH_4OH$ and then slightly acid with $HNO_3$ (1+3). Add 20–25 mL acidified molybdate soln for $P_2O_5$ content <5%; 30–35 mL for 5–20%; and enough acidified molybdate soln to ensure complete pptn for >20%. Shake or stir mech. 30 min at room temp.; decant *at once* thru filter and wash ppt twice by decanting with 25–30 mL portions $H_2O$, agitating thoroly and allowing to settle. Transfer ppt to filter and wash with cold $H_2O$ until filtrate from 2 fillings of filter yields pink color on adding phthln and 1 drop of the std alkali. Transfer ppt and filter to beaker or pptg vessel, dissolve ppt in small excess of the std alkali, add few drops of phthln, and titr. with std acid. Report as % P.

Ref.: JAOAC **47,** 420(1964).

CAS-7723-14-0 (phosphorus)

## 965.17 Phosphorus in Animal Feed

### Photometric Method

**First Action 1965**
**Final Action 1966**

(Not applicable to mineral-mix feeds. Dry ashing procedure is not applicable to feeds or mineral mixes contg monobasic Ca phosphate.)

### A. Apparatus

*Spectrophotometer.*—Capable of isolating 400 nm band and accepting ≤15 mm diam. cells.

### B. Reagents

(**a**) *Molybdovanadate reagent.*—Dissolve 40 g $NH_4$ molybdate.$4H_2O$ in 400 mL hot $H_2O$ and cool. Dissolve 2 g $NH_4$ metavanadate in 250 mL hot $H_2O$ and cool; add 250 mL 70% $HClO_4$. (*Caution:* See safety notes on perchloric acid.) Gradually add molybdate soln to vanadate soln with stirring, and dil. to 2 L.

(**b**) *Phosphorus std solns.*—(*1*) *Stock soln.*—2 mg P/mL. Dissolve 8.788 g $KH_2PO_4$ in $H_2O$ and dil. to 1 L. (*2*) *Working soln.*—0.1 mg P/mL. Dil. 50 mL stock soln to 1 L.

### C. Preparation of Standard Curve

Transfer aliquots of working std soln contg 0.5, 0.8, 1.0, and 1.5 mg P to 100 mL vol. flasks. Treat as in **965.17D**, beginning "Add 20 mL molybdovanadate reagent, . . ." Prep. std curve by plotting mg P against %*T* on semilog paper.

### D. Determination

Ash 2 g sample, in 150 mL beaker, 4 hr at 600°. Cool, add 40 mL HCl (1+3) and several drops $HNO_3$, and bring to bp. Cool, transfer to 200 mL vol. flask, and dil. to vol. with $H_2O$. Filter, and place aliquot contg 0.5–1.5 mg P in 100 mL vol. flask. Add 20 mL molybdovanadate reagent, dil. to vol. with $H_2O$, and mix well. Let stand 10 min; then read %*T* at 400 nm against 0.5 mg std set at 100% *T*. (Use ≤15 mm diam. cells.) Det. mg P from std curve.

$$\% \text{ P} = \text{mg P in aliquot}/(\text{g sample in aliquot} \times 10)$$

Ref.: JAOAC **48,** 654(1965); **59,** 937(1976).

CAS-7723-14-0 (phosphorus)

## 964.07 Microscopy of Animal Feed

### Basic Microscopic Examination

**First Action 1964**
**Final Action 1965**

### A. Apparatus

(**a**) *Magnifier-fluorescent illuminator with desk base, 3×, or reading glass.*

(**b**) *Microscopes and illuminator.*—*Illuminators:* Illuminator for this purpose should have: compactness and flexibility; transformer or resistor to vary light intensity; focusing adjustment to give uniformly lighted field of view; blue-white color from cool low-voltage source. (*1*) *Compound microscope.*—For mold counting and other filth and decomposition work, microscope should have following min. specifications: binocular body with inclined oculars; 4 parfocal achromatic objectives of ca 4, 10, 20, and 40×; revolving 4-place nosepiece; Abbe condenser with N.A. of 1.25; 10× Huygenian or widefield eyepieces; fine adjustment; mech. stage. (*2*) *Widefield stereoscopic microscope recommended for filth examination.*—Microscope should have following min. specifications: binocular body with inclined oculars; sliding or revolving nosepiece to accommodate 3 objectives; 3 parfocal objectives 1×, 3×, and 6 or 7.5×; paired 10× and paired 15× widefield oculars; mounted on base and capable of illumination by transmitted or reflected light. 30× is ordinarily used for routine examination of filter papers. Verification at higher magnification may be required. Following are preferred:

(*1*) *Widefield stereoscopic microscope.*—With arm rests, flat stage (remove spring holders), optional substage illumination, inclined eyepiece, and lenses to magnify ca 7–30×, 15× optimum.

(*2*) *Compound microscope.*—With mech. stage, substage condenser, inclined binocular eyepiece, 3 position rotating nosepiece, lenses to magnify ca 36–400×, 120× optimum.

(*3*) *Microscope illuminator.*—With iris diaphragm; movable stand holder with rod to permit adjusting light source as to ht and angle for substage or direct over-stage lighting; able to hold 2 blue glass filters or 1 blue and 1 ground glass; 60–100 watt bulb.

(**c**) *Sieves.*—Nest of 5″ No. 10, 20, 40, 60, 80, and bottom pan.

(**d**) *Stages.*—Dark Co glass plates 4 × 4″ (Fisher Scientific Co. No. 13-735); or blue paper and microscope slides.

(**e**) *Spot plates.*—Black and white.

(**f**) *Forceps.*—Fine pointed, curved. If necessary, bend and grind on emery wheel for good contact of points.

(**g**) *Dropping bottles.*—Amber, 30 mL, as reagent dispensers.

(**h**) *Microspatula; microstirring rods made by drawing out glass rods; spoon.*

### B. Reagents

(**a**) *Chloroform.*—Tech. Recover by filtration and distn.

(**b**) *Acetone.*—Tech.

(**c**) *Acetone, dilute.*—Dil. 75 mL acetone with 25 mL $H_2O$.

(**d**) *Dilute hydrochloric acid.*—Dil. 1 vol. HCl with 1 vol. $H_2O$.

(**e**) *Dilute sulfuric acid.*—Dil. 1 vol. $H_2SO_4$ with 1 vol. $H_2O$.

(**f**) *Iodine soln.*—Dissolve 0.75 g KI and 0.1 g I in 30 mL $H_2O$ and add 0.5 mL HCl. Store in amber dropping bottle.

(**g**) *Millon reagent.*—Dissolve, by gently warming, 1 part by wt Hg in 2 parts by wt $HNO_3$. Dil. with 2 vols $H_2O$. Let mixt. stand overnight and decant supernate. Soln contains $Hg(NO_3)_2$, $HgNO_3$, $HNO_3$, and some $HNO_2$. Store in g-s bottle. (*Caution:* See safety notes on mercury salts.)

(**h**) *Molybdate soln.*—Add 100 mL 10% $NH_4NO_3$ soln to 400 mL molybdate soln, **964.06A(a)**. Use only clear supernate to fill 30 mL amber dropping bottle. Discard and refill when crystn occurs.

(**i**) *Mountant I.*—Dissolve 10 g chloral hydrate in 10 mL $H_2O$ and add 10 mL glycerol. Store in amber dropping bottle.

(**j**) *Mountant II.*—Dissolve 160 g chloral hydrate in 100 mL $H_2O$ and add 10 mL HCl.

(**k**) *Silver nitrate soln.*—10%. Dissolve 10 g $AgNO_3$ in 100 mL $H_2O$.

### C. Standards

(**a**) *Feed ingredients.*—Collect ingredients used in grain and stock feeds known to conform to definitions of Association of American Feed Control Officials as stds. Store in 4 oz bottles. To control insects, add ca 1 mL $CS_2$, and stopper. Become thoroly familiar with structural appearance of stds before and after treatment with org. solvs.

(**b**) *Weed seeds.*—Collect common weed seeds occurring in grains. Most may be found in foreign material obtained after sieving com. whole grains with U.S. Grain Testing Sieve having $^5/_{64}$″ (2.5 mm) triangular holes. Identify from illustration in "Identification of Crop and Weed Seeds" (USDA Handbook 219 (1963), Government Printing Office, Washington, DC 20402). Store in numbered vials. Become familiar with those weed seeds designated as prohibited and restricted noxious under state laws of individual concern. (*See* "State Noxious-Weed Seed Requirements Recognized in the Administration of the Federal Seed Act" (USDA, Agricultural Marketing Service, Grain Div., Hyattsville, MD 20782).)

Ref.: JAOAC **47**, 504(1964).

## 970.08 Microscopy of Animal Feed
### Identification of Vegetable Tissues
### Final Action

#### *A. Principle*

Feeds are fractionated according to particle size and cleared where necessary for clear observation; conglomerates are disintegrated into constituents and fractions arranged on stage suitable for microscopic examination at lowest magnification that permits identification of components when compared to std feed ingredients.

#### *B. General Methods*

(**a**) *Scratch feeds.*—Spread representative portion of sample on white paper and examine under magnifier-fluorescent illuminator at 3× or with reading glass. Identify grains and weed seeds; note other foreign material, heat- and insect-damaged particles, live insects, and rodent excreta; examine for smut, ergot, and mold ("Grain Inspection Manual," USDA).

(**b**) *Mashes comparatively free from adhering fine particles.*—(*1*) *Low power microscopy.*—Arrange in nest form 3 sieves that will adequately fractionate feed according to particle size. Generally, for cattle feeds use No. 10, 20, and 40; for poultry feeds, No. 20, 40, and 60. Include bottom pan. Add ca 10 g unground feed (plastic tablespoon makes convenient scoop) to nest, and sieve thoroly. With spatula, spread portion from each sieve on 4 × 4″ Co glass stage and place under stereoscopic microscope. (Blue paper may also be used as stage.) Arrange illuminator above and near stage so light strikes sample at angle of ca 45° for shadow contrast. Adjust magnification (ca 15× optimum), illumination, and light filters to individual preference for clear observation. Blue light or northern daylight is preferred. Examine each fraction on stage sep. and systematically. Observe feed particles, continually probing, turning, and testing resistance to pressure with forceps. Note particle size, shape, color, resistance to pressure, texture, odor, and major structural features. Compare with stds. If desired, transfer individual particles with forceps to second glass plate for direct comparison with corresponding tissues from stds. Likewise transfer and break up conglomerates by gentle pressure with flat end of forceps. Make list of observed ingredients. Neglect trace grains which may be normal impurities in major grains. (Consult "Official Grain Standards of the United States," USDA, for amts of "other grains" permissible as impurities in whole grains.)

(*2*) *High power microscopy.*—Lower illuminator and select filters so adequate blue light is reflected thru substage condenser of high power microscope. With microspatula, transfer little of fine sievings from bottom sieve and pan to slide, add 2 drops mountant I, stir, and disperse with microstirring rod. Examine microscopically (120× optimum). Compare histologically with stds. Remove slide, add 1 drop I soln, stir, and re-examine. Starch cells are stained pale blue to black; yeasts and other protein cells, pale yellow to brown. If further tissue clarification is desired, boil little of same fine sievings 1 min with ca 5 mL mountant II. Cool, transfer drop or 2 of bottom settlings to slide, cover, and examine microscopically.

(**c**) *Oily feeds or those containing large particles obscured by adhering fine particles.*—(Most poultry feeds and unknowns are best examined by this technic.) Place ca 10 g unground feed in 100 mL tall-form beaker and nearly fill with $CHCl_3$ (hood). Stir briefly and let settle ca 1 min. With spoon, transfer floating (org.) material to 3.5″ (9 cm) watch glass, drain, and dry on steam bath. Sieve, and proceed as in (**b**). If desired, filter, dry, suspend fine particles in $CHCl_3$, and examine microscopically (rarely necessary).

(**d**) *Feeds in which molasses has caused lumpiness and otherwise obscured vision.*—Place ca 10 g unground feed in 100 mL tall-form beaker. Add 75 mL 75% acetone, stir few min to dissolve molasses, and let settle. Carefully decant and repeat extn. Wash residue twice with acetone by decantation, dry on steam bath, sieve, and proceed as in (**b**).

(**e**) *Pellets or crumbles.*—Gently grind few pellets at time in mortar with pestle with enough pressure to sep. pellet into its constituents, but not to break up constituents themselves. Sieve first grind thru No. 20 sieve and return particles remaining on sieve to mortar for further grinding. Depending on nature of pellet, proceed with ground material as in (**b**), (**c**), or (**d**).

## 970.09 Microscopy of Animal Feed
### Identification of Animal Tissues and Mineral Constituents
### Final Action

#### *A. Principle*

Feeds contg animal tissues and minerals when suspended in $CHCl_3$ readily sep. into 2 fractions: (*1*) Org. fraction which floats, consisting of muscle fibers, connective tissue, dried ground organs, feather remains, hoof and horn particles, etc. from either animal or marine products, plus all vegetable tissues. (*2*) Mineral fraction which sinks, consisting of bones, fish scales, teeth, and minerals.

#### *B. Preparation of Sample*

Perform $CHCl_3$ flotation sepn as in **970.08B(c)**. Collect floating material and dry on steam bath. Decant $CHCl_3$, collect mineral fraction, and dry on steam bath.

#### *C. Identification of Animal Tissue*

Examine dried floating material as in **970.08B(b)**.

#### *D. Identification of Major Mineral Constituents*

Place dried mineral fraction on nest of No. 40, 60, and 80 sieves and bottom pan. Sieve and place the 4 fractions in sep. groups on same Co glass plate or blue paper stage. Examine under stereoscopic microscope at ca 15×. Animal and fish bones, fish scales, and mollusc shells are generally recognizable. Salt usually occurs in cubes which may be dyed. Calcite form of limestone occurs as rhombohedrons.

#### *E. Confirmatory Tests*

With forceps, place unknown particle on glass plate and break up by applying gentle pressure with flat surface. Working under stereoscopic microscope, sep. particles ca 2.5 cm and place beside each a fractional drop of reagent solns listed by touching end of dropper to plate. Push particle into liq. with microstirring rod and observe what occurs at interface. Follow order given until pos. identification is obtained. If preferred, perform tests in black spot plate.

(**a**) *Silver nitrate soln.*—(*1*) Crystal immediately turns chalk white and slowly expands: chloride, probably salt. (*2*) Crystal turns yellow and yellow needles begin to grow: mono- or dibasic phosphate, generally dicalcium phosphate. (*3*) Sparingly sol. white needles form ($Ag_2SO_4$): sulfate, Mn-$MgSO_4$. (*4*) Particles slowly darken: bone.

(**b**) *Dilute hydrochloric acid.*—(*1*) Vigorous effervescence: $CaCO_3$. (*2*) Mild effervescence or none: make following tests.

(**c**) *Molybdate soln.*—Formation of minute yellow crystals at some distance from particle: tricalcium phosphate, either bone or rock phosphate. (All phosphates react, but mono- and dibasic phosphates have been identified with $AgNO_3$.)

(**d**) *Millon reagent.*—(*1*) Disintegrated particles mostly float, turn pink to red (protein), and fade in ca 5 min: bone phosphate. (*2*) Particles appear to swell and disintegrate but remain on bottom: defluorinated rock phosphate. (*3*) Particles merely disintegrate slowly: rock phosphate.

(**e**) *Dilute sulfuric acid.*—Long, thin white needles slowly form on addn of drop of $H_2SO_4$ (1+1) to HCl (1+1) soln of particle: confirms Ca.

## 975.09 Identification of Furazolidone, Tylosin, and Zoalene

*See* **973.80**.

## 963.07 Ethoxyquin in Animal Feed

**Fluorometric Method**

**First Action 1963**
**Final Action 1964**

### *A. Reagents and Apparatus*

(**a**) *Quinine sulfate reference soln.*—1 μg/mL 0.1*N* $H_2SO_4$. Dissolve 0.100 g quinine sulfate USP (dried at 120° for 3 hr before using) in 1 L 0.1*N* $H_2SO_4$. Dil. 10 mL aliquot of this soln to 1 L with 0.1*N* $H_2SO_4$. Use to calibrate photofluorometer.

(**b**) *Ethoxyquin std solns.*—Add 100.0 mg liq. ethoxyquin to 100 mL vol. flask and dil. to vol. with pet ether (*Soln A*). Dil. 5 mL *Soln A* to 100 mL with pet ether (*Soln B*, 50 μg/mL). Dil. 5 mL *Soln B* to 100 mL with pet ether (*Soln C*, 2.5 μg/mL). Dil. 10 mL *Soln C* to 20 mL with pet ether (1.25 μg/mL) and 5 mL to 25 mL (0.50 μg/mL).

(**c**) *Photofluorometer.*—Equipped with primary filter passing 365 nm Hg line (Corning Glass Works No. 5874 (CS7-39), or equiv.) and secondary filter passing 420–500 nm (Corning Glass Works 3389 + 5543 + 4784, half stock thickness, or equiv.).

### *B. Preparation of Standard Curve*

Adjust photofluorometer to read 0 with pet ether and 100 with quinine sulfate ref. soln. Obtain fluorescence readings for ethoxyquin std solns contg 0–2.5 μg/mL. Plot readings against μg ethoxyquin/mL on linear paper.

### *C. Determination*

Place 10 ± 0.1 g finely ground sample in 100 mL beaker and slurry with 50 mL MeOH. Stir and let stand 10 min. Decant thru plug of glass wool into 250 mL vol. flask. Reslurry residue with two 50 mL portions MeOH, decant, and filter, combining all filtrates. Dil. to vol. with MeOH. Transfer 25 mL aliquot to 250 mL separator, add 100 mL $H_2O$, and mix well. Add 50 mL pet ether, stopper, and shake moderately 1 min. Let stand few min to sep. (If emulsion forms, add ca 100 mg NaCl crystals. After emulsion breaks, drain aq. lower layer into 250 mL beaker.) Transfer pet ether layer to second 250 mL separator, return aq. layer to first separator, and re-ext with two 25 mL portions pet ether.

Add 50 mL $H_2O$ to combined pet ether exts in separator, stopper, and shake moderately. Let sep., drain lower aq. layer, and discard. Transfer pet ether layer to 100 mL vol. flask, and dil. to vol. with pet ether. Adjust photofluorometer as above and det. fluorescence readings. Obtain μg ethoxyquin/mL from std curve. Ppm ethoxyquin = 100 × μg/mL.

Add 50 mL $H_2O$ to combined pet ether exts in separator, stopper, and shake moderately. Let sep., drain lower aq. layer, and discard. Transfer pet ether layer to 100 mL vol. flask, and dil. to vol. with pet ether. Adjust photofluorometer as above and det. fluorescence readings. Obtain μg ethoxyquin/mL from std curve. Ppm ethoxyquin = 100 × μg/mL.

If untreated feed is available, prep. std curve from series of samples contg 0–250 μg ethoxyquin/10 g and carried thru detn.

Refs.: JAOAC **44**, 560(1961); **46**, 306(1963); **47**, 512(1964).

CAS-91-53-2 (ethoxyquin)

## 970.10 Cyanogenetic Glucosides in Animal Feed

*See* **936.11**.

## 970.11 Hydrocyanic Acid in Animal Feed

*See* **915.03**.

## Drugs in Animal Feed

*See* chapter on drugs in animal feed.

## Molasses and Molasses Products in Animal Feed

*See* chapter on sugars and sugar products.

# 5. Drugs in Feeds

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(Medicated feeds may deteriorate under improper storage conditions. When possible, use reasonably fresh samples, store them in the cold, and grind just before analysis.)

### 965.16 Sampling of Animal Feed

**Procedure**

Use slotted single or double tube, or slotted tube and rod, all with pointed ends.

Take ≥500 g sample, 1 kg preferred, as follows: Lay bag horizontally and remove core diagonally from end to end. Det. number of cores as follows: From lots of 1–10 bags, sample all bags; from lot of ≥11, sample 10 bags. Take 1 core from each bag sampled, except that for lots of 1–4 bags take enough diagonal cores from each bag to total ≥5 cores. For bulk feeds draw ≥10 cores from different regions; in sampling small containers (≤10 lb) 1 package is enough. Reduce composite sample to amt required, preferably by riffling, or by mixing thoroly on clean oil-cloth or paper and quartering. Place sample in air-tight container.

A sample from less than these numbers of bags may be declared an official sample if guarantor agrees. For samples that cannot be representatively taken with probe described, use other sampling means.

### 950.02 Animal Feed

**Preparation of Sample**

**Final Action**

Grind sample to pass sieve with circular openings 1 mm ($^1/_{25}$") diam. and mix thoroly. If sample cannot be ground, reduce to as fine condition as possible. Do not grind molasses feeds.

Refs.: JAOAC **33,** 424(1950); **41,** 223(1958); **48,** 658(1965).

### 963.32★ 2-Acetylamino-5-Nitrothiazole in Feeds

**Spectrophotometric Method**

**First Action 1963**
**Final Action 1964**
**Surplus 1977**

*See* **42.011–42.016**, 13th ed.

### 969.53 Aklomide in Feeds

**Spectrophotometric Method**

**First Action 1969**
**Final Action 1971**

(Applicable in presence of sulfanitran or roxarsone)

#### *A. Reagents*

(**a**) *Titanous chloride soln.*—4% aq. Prep. fresh on day of use from 20% soln or solid $TiCl_3$.

(**b**) *Sodium nitrite soln.*—0.1% aq. Prep. fresh on day of use.

(**c**) *Ammonium sulfamate soln.*—0.5%. Dissolve 500 mg $NH_4SO_3NH_2$ in $H_2O$ and dil. to 100 mL. Prep. fresh weekly.

(**d**) *Coupling reagent.*—0.1% aq. *N*-naphthylethylenediamine.2HCl. Prep. fresh weekly and store in dark glass bottle in refrigerator.

(**e**) *Aklomide std solns.*—2-Chloro-4-nitrobenzamide, purified for std use, available from Salsbury Laboratories, 2000 Rockford Rd, Charles City, IA 50616. (*1*) *Stock soln.*—1 mg/mL. Transfer 100 mg aklomide to 100 mL vol. flask, dissolve in ca 75 mL MeOH, dil. to vol. with MeOH, and mix well. (*2*) *Intermediate soln.*—10 μg/mL. Pipet 10 mL stock soln into 100 mL vol. flask, dil. to vol. with MeOH, and mix. Pipet 5 mL into 50 mL vol. flask. Evap. to dryness on $H_2O$ bath with aid of gentle air stream and cool to room temp. Add ca 30 mL 0.15*N* HCl, shake 10 min intermittently, dil. to vol. with 0.15*N* HCl, and mix well. (*3*) *Working solns.*—0, 0.4, 0.8, 1.2, 1.6, and 2.0 μg/mL. Transfer 0, 1, 2, 3, 4, and 5 mL intermediate soln to sep. 25 mL vol. flasks and dil. to vol. with 0.15*N* HCl.

#### *B. Preparation of Standard Curve*

Transfer 4 mL aliquot from each working std soln to sep. colorimetric tubes and proceed with reduction, color development, and measurement as in **969.53C**. Tubes contain 0, 1.6, 3.2, 4.8, 6.4, and 8.0 μg aklomide/tube, equiv. to 0, 0.008, 0.016, 0.024, 0.032, and 0.040% aklomide in feed when 5 g sample is taken. Plot *A* against % aklomide.

#### *C. Determination*

Weigh 5 g sample contg ca 0.025% aklomide into 100 mL vol. flask, add 75 mL MeOH, and heat 30 min in 60° $H_2O$ bath, shaking occasionally. Remove flask, cool to room temp., and dil. to vol. with MeOH. Mix thoroly and let stand 40 min to settle feed particles.

Pipet 5 mL clear supernate into 50 mL vol. flask and dil. to vol. with 0.15*N* HCl. Mix well and filter thru Whatman No. 4 paper into 125 mL erlenmeyer. (If filtrate is cloudy, refilter.) Pipet 4 mL filtrate into each of 2 tubes, add 2 drops 4% $TiCl_3$ from dropper, mix, and let stand 2 min. Add 2 drops 10*N* NaOH from dropper, mix until white ppt persists, and acidify with 2.0 mL HCl. Mix and let stand until soln clears. Add 0.5 mL $NaNO_2$ to one tube and 0.5 mL $H_2O$ to second tube as blank; mix. After 3 min, add 0.5 mL 0.5% $NH_4$ sulfamate to each tube and mix. After 2 min, add 0.5 mL coupling reagent to each, mix, and let color develop 15 min. Read *A* of soln at 545 nm in colorimeter or spectrophtr. Subtract reading of feed blank. Det. % aklomide in feed directly from std curve.

Ref.: JAOAC **52,** 438(1969).

CAS-3011-89-0 (aklomide)

### 969.54 Aklomide in Feeds

**Thin Layer Chromatographic Qualitative Test**

**Final Action 1971**

#### *A. Reagents*

(**a**) *Spray reagent.*—Dissolve and dil. 0.1 g *p*-(dimethylamino)cinnamaldehyde (DMC) (No. J436, J.T. Baker, Inc.)

to 100 mL with 1.0$N$ HCl. (Soln is stable ≥1 month.) Just before use, add 1 mL 20% $TiCl_3$ to 25 mL DMC soln and mix. Discard after 1 hr.

(**b**) *Aklomide reference std.*—1 mg/mL. *See* **969.53A(e)**(*1*).

### *B. Test*

Ext 10 g sample with 25 mL MeOH, shaking occasionally during 15 min. Filter thru Whatman No. 4 paper into 50 mL beaker. Conc. filtrate to ca 2 mL on steam bath. Spot ca 10 μL on 250 μm silica gel G TLC plate along with ref. std and develop ca 30 min, in ether. Remove from tank and air dry ca 15 min. Spray plate with DMC-$TiCl_3$ reagent. (*Caution:* See safety notes on spraying chromatograms.) Aklomide forms reddish pink spot. Compare $R_f$ value to that of ref. std.

Ref.: JAOAC **52,** 438(1969).

CAS-3011-89-0 (aklomide)

## 964.28 *p*-Aminobenzoic Acid in Feeds

**Spectrophotometric Method**

**Final Action 1965**

### *A. Preparation of Standard Solution*

Transfer 0.100 g *p*-aminobenzoic acid (99+% purity, available from ICN Pharmaceuticals Inc., Life Sciences Group) to 100 mL vol. flask, dissolve in 5 mL 1$N$ NaOH, and dil. to vol. with $H_2O$. Dil. 5 mL aliquot to 200 mL with $H_2O$ (1 mL = 25 μg). Place 2, 4, and 6 mL aliquots dild soln (50, 100, 150 μg) in 100 mL vol. flasks, add 3 mL HCl to each, dil. to vol. with $H_2O$, and mix.

### *B. Determination*

Transfer 5 g freshly ground feed to 250 mL vol. flask, add 135 mL $H_2O$, making slurry of first 10 mL to wet sample completely, and then add 15 mL HCl. Mix, and place on steam bath 25 min, swirling occasionally until soln darkens. Cool, dil. to 250 mL with $H_2O$, and let feed particles settle. Pipet 50 mL into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix thoroly. Pour soln into 250 mL beaker, add filter-aid, and filter thru 18.5 cm Whatman No. 2 paper, or equiv., discarding first 10–15 mL, if turbid.

Pipet two 10 mL aliquots into 50 mL beakers, add 5 mL $H_2O$ and 2 mL *fresh 0.10% $NaNO_2$ soln,* mix, and let stand 3 min. Add 2 mL *0.50% $NH_4$ sulfamate soln,* mix, and let stand 2 min. Then add, to one beaker only, 1 mL coupling reagent, **969.53A(d)**, and to other 1 mL $H_2O$. Mix solns and wait 10 min. Det. $A$ against $H_2O$ at 545 nm in spectrophtr. (Avoid false readings due to N bubbles on cell walls.) Subtract blank $A$ from sample $A$ and calc. μg found by ref. to std curve.

% *p*-aminobenzoic acid in feed = μg found/1000
K *p*-aminobenzoate = *p*-aminobenzoic acid × 1.278

Prep. stds by treating 10 mL aliquots of 3 final std solns, representing 5, 10, and 15 μg, as in detn, beginning ". . . add 5 mL $H_2O$ . . ." Plot $A$ at 5, 10, and 15 μg and draw straight line.

### *C. Qualitative Tests*

(To differentiate *p*-aminobenzoic acid, arsanilic acid, and sulfaquinoxaline)

Place 10 mL prepd sample filtrate in separator. Ext with 10 mL peroxide-free ether by vigorous shaking 30 sec. Let layers sep., and drain aq. layer into another separator. Re-ext with 10 mL ether and drain aq. layer into third separator for third extn with same vol. ether. After final extn, drain aq. layer into fourth separator, add 5 mL $H_2O$, mix, and couple soln as in **964.28B**, second par. Wait 10 min, add 5 drops HCl and 10 mL isoamyl alcohol, and ext gently ca 30 sec. Let stand until layers sep. Red color in solv. is due to *p*-aminobenzoic acid; that in lower layer, to arsanilic acid. Drain as much aq. layer as possible and again ext with 10 mL solv. Arsanilic acid remains as distinct color in aq. layer, not as mere trace due to incomplete removal of *p*-aminobenzoic acid. Combine ether exts, wash with 5 mL $H_2O$, discard, and ext with 10 mL 1% $Na_2CO_3$ soln; acidify, and couple again to prove presence of sulfaquinoxaline.

Ref.: JAOAC **47,** 214(1964).

CAS-150-13-0 (*p*-aminobenzoic acid)
CAS-98-50-0 (arsanilic acid)
CAS-59-40-5 (sulfaquinoxaline)

## 953.19★ 2-Amino-5-Nitrothiazole in Feeds

**Spectrophotometric Method**

**Final Action**

**Surplus 1980**

*See* **42.025–42.027**, 13th ed.

## 961.24 Amprolium in Feeds

**Spectrophotometric Method**

**First Action 1961**

**Final Action 1962**

### *A. Principle*

Amprolium is extd from feed with aq. MeOH. Ext is purified by chromatgy on alumina, and amprolium reacts with 2,7-naphthalenediol, $K_3Fe(CN)_6$, KCN, and NaOH in MeOH to form colored compd with absorption max. at 530 nm. There is no interference from usual components of com. feeds, vitamins, antibiotics, picolines, or pyrimidines. Nithiazide, Enheptin A, and nitrofurazone show some interference.

### *B. Reagents*

(*Caution:* See safety notes on cyanides.)

(**a**) *Alcoholic sodium hydroxide soln.*—Dil. 15.0 mL aq. NaOH soln, (**i**), with anhyd. MeOH to 200 mL. Stopper, and mix well.

(**b**) *Alumina.*—Reagent grade suitable for chromatgy. Should pass following test: Vigorously shake 10 g alumina with 100 mL $H_2O$ in 250 mL g-s flask ≥2 min. Let settle, decant, and det. pH potentiometrically. pH should be 9.5–10.5. Recovery of amprolium may vary among different brands of alumina. Test column recovery by spiking ext from nonmedicated feed.

(**c**) *Amprolium std soln.*—25 μg/mL. Weigh 25.0 mg Amprolium Ref. Std (available from Merck & Co.) into 50 mL vol. flask, dissolve in dil. MeOH, (**e**), dil. to vol., and mix. Dil. 5 mL to 100 mL in vol. flask with dil. MeOH. Soln is stable 1 week.

(**d**) *Color developing reagent.*—Add 5 mL $K_3Fe(CN)_6$ soln to 90 mL naphthalenediol soln, (**f**), in 250 mL g-s flask, and mix well. Add 5 mL KCN soln, (**g**), stopper, mix well, and let stand 30–35 min. Add 100 mL alc. NaOH soln, (**a**), and mix. Use within 75 min, filtering thru medium porosity fritted glass filter just before use.

(**e**) *Dilute methyl alcohol.*—Mix 2 vols anhyd. MeOH with 1 vol. $H_2O$. Cool to room temp. before use.

(**f**) *Naphthalenediol soln.*—Dissolve 25 mg 2,7-naphthalenediol (Eastman Kodak Co.) in 1 L anhyd. MeOH.

(**g**) *Potassium cyanide soln.*—Dissolve 1.0 g KCN in 100 mL $H_2O$. Kept tightly stoppered, soln is stable 2 weeks.

(**h**) *Potassium ferricyanide soln.*—Dissolve 200 mg

$K_3Fe(CN)_6$ in 100 mL $H_2O$. Kept tightly stoppered, soln is stable 2 weeks.

(**i**) *Sodium hydroxide soln.*—Dissolve 2.25 g NaOH in 200 mL $H_2O$.

### C. Extraction

Accurately weigh amt ground feed (≤15 g) contg 1.5–2.5 mg amprolium and transfer to 250 mL g-s flask. Add 100.0 mL dil. MeOH, stopper, and stir mag. or shake mech. 60 min. Filter thru Whatman No. 42, or equiv., paper and collect 25–40 mL clear filtrate, rejecting first 10–15 mL. Filtrate should be clear. Refilter, if necessary, thru fresh paper or centrf. until clear.

### D. Chromatography

(**a**) *Preparation of alumina.*—To 200 g $Al_2O_3$, (**b**), add 1 L $H_2O$. Stir mixt. 30 min. Filter slurry thru fast paper on buchner funnel. Wash $Al_2O_3$ on filter with three 100 mL portions of anhyd. MeOH. Air-dry under vac. until $Al_2O_3$ reaches room temp. Prepd $Al_2O_3$ should be free-flowing. Store in g-s bottle.

(**b**) *Preparation of column.*—Constrict end of 40 cm length of 9–10 mm id glass tubing by rotating in hot flame until opening is 4–5 mm. Insert small plug of Pyrex glass wool in lower end of tube and compress with glass rod to thickness ca 2–3 mm. Transfer 5.0 g prepd alumina to dry tube and pack by gentle tapping of tube. Prep. sep. column for each sample. *Note:* Column recovery of amprolium may vary among different brands of basic alumina. Test column recovery by spiking ext from nonmedicated feed.

(**c**) *Chromatography of feed extract.*—Pipet 25 mL clear ext onto column and let pass thru column by gravity. Reject first 3 mL eluate and collect next 5 mL for color development.

### E. Determination

Mark 3 sep. 15 mL centrf. tubes as $X$, $S$, and $B$. To $X$ add 4.00 mL clear eluate from column; to $S$ add 4.00 mL amprolium std soln, and to $B$ add 4.00 mL dil. MeOH as blank. Add 10.0 mL color developing reagent to each tube, stopper, mix, and let stand 20 min. Centrf. 2–3 min, decant into 1 cm cells, and cover. (If solns are not clear and free from suspended particles, decant into cells thru small plug of Pyrex glass wool.) Det. $A$ of solns $X$ and $S$ in spectrophtr or colorimeter at 530 nm against soln $B$ as ref. within 20–25 min after adding color developing reagent.

$$\% \text{ Amprolium in feed} = (2.5A \times C)/(A' \times W)$$

where $A$ and $A'$ refer to sample and std, resp., $C$ = mg amprolium in final aliquot of std soln (0.100 mg), and $W$ = g original sample.

Refs.: JAOAC **44,** 5(1961); **62,** 399(1979); **72,** 105(1989).

CAS-121-25-5 (amprolium)

## 965.47 Amprolium in Feeds

**Fluorometric Method**

**First Action 1965**
**Final Action 1967**

(Applicable in absence of antibiotics except procaine penicillin and chlortetracycline)

### A. Reagents

(**a**) *Amprolium std solns.*—(*1*) *Stock soln.*—0.20 mg/mL. Weigh 20.0 mg Amprolium Ref. Std (available from Merck & Co.) and dissolve in enough TCA soln, (**d**), to make 100.0 mL. (*2*) *Working soln.*—1 μg/mL. Dil. 5.00 mL stock soln to 100 mL with TCA soln and mix well. Further dil. 10 mL of this soln to 100 mL with $H_2O$, and mix well.

(**b**) *Potassium ferricyanide soln.*—Dissolve 2 g $K_3Fe(CN)_6$ in 100 mL $H_2O$.

(**c**) *Silver nitrate soln.*—Dissolve 5 g $AgNO_3$ in 100 mL $H_2O$.

(**d**) *Trichloroacetic acid (TCA) soln.*—Dissolve 5 g $CCl_3COOH$ in 100 mL $H_2O$.

### B. Extraction

Grind feed sample to pass No. 20 sieve and mix thoroly. (High-speed blender grinds most feeds to desired fineness in ca 3 min.) Weigh sample contg ca 750 μg amprolium and transfer to 250 mL g-s flask. Add 100.0 mL TCA soln, stopper, and agitate 30 min on mag. stirrer or mech. shaker.

Filter by gravity thru Whatman No. 42 paper, rejecting first 5 mL. Collect ≥10 mL clear filtrate. Transfer 5.00 mL clear ext to 50 mL vol. flask, dil. to vol. with $H_2O$, and mix well. This is dild sample ext.

### C. Development of Fluorophor

Mark three 50 mL centrf. tubes $X$, $Y$, and $Z$. To tube $X$ add 15.00 mL dild sample ext; to tube $Y$ add 1.50 mL TCA soln and 13.50 mL $H_2O$ as blank soln; and to tube $Z$ add 15.00 mL amprolium working std soln. To all tubes add 5.00 mL *NaOH soln* (3 + 10), stopper with polyethylene stoppers, and mix well. Immediately add 0.50 mL $AgNO_3$ soln to all tubes, stopper, and mix well. Let all tubes stand 2 min. Then to all tubes add 3.0 mL $K_3Fe(CN)_6$ soln, stopper, mix, and let stand 3.0 min.

During this 3 min wait, add 15 mL *n*-BuOH to all tubes, as overlay, and stopper. After 3 min, vigorously shake all tubes 1.0 min, and centrf. 1 min. Transfer 10.0 mL aliquots of upper BuOH layer from all tubes to test tubes. Add 1.00 mL absolute alcohol to each tube and mix well.

### D. Measurement of Fluorescence

(*Caution:* See safety notes on photofluorometers.)

(**a**) *For instruments designed to accommodate 10 × 10 mm cells and using monochromatic light for excitation.*—Set activation wavelength at 400 nm (uncorrected) and emission wavelength at 455 nm (uncorrected). Transfer ca 2.0 mL fluorophor BuOH ext to cell and read.

(**b**) *For instruments designed to accommodate 10 × 40 mm cells and using filters to adjust wavelengths for excitation and emission.*—Use Kopp Glass Co. No. C5840 filter placed after light source to adjust excitation wavelength and Kopp No. C3385 filter placed behind cell to adjust emission wavelength. Transfer entire contents of test tube contg extd fluorophor to cell and read.

### E. Calculations

$$\% \text{ Amprolium in feed} = (X - Y) \times C/[150 \times (Z - Y) \times W]$$

where $X$, $Y$, and $Z$ are fluorescence readings of sample, reagent blank, and std, resp.; $C$ = μg in 15 mL std soln (15.0); and $W$ = g sample.

Ref.: JAOAC **48,** 285(1965).

CAS-121-25-5 (amprolium)

## 981.27★ Arprinocid in Feeds

**Liquid Chromatographic Method**

**First Action 1981**
**Surplus 1988**

*See* **42.021–42.026**, 14th ed.

## 982.42★ Arprinocid in Premixes

### Spectrophotometric Method
### First Action
### Surplus 1988

*See* **42.027–42.032**, 14th ed.

## 954.17 Arsanilic Acid in Feeds

### Spectrophotometric Method
### Final Action 1960

(Applicable in absence of sulfonamides)

#### *A. Determination*

Transfer 4.0 g freshly ground sample to 200 mL vol. flask, and add ca 80 mL $H_2O$ and 4 mL 0.5*N* NaOH. Place flask on steam bath ca 5 min, swirling occasionally. Carefully add 20 mL HCl, mix, and cool to room temp. Dil. to vol. with $H_2O$, mix, pour into 250 mL beaker, add some Filter-Cel, or equiv., and filter thru Whatman No. 42, or equiv., paper, discarding first 5 mL.

Pipet 5 mL aliquots of clear filtrate into each of two 20 × 175 mm test tubes. To each tube add 2 mL *0.1% $NaNO_2$ soln*, mix, and let stand 5 min. Add 2 mL *0.5% $NH_4$ sulfamate soln* and let stand 2 min. Then add, to 1 tube only, 1 mL coupling reagent, **969.53A(d)**, mix, and let stand 10 min before dilg both solns to vol. of 15 mL. Mix well (if bubbles appear, filter thru glass wool), and det. *A* against $H_2O$ at 538 nm in spectrophtr or with 540 nm filter in photometer. Subtract *A* of blank from sample *A*. Det. μg arsanilic acid in aliquot (equiv. to 100 mg sample) from std curve.

#### *B. Preparation of Standard Curve*

Transfer 0.100 g pure *arsanilic acid* to 100 mL vol. flask, add ca 20 mL $H_2O$ and 2 mL 0.5*N* NaOH, and dissolve. Dil. to vol. with $H_2O$ and mix well. Transfer 10 mL to 100 mL vol. flask, dil. to vol. with $H_2O$, and mix well. Dil. 5 mL of this soln to 250 mL with $H_2O$ in vol. flask, and mix well (1 mL = 2 μg arsanilic acid). Pipet aliquots of 0, 2, 3, 5, and 8 mL of this std soln into 20 × 175 mm test tubes, add 1 mL HCl (1 + 1) to each tube, and continue as in **954.17A**, beginning "To each tube add 2 mL *0.1% $NaNO_2$* . . ." Subtract blank *A* from *A* of stds and plot differences against 4, 6, 10, and 16 μg arsanilic acid in aliquots.

Refs.: JAOAC **37,** 257(1954); **40,** 452(1957).

CAS-98-50-0 (arsanilic acid)

## 957.22 Arsenic (Total) in Feeds

### Colorimetric Test
### First Action 1957
### Final Action 1960

#### *A. Reagents*

(**a**) *Arsenic trioxide.*—NIST $As_2O_3$ SRM 83, or equiv.

(**b**) *Magnesium oxide-magnesium nitrate slurry.*—Suspend 75 g MgO and 105 g $Mg(NO_3)_2.6H_2O$ in enough $H_2O$ to make 1 L. Agitate vigorously before addn to sample. (Freshly prepd slurry gives ash which is easily disturbed by air currents.)

(**c**) *Stannous chloride soln.*—Dissolve 40 g As-free $SnCl_2$-$H_2O$ in HCl and dil. to 100 mL with HCl. Effective as long as it discharges yellow color in sample ext.

(**d**) *Absorbing soln.*—Transfer with graduate 25 mL 1.5% $HgCl_2$ soln, and with pipet 3.75 mL 6*N* $H_2SO_4$ and 3.75 mL 0.03*N* $KMnO_4$, into 250 mL graduate. Dil. to 250 mL with $H_2O$ and mix. Prep. fresh daily.

(**e**) *Ammonium molybdate reagent.*—Dissolve 1 g $(NH_4)_2MoO_4$ in 100 mL 5.4*N* $H_2SO_4$. Soln keeps several weeks. (Prep. 5.4*N* $H_2SO_4$ by dilg 6*N* (9 + 1).)

(**f**) *Hydrazine sulfate reagent.*—0.15%. Dissolve 0.15 g $N_2H_4.H_2SO_4$ in 100 mL $H_2O$. Soln keeps several weeks.

#### *B. Apparatus*

(Do not clean app. and glassware with detergents, as they interfere with color development. Haemo-Sol, available from Scientific Products, Inc., or equiv., is satisfactory.)

(**a**) *Evaporating dishes.*—70 mL; Coors No. 430, size 00A, or equiv.

(**b**) *Arsine evolution apparatus.*—Bend 6 mm id glass tubing at 120° angle ca 10 cm from one end and at 60° angle ca 15 cm from other end. Plug shorter end with glass wool impregnated with satd $Pb(OAc)_2$ soln and insert in rubber stopper, placed in top of 125 mL erlenmeyer, so that end of tube projects just below stopper. Plug other end with unimpregnated glass wool and connect thru rubber tubing to glass tube, constricted at lower end, that reaches to bottom of 50 mL large neck vol. flask, or if preferred, 50 mL centrf. tube, marked exactly at 50 mL and approx. at 20 mL.

#### *C. Preparation of Sample Solution*

Weigh ground sample contg ≤50 μg As (unless aliquot is to be taken from digested soln) into 70 mL ashing dish. If >2.5 g sample is used, increase amt of slurry and size of ashing dish. Add ca 10 mL well mixed slurry, (**b**), and enough $H_2O$ to permit thoro mixing with stirring rod. Rinse stirring rod, and dry sample at 100°. Ash 2–4 hr at 550–600°. (Slight C residue does not interfere. Use care to avoid loss of ash.)

Cool, and moisten residue with $H_2O$. Cover dish with watch glass and add ca 15 mL HCl (1 + 1). Let stand overnight, or heat on $H_2O$ bath with agitation until ash dissolves. Filter thru Whatman No. 30 paper into 125 mL erlenmeyer. Rinse filter with enough hot $H_2O$, in several portions, to obtain ca 60 mL filtrate.

#### *D. Preparation of Standard Curve*

Dissolve 0.660 g $As_2O_3$ in 25 mL 10% NaOH soln, dil. to 1 L with $H_2O$, and mix. Dil. 10 mL aliquot to 1 L with $H_2O$ (1 mL = 5 μg As). Transfer 0, 2, 4, 6, 8, 10, 12, and 14 mL aliquots from buret into 125 mL erlenmeyers. Dil. each to ca 60 mL with $H_2O$ and proceed as in **957.22E**. Plot *A* against μg As.

#### *E. Arsine Evolution*

Add ca 10 mL HCl, 2 mL KI soln, (15%: keep in dark; discard when soln turns yellow), and 0.5 mL $SnCl_2$ soln, (**c**). Swirl, heat in $H_2O$ bath 5 min, and cool. Have all parts of evolution app. ready for immediate assembly, with ca 20 mL absorbing soln, (**d**), in 50 mL vol. flask or centrf. tube marked at 50 mL. Add 5–6 g Zn, 30 mesh, to digested soln; quickly insert stopper contg glass tubing into erlenmeyer and place delivery tube against bottom of vol. flask or centrf. tube so that bubbles will be small. Use few drops of $H_2O$ to test for leaks between rubber stopper and erlenmeyer. Connecting glass tube must be large enough so bubbles will not carry over Pb compds from impregnated glass wool plug into absorption flask.

#### *F. Color Development*

After 30 min, disconnect rubber tubing, leaving delivery tube in receiving vessel so that any Hg arsenide on tube will be exposed to color-developing reagents. Add 1.0 mL $NH_4$ molybdate reagent, (**e**), and mix by forcing air thru delivery tube. Add 1.0 mL hydrazine sulfate reagent, (**f**), and again mix. Heat in boiling $H_2O$ bath 20 min. Rinse delivery tube with $H_2O$ and remove. Cool to room temp., dil. to 50 mL, and mix. Filter thru tight glass wool plug in funnel or centrf. (Do not use filter paper, as color will be adsorbed.) Read *A* against $H_2O$ at ≥750 nm. Max. *A* is at 840 nm. Det. As content from std curve.

As × 2.90 = arsanilic acid; As × 2.24 = arsenosobenzene; As × 3.51 = 3-nitro-4-hydroxyphenylarsonic acid; As × 3.3 = 4-nitrophenylarsonic acid; As × 3.47 = *p*-ureidobenzenearsonic acid.

Refs.: Ind. Eng. Chem. Anal. Ed. **15,** 408(1943); **24,** 1821(1952). Sandell, "Colorimetric Determination of Traces of Metals," 3rd ed., 1959. JAOAC **40,** 455(1957).

CAS-7440-38-2 (arsenic)

## 960.62★ Bithionol in Feeds

**Spectrophotometric Method**
**First Action 1960**
**Final Action 1961**
**Surplus 1970**

*See* **38.035–38.037**, 11th ed.

## 967.34 Buquinolate in Feeds

**Fluorometric Method**
**First Action 1967**
**Final Action 1972**

### A. Principle

Buquinolate is extd from feed with $CHCl_3$, concd to small vol., and sepd from interfering substances by TLC utilizing 2 solv. systems. Buquinolate is eluted from substrate and detd fluorometrically.

### B. Reagents

(**a**) *Alcohol, 80%.*—Dil. 84.3 mL alcohol to 100 mL with $H_2O$.

(**b**) *Developing solvent.*—Mix $CHCl_3$ with alcohol (10 + 1). Prep. fresh daily.

(**c**) *Buquinolate std solns.*—(*1*) *Stock soln.*—0.5 mg/mL. Dissolve 50.0 mg Buquinolate Ref. Std (available from Norwich Eaton Pharmaceuticals, Inc., 17 Eaton Ave, Norwich, NY 13815) in $CHCl_3$ to make 100 mL. Warm mixt. on steam bath as necessary. Soln is stable 1 month if protected from evapn. (*2*) *Working soln.*—100 μg/mL. Pipet 5 mL stock soln into 25 mL vol. flask, dil. to vol. with $CHCl_3$, and mix well. Prep. fresh daily.

### C. Apparatus

(**a**) *Developing tanks.*—Line developing tanks (for plates ≤20 × 20 cm) with Whatman 3 MM paper. Add 100 mL $CHCl_3$ to one tank; add 100 mL developing solv., (**b**), to second tank. Prep. each tank fresh daily.

(**b**) *Plates for TLC.*—Clean plates thoroly with alkyl benzene sulfonate-type detergent (Ajax, or equiv.) and brush; rinse plates with $H_2O$ and then with acetone. Let plates air dry. Slurry 60 g silica gel G (Brinkmann No. 68-00-261-3) with 120 mL $H_2O$. Pour into suitable applicator and spread 0.500 mm layer on 20 × 20 cm plates. Air dry 15–30 min; then dry 2 hr at 110°. Cool and store plates in desiccator until used.

(**c**) *Fluorometer.*—Either spectrophotofluorometer or filter fluorometer may be used. (Suitable filters are: excitation, PTR Optics type UV-7E (UV Spectrum Filter, No. 59-07-9, PTR Optics Corp., 145 Newton St, Waltham MA 02154); emission, Kopp Glass Co. filters C7380 and C5840.) (*Caution*: See safety notes on photofluorometers.)

### D. Determination

(*Caution:* See safety notes on hazardous radiations.)

Grind ca 100 g sample to pass No. 30 sieve and mix thoroly. Accurately weigh sample contg 1.25 mg buquinolate into 250 mL g-s erlenmeyer. Pipet 100 mL $CHCl_3$ into sample flask. Shake mech. 1 hr. Filter ext thru Whatman No. 54 paper on buchner with mild vac. (Take care to prevent solv. loss by evapn.) Transfer exactly 80 mL ext to 150 mL beaker and evap. almost to dryness on steam bath. Take up residue in small portion $CHCl_3$ and transfer to 10 mL vol. flask with small portions $CHCl_3$. Dil. to vol. with $CHCl_3$ and mix well.

Apply 250 μL sample ext and 250 μL working std soln to TLC plate. Place spots ca 25 mm from bottom of plate and 40 mm apart. (Do not touch pipet to plate.) Develop plate in $CHCl_3$ developing tank, (**a**), until solv. front nearly reaches top of plate (ca 1 hr). Observe plate under short wavelength UV light: Buquinolate remains at origin; feed background migrates. Transfer air-dried (5–10 min) plate to tank contg developing solv., (**b**). Let plate develop until solv. front advances 12 cm. Air dry 5–10 min. Examine plates under short wavelength UV light. Buquinolate migrates from origin ($R_f$, 0.4–0.6). With spatula, outline each buquinolate spot plus blank spot of equiv. area and $R_f$. Remove adsorbent from around buquinolate spots and discard. Quant. transfer each spot to sep. g-s 25 mL erlenmeyers. Pipet 10 mL 80% alcohol, (**a**), into each flask, shake mech. 20 min, and centrf.

Det. intensity of fluorescent radiation (*I*) of sample, std, and blank in 10 × 10 mm silica cells, at excitation and emission wavelengths of 265 and 375 nm, resp.

$$\% \text{ Buquinolate} = [(I_{\text{sample}} - I_{\text{blank}})/(I_{\text{std}} - I_{\text{blank}})] \times (0.125/\text{g sample})$$

Ref.: JAOAC **50,** 264(1967).

CAS-5486-03-3 (buquinolate)

## 963.33★ Cadmium Anthranilate in Feeds

**Spectrophotometric Method**
**First Action 1963**
**Final Action 1964**
**Surplus 1974**

*See* **42.046–42.047**, 12th ed.

## 977.35 Carbadox in Feeds

**Spectrophotometric Method**
**Final Action 1981**

(Applicable to levels ≥0.0055%. Carbadox solns are light sensitive. Exts must be protected from direct sunlight or artificial light.)

### A. Apparatus

(**a**) *Filter aid.*—Celite 545 (Manville Filtration and Minerals) or Millipore prefilter pad (No. AP2504700, Millipore Corp., Ashby Rd, Bedford, MA 01730), or equiv.

(**b**) *Spectrophotometer.*—For use at 520 nm.

### B. Reagents

(**a**) *Carbadox std solns.*—(*1*) *Stock soln.*—1.10 mg/mL. Weigh 110.0 mg Carbadox Ref. Std (available from Pfizer, Inc., Quality Control, Agricultural Div., 1107 S Rt 291, Lee's Summit, MO 64048) into 100 mL vol. flask, dissolve in $CHCl_3$-MeOH (3 + 1), and dil. to vol. with same solv. Ultrasonic bath speeds dissoln. Prep. fresh daily. (*2*) *Working soln.*—0.110 mg/mL. Pipet 10 mL stock soln into 100 mL vol. flask, dil. to vol. with $CHCl_3$-MeOH (3 + 1), and mix well. Prep. fresh daily.

(**b**) *Methanolic hydrochloric acid soln.*—1*N*. Dil. 85 mL HCl to 1 L with MeOH.

(**c**) *Methanolic sodium hydroxide soln.*—0.05*N*. Dissolve 2.0 g NaOH in MeOH and dil. to 1 L with MeOH. Prep. fresh weekly or sooner if ppt forms.

(**d**) *Potassium phosphate soln.*—1*M*. Dissolve 136 g $KH_2PO_4$ in $H_2O$ and dil. to 1 L.

(**e**) *Sodium hydroxide-sodium chloride soln.*—Dissolve 100 g NaCl in 0.1*N* NaOH and dil. to 1 L with 0.1*N* NaOH.

(**f**) *Stannous chloride soln.*—Prep. immediately before use. Add 8.0 g $SnCl_2.2H_2O$ to 100 mL methanolic 1*N* HCl. Place in 55–60° $H_2O$ bath and swirl intermittently until soln is clear (ca 20 min). Stopper and cool to room temp. Use within 2 hr.

### C. Preparation of Samples

(*Caution:* See safety notes on chloroform and methanol.)

Weigh duplicate portions ground feed into 250 mL erlenmeyers: 2.000 g for 0.0330–0.0606% carbadox; 5.000 g, 0.0110–0.0330%; and 20.00 g, 0.0055–0.0110%. Wet each portion with 10 mL $H_2O$, let stand 5 min, and add 140 mL $CHCl_3$-MeOH (3 + 1). Add 15.0 mL working std soln to 1 portion. Stopper both flasks loosely or with polyethylene stopper with pinhole, and boil gently 1 hr. Cool to room temp.

Using three 25 mL portions $CHCl_3$-MeOH (3 + 1), quant. transfer mixt. to buchner precoated with Celite or contg prefilter pad, collecting filtrate under vac. in 250 mL vol. flask. Dil. to vol. with $CHCl_3$-MeOH (3 + 1), and mix well. Pipet 100 mL aliquot into 250 mL separator contg 50 mL NaOH-NaCl soln. Shake 10 sec and discard lower $CHCl_3$ layer. Add 50 mL $CHCl_3$, shake 10 sec, and discard $CHCl_3$ layer. Add 10 mL $KH_2PO_4$ soln, and ext with three 50 mL portions $CHCl_3$, combining exts in r-b flask. Do not let any solids at interface drain into flask. Evap. to dryness, using rotary evaporator and 60° $H_2O$ bath.

Conduct reagent blank of $H_2O$ and $CHCl_3$-MeOH (3 + 1) thru boiling, filtration, extns, and evapn, omitting addn of feed and carbadox.

Alternatively, weigh samples as above and ext as follows: Wet each portion with 10 mL $H_2O$, let stand 5 min, and add 140 mL $CHCl_3$-MeOH (3 + 1) to one flask. Prep. spiked sample by adding 130 mL $CHCl_3$-MeOH (3 + 1) and 10 mL carbadox working std soln to other flask. Break up any clumps with spatula. Stopper tightly and let stand overnight in dark at room temp. Using three 25 mL portions of $CHCl_3$-MeOH (3 + 1) quant. transfer mixt. to buchner precoated with Celite or contg prefilter pad, collecting filtrate under vac. in 250 mL vol. flask for unspiked sample and in 500 mL vol. flask for spiked sample. Proceed as above, beginning "Dil. to vol. with $CHCl_3$-MeOH . . ."

### D. Determination

Dissolve residue in flask from sample, sample plus std, and blank in 5.00 mL 0.05*N* methanolic NaOH. Add 20.0 mL $SnCl_2$ soln, swirl gently, and let stand 10 min for complete color development. If necessary, clarify soln by filtration thru small glass wool plug. If alternative overnight leach was used, clarify soln by centrifg 10 min at 100 rpm. Within 15 min after completion of color development, det. *A* of clear solns at 520 nm against MeOH as ref. solv. Subtract *A* of blank from *A* of sample and *A'* of sample plus std.

$$\% \text{ Carbadox} = (A/\text{g sample}) \times [1/(A' - A)] \times (\text{mg carbadox/mL working std soln}) \times (1 \text{ g}/1000 \text{ mg}) \times 15 \text{ mL aliquot} \times 100$$

When alternative overnight leach was used, change calcn to:

$$\% \text{ Carbadox} = (A/\text{g sample}) \times [1/(2A' - A)] \times (\text{mg carbadox/mL working std soln}) \times (1 \text{ g}/1000 \text{ mg}) \times 10 \text{ mL aliquot} \times 100$$

Refs.: JAOAC **60,** 1059(1977); **62,** 982(1979).

CAS-6804-07-5 (carbadox)

## 969.55 Decoquinate in Feeds

**Fluorometric Method**

**First Action 1969**
**Final Action 1972**

### A. Principle

Decoquinate is extd from feed with 1% $CaCl_2$-MeOH soln. After addn of $H_2O$ and acid, drug is extd into $CHCl_3$, then sepd from interfering materials by chromatgy on Florisil. Decoquinate is eluted from column with 1% $CaCl_2$-MeOH and detd by fluorometry against std treated similarly.

### B. Reagents

(**a**) *Calcium chloride-methanol soln.*—1%. Dissolve 10 g anhyd. $CaCl_2$, reagent grade, in 1 L MeOH, spectral grade (EM Science OmniSolv No. MX0488), or equiv. redistd, reagent grade, anhyd. MeOH. Filter thru Whatman No. 2 paper.

(**b**) *Decoquinate std solns.*—(*1*) *Stock soln.*—300 μg/mL. Weigh 30 mg Decoquinate Ref. Std (available from Hess & Clark Inc.). Dissolve and dil. to 100 mL with 1% $CaCl_2$-MeOH soln. Prep. fresh monthly. (*2*) *Working soln.*—6 μg/mL. Pipet 5 mL stock soln into 250 mL vol. flask and dil. to vol. with 1% $CaCl_2$-MeOH soln. Check *A* of this soln in 1 cm quartz cells at 265 nm against spectral grade MeOH (ca 0.660). Prep. fresh std when *A* is outside range 0.620–0.700. Soln is stable ≥1 week. (*3*) *Fluorescence reference soln.*—1.5 μg/mL. Pipet 25 mL working std soln into 100 mL vol. flask and dil. to vol. with spectral grade MeOH. Check *A* at 265 nm as above. Prep. fresh std when *A* is outside range 0.150–0.190.

(**c**) *Florisil.*—100–200 mesh (Fisher No. F-101).

### C. Apparatus

(**a**) *Chromatographic columns.*—Draw 30 cm length of 9 mm tubing (7 mm id) to drip tip. Insert small glass wool plug to support adsorbent. Close drip end with short piece of tubing and pinch clamp. Add 5 mL $CHCl_3$ to column, then 0.5 ± 0.01 g Florisil. Add 2 mL addnl $CHCl_3$ and stir with thin glass rod to settle adsorbent. Remove tubing and wash down sides of tube with $CHCl_3$. Prep. just before use.

(**b**) *Separators.*—125 or 250 mL with Teflon stopcocks.

(**c**) *Fluorometer.*—(*Caution:* See safety notes on photofluorometers.) Either spectrofluorometer or filter fluorometer may be used. Excitation filter: UV-2 (UV Spectrum Filters No. 14-16-8, 325 nm); emission filter: S/UV (UV Spectrum Filters No. 14-01-4, 390 nm) (PTR Optics Corp., 145 Newton St, Waltham, MA 02154).

### D. Determination

Weigh 10 g sample into 125 mL erlenmeyer, add exactly 50 mL 1% $CaCl_2$-MeOH soln, stopper, and shake mech. 20

min. Decant soln into centrf. tube and centrf. 5 min at moderate speed. Pipet 10 mL clear supernate into 125 mL separator. Prep. std by pipetting 10 mL working std soln into another separator. Add exactly 10 mL $CHCl_3$ to each funnel by pipet and swirl to mix. Add 100 mL dil. HCl (1 + 19) to each funnel. Shake gently by inverting 25 times; then allow 15 min for phases to sep. Drain $CHCl_3$ layer into centrf. tube and centrf. 5 min. Remove by aspiration any droplets of floating $H_2O$ phase that seps.

Pipet 5 mL $CHCl_3$ sample soln onto Florisil column. Pipet 5 mL $CHCl_3$ std soln onto another column. Pipet 5 mL $CHCl_3$ onto third column (reagent blank). Pass two 10 mL portions anhyd. MeOH thru each column. Let MeOH drain to surface of Florisil and discard column effluent. Elute with 15 mL 1% $CaCl_2$-MeOH soln, collecting in tube marked at 15 mL. Mix well, centrf. if not clear, and transfer to fluorometer cells. Set activation wavelength of fluorometer at 325 nm and emission wavelength at 390 nm. Set fluorometer sensitivity with fluorescence ref. std to give convenient scale reading (e.g., 100). Det. fluorescence of samples, std, and reagent blank. Subtract reagent blank correction, if any, from reading of std and samples.

% Decoquinate in feed = (0.003 × corrected fluorescence of sample)/corrected fluorescence of std.

Ref.: JAOAC **51,** 1279(1968).

CAS-18507-89-6 (decoquinate)

## 977.36 Dibutyltin Dilaurate in Feeds

### Atomic Absorption Spectrophotometric Method

**First Action 1977**
**Final Action 1979**

#### A. Principle

DBTD is extd from feed with $CHCl_3$, ext is filtered to remove feed particles, and aliquot is concd in presence of MeOH until $CHCl_3$ is removed. MeOH soln is dild and filtered to remove feed interference, and Sn is detd by AA using air-$C_2H_2$ flame.

#### B. Apparatus and Reagents

(**a**) *Atomic absorption spectrophotometer.*—Double beam, operated at 286.3 nm with air-$C_2H_2$ flame and direct readout using 10 mv recorder. Optimize instrument according to manufacturer's instructions.

(**b**) *Hot plate.*—Regulated to ±3°.

(**c**) *Mechanical shaker.*—Wrist-action type (Burrell Corp., or equiv.).

(**d**) *Tin std solns.*—(*1*) *Stock soln.*—500 μg/mL. Accurately weigh ca 0.217 g dibutylin bis(2-ethylhexanoate) (NIST SRM No. 1057 or Eastman Kodak No. 10427, % Sn certified) into 100 mL vol. flask, and dissolve and dil. to vol. with MeOH. (*2*) *Working soln.*—10 μg/mL. Pipet 2 mL stock soln into 100 mL vol. flask, add 1.0 mL HCl, and dil. to vol. with MeOH.

#### C. Preparation of Sample and Extraction

Grind sample in high-speed blender to pass No. 20 sieve (ca 3 min), and mix thoroly. Accurately weigh aliquot contg ca 10 μg Sn/mL in final soln (*see* Table **977.36**), and transfer to 125 mL erlenmeyer. Add 50 mL $CHCl_3$, mix, and place flask in 55–60° $H_2O$ bath. Let sample reach bath temp.; then stopper tightly. Continue heating addnl 30 min, swirling occasionally. Remove from $H_2O$ bath and shake mech. 20 min. Filter thru Whatman No. 4 paper, and collect ≥30 mL filtrate in 50 mL erlenmeyer.

**Table 977.36 Sample Weights for DBTD-Containing Feeds**

| %DBTD | Feed Sample, g |
|---|---|
| 0.020 (Polystat) | 13.00 |
| 0.0375 (Tinostat) | 7.00 |
| 0.0700 (Wormal) | 3.75 |
| 0.1400 (Wormal) | 2.00 |

Pipet 25 mL filtrate into 100 mL graduated beaker, add 2 boiling chips and 0.25 mL HCl, and conc. to ca 10 mL at gentle boil on hot plate. Add 20 mL MeOH and conc. sample to ca 10 mL again; then repeat with addnl 20 mL and 30 mL portions MeOH. (Raise temp. of hot plate to maintain gentle boiling as ratio of MeOH to $CHCl_3$ increases.) Remove from heat and let cool to room temp. Transfer MeOH soln to 25 mL vol. flask, washing beaker and funnel with 2–3 five mL portions MeOH, dil. to vol. with MeOH, and mix thoroly. Filter thru Whatman No. 42 paper and collect filtrate in another 25 mL vol. flask.

Prep. blank by dilg 1 mL HCl to 100 mL with MeOH.

#### D. Determination

(*Caution:* See safety notes on AAS.)

Let spectrophtr warm up thoroly and equilibrate by aspirating MeOH 15 min, using air-$C_2H_2$ flame and triple slot burner head. Zero spectrophtr by aspirating blank; then aspirate sample and std solns, using conditions given in (**a**). Repeat sequence for each sample.

$$\% \text{ DBTD} = A \times C \times 50 \times 5.32 \times 10^{-6} \times (100/A') \times W = (A/A') \times (0.266/W)$$

where $A$ and $A'$ refer to sample and std, resp.; $C$ = g std/mL; and $W$ = g sample.

Ref.: JAOAC **60,** 1054(1977).

CAS-77-58-7 (dibutyltin dilaurate)

## 956.10★ Diethylstilbestrol in Feeds

### Spectrophotometric Method

**First Action**
**Surplus 1988**

*See* **42.059–42.062**, 14th ed.

## 970.85 Dimetridazole in Feeds

### Spectrophotometric Method

**First Action 1970**
**Final Action 1988**

#### A. Principle

Dimetridazole is extd from feeds with MeOH, sepd from interfering substances by two alumina chromatgc steps, and detd spectrophtric at its UV wavelength max. Nihydrazone, furazolidone, zoalene, 2-chloro-4-nitrobenzamide, tylosin, and large amts procaine (from procaine penicillin) interfere.

#### B. Apparatus and Reagents

(**a**) *Spectrophotometer.*—For use in UV.

(**b**) *Chromatographic tubes.*—13 × 150 mm and 15 × 250 mm, constricted at bottom to hold glass wool plug and 6 mm od delivery tube.

(**c**) *Aluminum oxide.*—Suitable for chromatgy, **961.24B(b)**. To det. suitability of alumina, perform detn on feed that does not contain dimetridazole or other imidazole drugs. If feed appears to contain >0.004% dimetridazole, use another batch of alumina.

**(d)** *1,2-Dimethyl-5-nitroimidazole (dimetridazole) std solns.*—*(1) Stock soln.*—0.1 mg/mL. Weigh 100 mg dimetridazole std into 100 mL vol. flask. Dissolve in $H_2O$ by shaking frequently ca 20 min. Dil. to vol. with $H_2O$ and mix. Pipet 20 mL into 200 mL vol. flask, dil. to vol. with $H_2O$, and mix. *(2) Working solns.*—Pipet 5, 10, 20, 30, and 40 mL stock soln into sep. 100 mL vol. flasks. Add 5.0 mL 3*N* HCl to each, immediately dil. to vol. with $H_2O$, and mix. Pipet 5 mL each soln and 5 mL 0.10*N* NaOH into sep. 50 mL erlenmeyers. Stopper and mix. These solns contain 2.5, 5, 10, 15, and 20 μg dimetridazole/mL.

### C. Preparation of Standard Curve

Proceed as in **970.85F**, using working std solns and blank prepd by mixing 5 mL 0.15*N* HCl with 5 mL 0.10*N* NaOH.

Read *A* against blank for recording or manual spectrophtrs. Construct std curve by plotting *A* against μg dimetridazole/mL.

### D. Preparation of Sample

Weigh portion finely ground feed contg 0.5–2.0 mg dimetridazole (usually 5 g) into 100 mL vol. flask. Add 70–75 mL MeOH and place in 60° const temp. bath 30 min. Make certain that $H_2O$ level covers flask to ca 3 mm below MeOH level. Swirl flask 2 or 3 times during first 5 min to heat evenly. Cool to room temp., dil. to vol. with MeOH, and mix. Let stand 5–10 min to let coarse feed particles settle.

### E. Chromatography

Place small glass wool plug in bottom of 250 × 15 mm chromatgc tube and add 8 cm layer alumina; pack column tightly to prevent streaking. (If streaks enter effluent, pos. bias is introduced.) Decant methanolic ext onto column so that settled feed particles are not disturbed. Collect ca 30 mL eluate in 50 mL vol. flask. Stopper until ready for use.

(*Note:* Dimetridazole sublimes at temps >70°; manner of solv. removal is critical.) For feed contg 0.015% dimetridazole, pipet 15 mL effluent (4 mL if feed contains 0.06%; 3 mL if feed contains 0.10%) into 125 mL suction or r-b flask and evap. under reduced pressure from $H_2O$ aspirator. If 15 mL is taken, use hot plate (low heat) or $H_2O$-bath to reduce to 3–4 mL. Shake to prevent bumping. When vol. approaches 3–4 mL remove flask from heat and remove last 3–4 mL only with heat from palm of hand. Continue shaking to prevent bumping. Do not attempt to attain complete dryness because part of the 2–3 drops of oily residue is dimetridazole.

Wash down walls of flask, beginning at base of neck, with 5.0 mL 0.10*N* NaOH. Swirl to wash walls. Let stand 5 min and add 5.0 mL 0.15*N* HCl. Swirl to mix and wash flask walls. Stopper until ready for chromatgy.

### F. Determination

Prep. second alumina column by inserting small glass wool plug into bottom of 150 × 13 mm chromatgc tube, add 4 cm layer alumina, and tap gently to pack column lightly. Pour entire 10 mL soln onto column and let pass thru by gravity. Collect effluent in 50 mL erlenmeyer. Force out liq. adhering to column by applying air pressure with rubber bulb. Swirl flask to mix. Stopper until ready to read. Pass blank soln of 5 mL 0.15*N* HCl and 5 mL 0.10*N* NaOH thru sep. 4 cm alumina column as above.

**(a)** *Using recording spectrophotometer.*—Fill matched pair silica cells with reagent blank and with sample soln (always use same cell for blank) and scan from 330 to 310 nm. Read *A* at peak and obtain concn of soln in μg/mL from std curve.

$$\% \text{ Dimetridazole} = [(\mu g/mL \text{ from std curve}) \times \text{diln factor} \times 100]/(\text{g sample} \times 10^6)$$

where diln factor = 66.67 for feeds contg 0.015%; 250.0, 0.06%; and 333.3, 0.1%.

**(b)** *Using manual spectrophotometer.*—Locate peak *A* of sample soln (ca 318 nm), using matched pair silica cells, and set wavelength at peak. Read *A* of sample and blank solns and correct sample for blank. Obtain concn of soln in μg/mL from std curve, and calc. % in feed as above.

Refs.: JAOAC **48**, 301(1965); **53**, 646(1970).

CAS-551-92-8 (dimetridazole)

## 964.29 Ethopabate in Feeds

**Colorimetric Method**

**First Action 1964**
**Final Action 1967**

### A. Principle

Ethopabate is extd from feed by 50% MeOH at room temp. Clear filtrate is acidified with dil. HCl and extd with $CHCl_3$. Most interfering substances (amines, *p*-aminobenzoic acid, procaine) are sepd. $CHCl_3$ ext is washed with $Na_2CO_3$ soln to remove sulfaquinoxaline, acetyl-(*p*-nitrophenyl) sulfanilamide, and chlortetracycline. Ethopabate is converted to free amine by controlled acid hydrolysis. Free amine is diazotized and coupled; colored complex is extd with *n*-BuOH and read at 550 nm.

### B. Reagents

**(a)** *Dilute hydrochloric acid.*—0.3*N*. Dil. 25 mL HCl with $H_2O$ to 1 L.

**(b)** *Sodium carbonate soln.*—Dissolve 40 g anhyd. $Na_2CO_3$ in $H_2O$ and dil. to 1 L.

**(c)** *Coupling reagent (NED).*—Dissolve 50 mg *N*-(1-naphthyl)ethylenediamine.2HCl in 25 mL $H_2O$. Prep. fresh as needed.

**(d)** *Ethopabate std solns.*—*(1) Stock soln.*—0.400 mg/mL. Weigh 40.0 mg Ethopabate Ref. Std (available from Merck & Co.) into 100 mL vol. flask, dissolve in MeOH, and dil. to vol. *(2) Intermediate soln.*—40 μg/mL. Pipet 10 mL stock soln into 100 mL vol. flask, dil. to vol. with aq. MeOH (1 + 1), and mix well. Stored in tightly stoppered flasks, solns are stable ≥1 month. *(3) Working soln.*—16.0 μg/20.0 mL. Pipet 5 mL intermediate soln into 250 mL vol. flask, dil. to vol. with aq. MeOH (1 + 1), and mix well.

### C. Extraction

Grind feed sample to pass No. 20 sieve and mix thoroly. (High-speed blender grinds most feeds to desired fineness in ca 3 min.) Accurately weigh sample contg ca 80 μg ethopabate (do not exceed 20 g). Transfer to 250 mL g-s flask. Add 100.0 mL aq. MeOH (1 + 1) and mag. stirrer bar, stopper tightly, and stir 1 hr. (Mech. shaker that provides vigorous agitation may be used.) Centrf., or filter portion of ext thru fast paper. Collect only enough filtrate to supply aliquot for test. If necessary, store exts overnight at room temp. in tightly stoppered flasks.

### D. Removal of Interferences

Pipet 20 mL clear ext into 50 mL centrf. tube. Add 5.0 mL dil. HCl (1 + 9) and 10 mL $CHCl_3$, stopper with polyethylene stopper, and shake vigorously 3 min on mech. shaker. Centrf., and carefully transfer bottom $CHCl_3$ layer into clean 50 mL centrf. tube, using syringe with long needle. Repeat extn with two more 10 mL portions $CHCl_3$. Add 10 mL $Na_2CO_3$ soln to combined $CHCl_3$ exts, stopper, and shake 3 min. Centrf., and without disturbing interface, draw off most of top aq. layer, using syringe, and discard. Repeat washing with another 10 mL $Na_2CO_3$ soln, discarding washing. Add 10 mL $H_2O$ to $CHCl_3$ ext, stopper, shake vigorously ca 1 min, and centrf. Draw off aq. layer and discard. Repeat with another 10 mL $H_2O$. (To avoid loss of drug and low results, do not disturb interface on $CHCl_3$, and complete extn and washings in shortest time possible. Prolonged contact with HCl and $Na_2CO_3$ may cause partial hydrolysis of ethopabate.)

Pipet 20 mL aq. MeOH (1 + 1) into 50 mL centrf. tube, add 5.0 mL dil. HCl (1 + 9), and proceed as for sample (reagent blank).

Pipet 20 mL ethopabate working std soln (16.0 μg) into 50 mL centrf. tube, add 5.0 mL dil. HCl (1 + 9), and proceed as for sample (std).

### *E. Conversion of Ethopabate to Free Amine*

Quant. transfer washed $CHCl_3$ exts to sep. 100 mL beakers. Rinse each centrf. tube with two 3 mL portions aq. MeOH (1 + 1), adding rinsings to beaker. Place beaker on steam bath and evap. $CHCl_3$ to vol. of ca 2 mL. Add 5.0 mL aq. MeOH (1 + 1) and swirl beaker to dissolve completely.

Quant. transfer soln to r-b centrf. tube. Rinse beaker with 10, 10, and 5 mL portions 0.3*N* HCl. Immerse tube in boiling $H_2O$ bath so that level of liq. in tube is just below level of $H_2O$ bath. Heat 45 min. Remove tube from hot $H_2O$ bath and cool to 10–15° in cold $H_2O$ bath.

### *F. Development and Measurement of Color*

Remove tubes from cold $H_2O$ bath. Add 1.0 mL *freshly prepd 0.2% $NaNO_2$ soln* to each tube, mix, and let stand 2 min. Add 1.0 mL 1.0% $NH_4$ sulfamate soln, mix, and let stand 2 min. Add 1.0 mL NED soln, mix, and let stand 10 min. Add 5.0 g NaCl and 5.00 mL *n*-BuOH, stopper, and shake vigorously until NaCl dissolves. Centrf., carefully transfer portion of clear, colored alc. layer to 1 cm cell, and read *A* at 555 nm against *n*-BuOH. Correct for reagent blank.

$$\%\ \text{Ethopabate in feed} = 0.008 \times (A - A_B)/[(A' - A_B) \times W]$$

where $A$, $A_B$, and $A'$ refer to sample, reagent blank, and std, resp., and $W$ = g original sample.

Refs.: JAOAC **47,** 221(1964); **48,** 280(1965).

CAS-59-06-3 (ethopabate)

## 985.51 Furazolidone in Feeds and Premixes

**Liquid Chromatographic Method**

**First Action 1985**

**Final Action 1988**

(Applicable to premixes contg 2–22% furazolidone and to feeds contg 0.005–0.05% furazolidone. *Note:* Furazolidone solns are light-sensitive. Protect exts and stds from direct sun and artificial light.)

(*Caution:* See safety notes on centrifuges, distillation, pipets, acetonitrile, acetone, and dimethylformamide.)

### *A. Principle*

Unground premix is extd with DMF, and concn of ext is adjusted with 5% tetraethylammonium bromide (TEAB) to ca 55 μg furazolidone/mL for LC. Complete feed is extd with $H_2O$–acetone on continuous extn app., solv. is evapd, and residue is dissolved in DMF. 5% TEAB is added to sep. fat. Ext is cooled and clarified for LC.

### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Instrument capable of maintaining const pulseless flow of mobile phase at 0.5–1.5 mL/min. Operating conditions: flow rate 1.5 mL/min; loop injection vol. 20 μL; detector sensitivity 0.32 AUFS or adjusted to produce working std peak response 60–80% full scale; detector wavelength 365 nm (settings from 390 to 405 nm may be used to improve selectivity for very low level samples if chromatgc column does not adequately resolve interfering peaks).

(**b**) *Chromatographic column.*—Any reverse phase column, $C_{18}$ or $C_8$, with particle size $\leq$10 μm that will produce single, sharp furazolidone peak with peak skew <1.4. Guard column may be used.

(**c**) *Continuous extraction apparatus.*—Goldfisch (Labconco Corp., 8811 Prospect Ave, Kansas City, MO 64132, No. 3001), or equiv.

(**d**) *Extraction thimbles.*—Whatman, single thickness, 19 × 90 mm (Scientific Products, Inc., No. E6480-4).

(**e**) *Sample clarification filter.*—13 mm glass fiber pre-filter pads (Gelman No. 66073, available from Fisher Scientific Co., No. 09-731A) inserted in 0.5 in. id 5 mL syringe barrel (Pharmseal, Scientific Products, No. S9504-5) or any filtration device designed for clarification of aq. chromatgc samples.

### *C. Reagents*

(**a**) *Extractants.*—DMF, reagent grade, for premixes. Acetone (reagent grade)–$H_2O$ (93 + 7) for complete feeds.

(**b**) *Diluent.*—5% tetraethylammonium bromide (5% TEAB, w/v) (Eastman Kodak Co., No. 1516) in distd, deionized $H_2O$. Keep in refrigerator. For correct final concns, warm to room temp. before pipetting.

(**c**) *Furazolidone std solns.*—(*1*) *Stock soln.*—Ca 1.1 mg/mL. Accurately weigh 0.110 ± 0.005 g furazolidone std (Hess and Clark, Inc., 7th & Orange Sts, Ashland, OH 44805) and record exact wt to nearest 0.1 mg ($W_s$). Transfer into 100 mL vol. flask, dissolve and dil. to vol. with DMF (sonication aids dissolution). Soln is stable if stored in dark. (*2*) *Intermediate soln.*—Ca 110 μg/mL. Dil. 10.0 mL stock soln to 100 mL with DMF. Soln is stable if stored in dark. (*3*) *Working std soln.*—Ca 55 μg/mL. Mix 10.0 mL intermediate soln with equal vol. 5% TEAB. (Mix equal vols. Do not dil. to vol.) Let soln cool to room temp. Prep. daily. Diln of std ($D_s$) = 2000.

(**d**) *Mobile phase.*—$CH_3CN$ (LC quality)–2% HOAc in distd, deionized $H_2O$ (20 + 80), or as adjusted to give capacity factor ($k$) of ca 2.5 for furazolidone.

### *D. Extraction*

(**a**) *Complete feeds.*—Det. approx. sample wt to contain ca 550 μg furazolidone by using formula, sample wt, g = 0.555/% guarantee. Accurately weigh (to nearest 0.01 g) calcd wt of ground, mixed sample (±5%) into extn thimble ($W_u$ = actual sample wt). Press cotton plug down onto top of feed to prevent channeling. Add 45–50 mL acetone–$H_2O$ (93 + 7) extractant and 2 or 3 boiling chips to extn beaker. Ext 8–18 h on extn app. at "Hi" setting. Evap. solv. on steam bath (stream of air directed into beakers or blowing across beakers, as with partially closed hood door, hastens evapn). If any $H_2O$ is evident in beaker after initial evapn, add ca 25 mL acetone, swirl to mix, and re-evaporate (repeat as necessary to remove all $H_2O$). Remove from steam bath as soon as evapn is complete. Add 5.00 mL DMF, heat on steam bath just until bottom of beaker is hot (15–30 s) and swirl, washing sides, to dissolve residue (generally all residue dissolves in warm DMF). Add 5.00 mL 5% TEAB, mix, pour soln into 15 mL centrifuge tube, and let cool. Centrifuge 5–10 min at 2000 rpm (2500 × *g*). Using disposable pipet attached to aspirator and trap, re-

**Table 985.51 Sample Sizes, Dilutions, and Total Sample Dilutions for Assay of Furazolidone in Feeds**

| Label Claim, % | Sample Wt, g | Dilns with DMF, mL | Total Sample Diln ($D_u$), mL |
|---|---|---|---|
| 2.2 | 1.00 | none | 400 |
| 3.3 | 1.00 | 30/50 | 666.7 |
| 11.0 | 1.00 | 20/100 | 2000 |
| 22.0 | 1.00 | 10/100 | 4000 |

move fat layer floating on supernate. Diln for complete feed samples ($D_u$) = 10.

(**b**) *Premixes*.—Accurately weigh (±5%) amt unground sample indicated in Table **985.51** into 500 mL g-s erlenmeyer ($W_u$ = actual sample wt). Add by pipet 200.0 mL DMF, stopper, and shake flask 30 min. Either let suspended material settle or centrf. or filter portion of ext. Dil. with DMF to ca 110 μg furazolidone/mL. To 5.00 mL 5% TEAB soln add 5.00 mL dild ext, mix, and let soln cool to room temp. Clarify as for complete feeds, (**a**). (Total sample diln = $D_u$, Table **985.51**.)

### *E. Determination*

(**a**) *Complete feeds*.—Make several injections of furazolidone working std soln, adjusting mobile phase strength to give $k$ ca 2.5 and peak ht 60–80% full scale ($k = (t_1 - t_0)/t_0$, where $t_0$ = distance from injection to first perturbation of std chromatogram). Make 2 or more injections of std to ensure 1–2% repeatability of peak responses. Bracket each 2 sample injections by std injections. Use av. peak ht $P_s$ (or av. peak area) of stds bracketing each pair of samples to calc. furazolidone concn in samples (sample peak response = $P_u$). If no drift in std peak hts is evident thruout run, then use av. for all std injections in calcns.

(**b**) *Premixes*.—Det. furazolidone as for complete feeds (**a**). Diln ($D_u$) is shown in Table **985.51**.

### *F. Calculation*

% Furazolidone

$$= (P_u \times W_s \times D_u \times 100)/(P_s \times W_u \times D_s)$$

where $P_u$ and $P_s$ = peak response of sample (unknown) and std, resp.; $W_u$ and $W_s$ = g sample and std, resp.; and $D_u$ and $D_s$ = mL total dilns of sample and stds, resp. Det. total dilns as in following example: If extn of 1 g sample in 200 mL solv. is followed by serial dilns of 20/100 and 5/10, then total diln is 1 g/200 mL × 20/100 × 5/10 = 1 g/2000 mL, and $D_u$ = 2000 mL.

Ref.: JAOAC **68,** 1033(1985).

CAS-67-45-8 (furazolidone)

## 960.63 Furazolidone, Nitrofurazone, or Bifuran in Feeds

**Colorimetric Method**

**First Action 1960**
**Final Action 1961**

### *A. Reagents*

(**a**) *Phenylhydrazine hydrochloride soln*.—Dissolve 0.5 g phenylhydrazine.HCl in 50 mL $H_2O$. Prep. fresh daily. Mix equal vol. of this soln with HCl.

(**b**) *Furazolidone std solns*.—(*1*) *Stock soln*.—0.55 mg/mL. Weigh 55 mg furazolidone std (available from Hess & Clark, Inc.) into 100 mL vol. flask, dil. to vol. with dimethylformamide (DMF), and mix. Soln is stable several months when protected from light. (*2*) *Working soln*.—Prep. working std corresponding to label declaration. For feeds contg 0.011% furazolidone, pipet 2 mL stock soln into 100 mL vol. flask, add 48 mL DMF, and dil. to vol. with $H_2O$. For feeds contg 0.00275% furazolidone, pipet 0.5 mL stock soln into 100 mL vol. flask, add 49.5 mL DMF, and dil. to vol. with $H_2O$.

(**c**) *Nitrofurazone std solns*.—(*1*) *Stock soln*.—0.56 mg/mL. Weigh 56 mg nitrofurazone std (available from Hess & Clark, Inc.) into 100 mL vol. flask, dil. to vol. with DMF, and mix. Soln is stable several months when protected from light. (*2*) *Working soln*.—Prep. working std corresponding to label declaration. For feeds contg 0.0056% nitrofurazone, pipet 1 mL stock soln into 100 mL vol. flask, add 49 mL DMF, and dil. to vol. with $H_2O$. For feeds contg 0.0112% nitrofurazone, pipet 2 mL stock soln into 100 mL vol. flask, add 48 mL DMF, and dil. to vol. with $H_2O$.

(**d**) *Bifuran std solns*.—(*1*) *Stock soln*.—0.1285 mg/mL. Pipet 20 mL nitrofurazone stock soln and 3 mL furazolidone stock soln into 100 mL vol. flask and dil. to vol. with DMF. (*2*) *Working soln*.—For feeds contg 0.0064% total nitrofurans, prep. working std by pipetting 5 mL bifuran stock soln into 100 mL vol. flask, adding 45 mL DMF, and dilg to vol. with $H_2O$.

(**e**) *Adsorbent*.—To 100 parts alumina, **961.24B(b)**, in screw cap bottle, add 4 parts $Mg(OH)_2$, shake until thoroly mixed, then add 5 parts $H_2O$, and mix until all lumps disappear. Store in tightly sealed container.

### *B. Determination*

(*Caution:* See safety notes on pipets, toxic solvents, and dimethylformamide.)

Grind coarse or pelleted feeds to "20 mesh" thru cutting-type mill such as Wiley Intermediate. Finer feeds need not be ground. Weigh 10 g sample into 125 mL erlenmeyer, add exactly 50 mL DMF, stopper loosely, and place in boiling $H_2O$ bath 5 min. Mech. shake 10 min and filter thru rapid paper. To 25 mL filtrate add 25 mL $H_2O$ and mix.

Prep. ca 20 mm diam. adsorption column, contg adsorbent, to ht of 5 cm. Pass the 50% DMF sample soln thru column, discarding first 3 mL eluate. (If column flow stops, break up gummy film at top of adsorbent, using long thin glass rod.) Pipet 5 mL aliquots of eluate into each of 2 numbered test tubes. Protect one tube from light. To other tube, add 3 drops *freshly prepd 2% soln of Na hydrosulfite* and let stand 20 min, shaking at ca 5 min intervals. Treat 5 mL aliquots of working std soln in exactly same manner.

Pipet 5 mL phenylhydrazine.HCl soln into each of the numbered test tubes contg samples and stds. Mix and place tubes in 70° $H_2O$ bath 25 min; cool in 15° $H_2O$ bath 5 min. Add exactly 10 mL toluene to each tube, stopper, and shake vigorously 40 times. Centrf. or filter toluene soln directly into absorption cell thru cotton wad inserted in stem of small funnel. Read $A$ of solns at 440 nm.

% Furazolidone

$$= [(A_{samp.} - A_{red.samp.}) \times 0.011 \text{ (or } 0.00275)]/(A_{std} - A_{red.std})$$

% Total nitrofurans (bifuran)

$$= [(A_{samp.} - A_{red.samp.}) \times 0.0064]/(A_{std} - A_{red.std})$$

% Nitrofurazone

$$= [(A_{samp.} - A_{red.samp.}) \times 0.0056 \text{ (or } 0.011)]/(A_{std} - A_{red.std})$$

Refs.: JAOAC **40,** 463(1957); **41,** 333(1958); **43,** 310(1960); **44,** 30(1961); **52,** 233(1969).

CAS-67-45-8 (furazolidone)
CAS-59-87-0 (nitrofurazone)

## 973.80 Furazolidone and Zoalene in Feeds

**Qualitative Tests**

**First Action 1973**
**Final Action 1988**

### *A. Apparatus*

*See* **964.07A**.

### *B. Reagents*

(**a**) *Dimethylformamide (DMF)*.—Reagent grade.

(**b**) *Alcoholic potassium hydroxide soln*.—4%. Dissolve 4 g KOH in 100 mL alcohol. If premixed with DMF (1 + 9), prep. fresh daily.

(**c**) *Ethylenediamine*.—Use in hood.

### *C. Preparation of Sample*

Gently grind pellet, cube, or crumble forms with mortar and pestle. Sieve thru nest of Nos. 10, 20, and 30 sieves with pan. Drugs usually are concd in portion in pan.

### *D. Identification*

(**a**) *DMF test*.—Place 9 drops DMF and 1 drop alc. KOH soln in each of 3 depressions of white spot plate. Sprinkle ca 0.01 g fine feed material into each soln from tip of spatula while observing reaction under microscope. Furazolidone produces intense blue color, easily detected at ≥0.0025%. Zoalene gives bright green, easily detected at ≥0.0025%. Color of minute particles of zoalene fades rapidly; color of larger particles persists 3–5 min.

(**b**) *Ethylenediamine test*.—Place dry filter paper at bottom of petri dish and sprinkle ca 0.5 g fine feed evenly over paper. Dispense 2–4 mL ethylenediamine under edge of paper so as to wet entire paper and sample. Examine under stereoscopic microscope at 10× for particles developing bright purple, indicating zoalene, or deep red, indicating furazolidone. (Blood meal, frequently used in livestock feed, also gives deep red color with reagent.)

Refs.: JAOAC **56,** 762(1973); **60,** 389(1970).

CAS-67-45-8 (furazolidone)
CAS-148-01-6 (zoalene)

## 960.64★ Glycarbylamide in Feeds

**Colorimetric Method**

**First Action 1960**
**Final Action 1961**
**Surplus 1970**

*See* **38.066–38.071**, 11th ed.

## 971.44 Ipronidazole in Feeds

**Gas Chromatographic Method**

**First Action 1971**

### *A. Principle*

Ipronidazole is extd from feed with warm 0.2*N* HCl, transferred to benzene after alkalinization, and measured by GC with electron capture detector.

### *B. Apparatus*

(**a**) *Gas chromatograph*.—With electron capture detector. Conditions: temps (°)—column 180 ± 2 (isothermal), injection 240 ± 2, detector 210 ± 2; N flow 60 mL/min (install 3′ (0.9 m) × 1/4″ molecular sieve 5A trap in N line).

(**b**) *Recorder*.—0–1 mv input and 1.0 sec full scale deflection, chart speed 0.75″/min, or equiv.

(**c**) *Gas chromatographic column*.—0.9 m (3′) × 1/8″ od stainless steel tubing packed with 5% Carbowax 20M-terephthalic acid (TPA) on 100–120 mesh Chromosorb G, acid-washed and dichlorodimethylsilane-treated (Applied Science).

(**d**) *Column preparation*.—Dissolve 1.0 g Carbowax 20M-TPA in 30 mL $CHCl_3$ and let stand over 19.0 g Chromosorb G 3 days with occasional swirling. After standing, evap. $CHCl_3$ under N and dry 1 hr in 70° oven. After packing, condition column 2 days at 200° in slow stream of N before use.

### *C. Reagents*

(**a**) *Benzene*.—Redistil from all-glass app., discarding first and last 10%. (*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, and benzene.)

(**b**) *Ipronidazole*.—Available from Hoffmann-La Roche, Inc.; prep. std soln contg 0.3 μg ipronidazole/mL benzene.

### *D. Chromatography of Standard*

Inject 5 μL std ipronidazole soln into gas chromatograph and adjust conditions to obtain ca 5 min retention time. Det. area under ipronidazole peak as product of peak ht (cm) and width (cm) at half ht, using slope baseline technic. After satisfactory performance of column is established, inject at least duplicate 5 μL aliquots of std soln (1.5 ng/5 μL) at beginning of work day and periodically thereafter (e.g., after each 5–6 sample injections). Measure ipronidazole peak area in each case.

### *E. Preparation of Sample*

(*Caution:* See safety notes on pipets.)

Ext mash-type feeds without prior grinding. Grind pelleted feeds to pass No. 30 sieve before extn. (Method for feeds is described for 0.0060% level of ipronidazole with modifications indicated for 0.0030 and 0.0090% levels.)

Transfer 10 g sample into 500 mL vol. flask (for 0.0030% level, use 20 g sample) and add exactly 200 mL 0.2*N* HCl previously warmed to 40 ± 3°. Stopper flask and shake mech. 20 min. Let settle few min. Centrf. portion of supernate and pipet 10 mL (for 0.0090% level, transfer 5.0 mL and add 5 mL 0.2*N* HCl) to g-s centrf. tube. Place tube in cold $H_2O$ bath and add 0.5 mL 5*N* NaOH to make alk. (pH = 10–12.5; check with Accutint pH paper; do not hold eluate at high pH for prolonged time). Pipet 20 mL benzene into tube, stopper, shake mech. 5 min, and centrf. 10 min at 2500 rpm. Pipet 5 mL clear benzene ext into 25 mL vol. flask, dil. to vol. with benzene, and mix. This is sample soln.

### *F. Determination*

Inject 5 μL sample soln into chromatograph. Measure area of ipronidazole peak obtained from chromatogram.

$$\% \text{ Ipronidazole} = (B \times C \times D)/(B' \times 10{,}000)$$

where $B$ and $B'$ = areas under ipronidazole peak for sample and std solns, resp.; $C$ = μg ipronidazole/mL in std soln (0.3); and $D$ = diln factor (0.0030% level, $D$ = 100; 0.0060%, 200; and 0.0090%, 400).

Ref.: JAOAC **54,** 72(1971); **57,** 29(1974).

CAS-14885-29-1 (ipronidazole)

## 980.36 Melengestrol Acetate in Feed Supplements

**Gas Chromatographic Method**

**First Action 1980**
**Final Action 1988**

(*Caution:* See safety notes on chloroform, hexane, and methanol.)

### A. Principle

MGA is extd from aq. slurry of supplement sample with hexane, partitioned from hexane into aq. MeOH, and then from aq. MeOH into $CH_2Cl_2$. After evapn of $CH_2Cl_2$, dried ext is transferred to alumina column with hexane, and eluted with $CHCl_3$-hexane soln. MGA in eluate is detd by GC. MGA can be assayed in cattle feed supplements contg 0.125 mg/lb to 1.00 mg MGA/lb supplement (0.28–2.2 mg/kg).

### B. Apparatus

**(a)** *Extractor.*—Liq.-liq. (Ace Glass No. 6840-96 or equiv.).

**(b)** *Gas chromatograph.*—Tracor Model MT-220 (replacement Model 540, Tracor Instruments Inc.), or equiv., with $^{63}Ni$ pulsed electron affinity detector, 0.6 m (2′) × 3 mm id glass column packed with 1% OV-17 on 100–200 mesh Gas-Chrom Q (Applied Science), and Tracor 1.0 mv recorder with chart speed of 0.5″/min. *Operating conditions.*—Linde 99.996% high purity N, or equiv., carrier gas 50 mL/min; purge, off; temps (°)—injector 235, detector 300, column 225; detector pulse ht 60 V, pulse interval 300 μsec, sensitivity 80 × $10^{-11}$ AFS.

**(c)** *Rotary-evaporator.*—Valley Electromagnetics, One Wolfer Park, Spring Valley, IL 61362, or equiv., **976.36B(m)**.

**(d)** *Chromatographic tube.*—18 × 500 mm, fitted with coarse porosity fritted glass disc and Teflon stopcock (Fischer and Porter Co., or equiv.).

### C. Reagents

**(a)** *Aluminum oxide.*—Woelm acid, anionotropic, activity grade 1 for column chromatography (ICN Pharmaceuticals or equiv.).

**(b)** *Cholesteryl chloroacetate (CCA) internal std soln.*—*(1) Stock soln.*—500 μg/mL. Dissolve 125 mg (Aldrich Chemical Co., C7680-9) in 250 mL absolute ethanol-hexane (5 + 95). *(2) Working soln I.*—50 μg/mL. Pipet 10 mL stock soln and dil. to 100 mL with absolute ethanol-hexane. *(3) Working soln II.*—5 μg/mL. Pipet 10 mL stock soln and dil. to 1 L with absolute ethanol-hexane (5 + 95).

**(c)** *Medroxyprogesterone acetate (MAP) extraction std soln.*—*(1) Stock soln.*—2 mg/mL. Dissolve 200 mg (The Upjohn Co., 7000 Portage Rd, Kalamazoo, MI 49001) in 100 mL absolute alcohol (requires overnight shaking). *(2) Working solns.*—16, 20, 30 and 60 μg/mL. Pipet 8, 10, 15 and 30 mL aliquots stock soln and dil. to 1 L with absolute alcohol for use with 0.125–0.250, 0.250–0.450, 0.450–0.750, 0.750–1.00 mg MGA/lb samples, resp.

**(d)** *Melengestrol acetate (MGA).*—*(1) Stock soln.*—25 μg/mL. Dissolve 100 mg Ref. Std (The Upjohn Co.) in 100 mL absolute alcohol. Dil. 5.0 mL to 200 mL with hexane. *(2) Working soln.*—1.25 μg/mL. Dil. 10 mL stock soln to 200 mL with absolute alcohol-hexane (5 + 95).

**(e)** *Gas chromatography reference soln.*—CCA, 5 μg/mL; MAP 2.4 μg/mL; MGA, 0.125 μg/mL. Pipet 10 mL each CCA (50 μg/mL) and MGA (1.25 μg/mL) and 4 mL MAP (60 μg/mL) and dil. to 100 mL with hexane.

**(f)** *Solvents.*—Distd-in-glass hexane and $CH_2Cl_2$, pesticide and gas chromatgy grade (Burdick & Jackson Laboratories, or equiv.).

**(g)** *Solvent partition soln.*—Mix 0.25% aq. $Na_2SO_4$ soln with MeOH (30 + 70).

### D. Extraction

Grind dry samples to pass 1 mm screen. Thoroly mix sample and place ca 15 g sample, weighed to nearest 0.01 g, into extractor, and pipet 10 mL appropriate extn std soln, (**c**), for level MGA being assayed. Rinse sample to bottom of extractor with 30 mL $H_2O$. Place mag. stirring bar in extractor. Fill to side arm with hexane and insert solv. return tube in position. Attach extractor to condenser and 500 mL receiving flask contg 100 mL hexane and several SiC boiling chips. Ext sample 3 hr by heating receiving flask enough to have rapid reflux (≥20 mL/min) at condenser while stirring sample vigorously. *It is imperative that reflux rate be fast enough to completely ext sample in 3 hr.* 250 watt Glas-Col heating mantle operated at 120 v will do this. Control emulsions by regulating stirring rate. *Sample must be stirred vigorously and continuously.* Up to 30 mL addnl $H_2O$ may be added thru condenser to aid stirring action, if necessary. Let apparatus cool, set receiving flask aside, and transfer extractor contents to 1 or 2 L separator. Let phases sep. clearly; discard aq. (lower) layer and transfer hexane layer to 2 L r-b flask and roto-evap. just to dryness. Dissolve residue in 100 mL solv. partition soln, (**g**).

### E. Solvent Partition

Quant. transfer ext in 500 mL receiving flask to 500 mL separator. Rinse 2 L flask with 100 mL solv. partition soln, (**g**), into receiving flask and then quant. transfer receiving flask contents into same separator. Gently shake funnel by inverting 15 times, let solv. layers sep. clearly, and drain lower layer into 1 L separator contg 200 mL $CH_2Cl_2$. Repeat rinsing flasks, transfers, and partitioning with 3 or more 100 mL portions solv. partition soln. Vigorously shake combined exts in 1 L separator 15 sec, let phases sep. clearly, and drain lower ($CH_2Cl_2$) layer into original 500 mL receiving flask. Repeat partition into $CH_2Cl_2$ 2 more times, using 40 mL $CH_2Cl_2$ each time. Roto-evap. combined exts to near dryness. Remove residual $H_2O$ with ≥2 sep. addns of 10 mL absolute alcohol and roto-evap. solv. Remove residual alcohol with two 10 mL portions hexane, roto-evapg each portion. Dissolve residue in 10 mL hexane.

### F. Alumina Column Chromatography

Prep. column by slurrying 20 g aluminum oxide in $CHCl_3$ and transfer with $CHCl_3$ wash bottle to tube, (**d**), contg ca 100 mL $CHCl_3$. While column drains, let alumina settle and add 5 g $Na_2SO_4$ to column while letting $CHCl_3$ slowly drain. Use ca 100–150 mL total $CHCl_3$ in this step. *In this and following steps, it is important to let each portion of solv. added drain to top of $Na_2SO_4$ layer before adding next portion.* $CHCl_3$ must contain 0.5–1.0% alcohol. Rinse column with 10–15 mL hexane, followed by three 50 mL hexane washes. While column drains, quant. transfer ext to column with four 10 mL portions and one 75 mL portion hexane. Discard all washings collected to this point. Elute MGA with five 50 mL portions $CHCl_3$-hexane (33 + 67) solv., using each portion to rinse flask contg ext; collect 250 mL eluate in 250 mL vol. flask.

### G. Determination

Thoroly mix column eluate and pipet aliquot for analysis as follows: 0.125–0.250 mg MGA/lb, 40 mL; 0.250–0.450 mg MGA/lb, 30 mL; 0.450–0.750 mg MGA/lb, 20 mL; 0.750–1.00 mg MGA/lb, 10 mL, and evap. to dryness in 50 mL g-s r-b flask, using warm (45–60°) hot plate or $H_2O$ bath and stream of N or air. When aliquot approaches dryness, add ca 5 mL absolute alcohol and evap. to remove residual $H_2O$. Repeat addn of alcohol if necessary until aliquot dries completely. Dissolve in 10.0 mL 5 μg/mL CCA working soln II, (**b**)*(3)*. Inject 3 μL each of sample and std into gas chromatograph operated as in **980.36B(b)**. Order of elution, min: MAP, 4–6; MGA, 6–8; CCA, 8–11. Make several injections to achieve reproducible (< ± 5%) peak hts or areas depending on type and condition of gas chromatograph. *It is imperative that GC response be linear.* Check linearity using MGA concs 0.5 and 2.0 times GC ref. soln, (**e**), concn (0.0625, 0.125, and 0.25 μg/mL). *Keep CCA concn const.*

### H. Calculations

Calc. mg MGA/lb sample:

$$\text{mg MGA/lb} = R \times R' \times 1.25 \times (250/\text{mL aliquot}) \times (0.4536/W)$$

where $R$ and $R'$ = peak ht ratios of MGA/CCA in sample and CCA/MGA in std, resp.; 1.25 = μg MGA in 10 mL std soln (**e**); 250 = mL eluate collected; mL aliquot = 10–40 mL aliquot taken; 0.4536 = conversion factor to obtain mg/lb; $W$ = g sample extd. MAP is added to sample prior to extn to indicate magnitude of extn efficiency and cleanup losses. Reassay samples showing <85% MAP recovery after reasons for low MAP recovery have been detd.

$$\%\ \text{MAP recovered} = R_1 \times R_2 \times 24 \times (100/M) \times (250/\text{mL aliquot})$$

where $R_1$ and $R_2$ = peak ht ratios of MAP/CCA in sample and CCA/MAP in std, resp.; 24 = μg MAP in 10 mL std soln (**e**); $M$ = μg MAP added to sample; and (250/mL aliquot) are defined above.

Ref.: JAOAC **63,** 425(1980).

CAS-2919-66-6 (melengestrol acetate)

## 971.45★ Nequinate in Feeds

**Spectrophotofluorometric Method**
**First Action 1971**
**Final Action 1974**
**Surplus 1975**

*See* **42.084–42.087,** 13th ed.

## 956.11 Nicarbazin in Feeds

**Spectrophotometric Method**
**Final Action**

(Presence of furazolidone, nitrofurazone, or nihydrazone may cause high results. Confirm presence of nicarbazin by Identification Test, **956.11F**.)

### A. Reagents

(**a**) *Dimethylformamide* (*DMF*).—Reagent grade.

(**b**) *Alumina*.—*See* **961.24B(b)**.

(**c**) *Alcohol*.—Formulas SDA Nos. 2B, 3A, or 30 may be used.

(**d**) *Alcoholic sodium hydroxide soln*.—Dil. 2.0 mL clear 50% NaOH soln, **936.16B(b)**, to 100 mL with alcohol. Centrf. in stoppered tube. Prep. fresh daily.

(**e**) *Nicarbazin std solns*.—(*1*) *Stock soln*.—Weigh 25.0 mg Nicarbazin Ref. Std (available from Merck & Co.) into 500 mL vol. flask, and dissolve in ca 150 mL DMF with aid of gentle heat. Cool, dil. to vol. with DMF, and mix well. Store protected from light. (*2*) *Working soln*.—12.5 μg/mL. Transfer 25.0 mL stock soln to 100 mL vol. flask and dil. to vol. with DMF. Mix well.

### B. Preparation of Column

Use glass tube 22 mm id, ca 50 cm long, constricted at lower end. Place plug of glass wool in constricted end and add 30 g alumina in 3 portions. Tamp each portion with glass rod while applying gentle suction. Wash column with 25 mL DMF, draining to point 1–2 cm above bed level before adding sample to column. Prep. column for each sample and std.

Never let column run dry; keep head of liq. at all times.

### C. Preparation of Sample

(*Caution:* See safety notes on distillation, toxic solvents, and dimethylformamide.)

Weigh 10.0 g sample into 250 mL erlenmeyer and add 100.0 mL DMF. Heat *just to bp* on hot plate in hood with intermittent stirring. Cool to room temp. by immersing in $H_2O$ bath. Decant supernate into centrf. tubes and centrf. 3 min.

### D. Determination

(*Caution:* See safety notes on pipets.)

Pipet 25.0 mL clear ext onto column and let pass thru column with aid of gentle suction. Wash column with three 10 mL portions DMF and reject washings. Elute with nine 5 mL portions alcohol, discarding first 15 mL eluate and collecting next 25 mL eluate in 25 × 200 mm tube. Quant. transfer eluate into 50 mL vol. flask and dil. to vol. with alcohol. Mix well.

Pipet 25.0 mL working std soln onto another column and proceed as for sample.

Pipet two 15.0 mL portions sample soln into sep. 25 mL vol. flasks. To one add 5.0 mL alc. NaOH soln and adjust vol. of both solns to 25 mL with alcohol. Read $A$ of yellow soln formed in first flask within 5 min in spectrophtr or colorimeter at 430 nm against second soln as blank. Calc. wt nicarbazin from std curve.

### E. Preparation of Standard Curve

Pipet 10, 15, and 20 mL aliquots of chromatgd working std soln into sep. 25 mL vol. flasks, add 5 mL alc. NaOH, and dil. to vol. with alcohol. Mix well. Measure $A$ within 5 min at 430 nm against alcohol.

Prep. std curve by plotting $A$ against μg nicarbazin.

### F. Identification Test

Place alcohol in 1 cm quartz cell and clear chromatgd sample soln in matched cell. Det. $A$ at 2 nm intervals from 340 to 349 nm with Beckman Model DU spectrophtr, or equiv., at min. slit width. Absorption max. at 344 ± 4 nm confirms presence of nicarbazin.

Refs.: JAOAC **39,** 321(1956); **40,** 469(1957); **41,** 326(1958).

CAS-330-95-0 (nicarbazin)

## 967.35 Nicotine in Feeds

**Spectrophotometric Method**
**First Action 1965**
**Final Action 1967**

(Applicable in the presence of phenothiazine, dibutyltin dilaurate, and 2,2′-dihydroxy-5,5′-dichlorodiphenylmethane)

### A. Principle

Nicotine is extd with alkali, steam distd, extd with $CHCl_3$, and detd spectrophtric in acidic soln.

### B. Reagents

(**a**) *Dilute hydrochloric acid*.—0.05*N*. Dil. 4.1 mL HCl to 1 L with $H_2O$.

(**b**) *Nicotine std soln*.—0.012 mg/mL. Accurately weigh ca 100 mg nicotine and dil. to 100 mL in vol. flask with 0.05*N* HCl. Transfer 3.0 mL aliquot to 250 mL vol. flask and dil. to vol. with 0.05*N* HCl. (*Caution:* Nicotine is very toxic.)

(**c**) *Antifoam*.—Antifoam A (Dow Corning Corp.), or equiv.

### C. Apparatus

(**a**) *Distillation flask*.—250 mL r-b flask and Claisen distg head or 250 mL Claisen flask.

(**b**) *Condenser*.—Graham coil type with 300 mm jacket (Corning No. 2500, or equiv.). Must be used in vertical position.

(**c**) *Ultraviolet recording spectrophotometer*.—Double beam, capable of scanning UV spectrum from 220 to 300 nm, with 1 cm cells.

### D. Determination

(Detn can be interrupted at any step where soln is acidic.)

Accurately weigh representative portion of feed, ground thru No. 20 sieve, contg ca 3 mg nicotine and transfer to 250 mL centrf. bottle. Add 100 mL 0.5% NaOH soln, stopper (Neoprene or rubber), and shake vigorously 1 min. Centrf. ca 5 min at 1500 rpm. Decant free flowing and viscous liq. into 400–600 mL beaker. Rinse lip and centrf. bottle into beaker with few mL $H_2O$, being careful not to dislodge solid material. Repeat extn, centrfg, and rinsing twice, combining supernates in beaker. Adjust soln to pH 2–3 with HCl and evap. on hot plate to ca 100 mL. Cool, adjust to pH 10–14 with NaOH (1 + 1), and transfer quant. to distn flask, using min. of $H_2O$. Vol. must be ≤125 mL. Add 10 drops antifoam to flask. Place tip of condenser below surface of 7 mL $H_2SO_4$ (1 + 5) in 500 mL flask or beaker (container must be tilted at first to obtain sufficient depth). Steam distil at rate of ≥8 mL/min. (It will be necessary to heat Claisen flask as distn proceeds to avoid condensation of steam in flask.) Collect ca 300 mL distillate. When distn is complete, rinse condenser into receiver with ca 5 mL $H_2O$.

Transfer distillate to 500 mL separator, rinse with $H_2O$, make distinctly alk. (pH 10–14) with NaOH (1 + 1), and ext with five 20 mL portions $CHCl_3$. Combine $CHCl_3$ exts in 250 mL separator. Ext nicotine from $CHCl_3$ with 20, 20, 15, 15, and 15 mL portions 0.05*N* HCl. Combine HCl exts in 125 mL separator and shake gently few sec with 15 mL pet ether to remove any remaining $CHCl_3$. Drain clear HCl layer into 250 mL vol. flask and wash pet ether with addnl 10 mL 0.05*N* HCl. Drain acid layer into vol. flask, dil. to vol. with 0.05*N* HCl, and record UV spectrum from 220 to 300 nm in 1 cm cell against 0.05*N* HCl in recording spectrophtr. Draw line tangent to 2 min. obtained (ca 226 and 280 nm), drop perpendicular from point of max. *A* (ca 259 nm) to tangent line, and det. net *A*. Similarly det. net *A'* of std soln.

$$\% \text{ Nicotine in feed} = (\text{Net } A \times \text{mg nicotine in final std soln} \times 100)/(\text{Net } A' \times \text{g sample} \times 1000)$$

Ref.: JAOAC **47**, 226(1964).

CAS-54-11-5 (nicotine)

## 971.46 Nifursol in Feeds

**Spectrophotometric Method**

**First Action 1971**
**Final Action 1984**

### A. Principle

Nifursol is extd from feed with dimethylformamide (DMF). Feed interferences are removed from DMF soln by column chromatgy on alumina. Drug is reacted with phenylhydrazine to form 5-nitrofurfural phenylhydrazone, which is extd into toluene and detd spectrophtric at 555 nm immediately after addn of methylbenzethonium hydroxide.

### B. Apparatus

(**a**) *Chromatographic tubes*.—400 × 20 mm id, with glass wool plug.

(**b**) *Spectrophotometer*.—For use at 555 nm.

### C. Reagents

(**a**) *N,N-Dimethylformamide (DMF) solns*.—(*1*) *95% DMF*.—Dil. 95 parts DMF (No. DX1730, EM Science or equiv.) with 5 parts $H_2O$. (*2*) *50% DMF*.—Dil. 50 parts DMF with 50 parts $H_2O$. (*Caution:* See safety notes on dimethylformamide.)

(**b**) *Alumina*.—To 100 parts 80–200 mesh alumina (Alcoa F-20, Fisher Scientific Co., A-540), add 6 parts powd $Mg(OH)_2$. Shake mixt. in screw-cap bottle until thoroly mixed. Add 8 parts $H_2O$ and immediately mix until all lumps disappear.

(**c**) *Phenylhydrazine soln*.—Prep. immediately before use by dissolving 0.25 g phenylhydrazine.HCl crystals in 25 mL $H_2O$. Add 25 mL HCl.

(**d**) *Methylbenzethonium hydroxide*.—1*M* soln in MeOH (No. 23, 394-3, Aldrich Chemical Co., Inc.).

(**e**) *Nifursol std solns*.—(*1*) *Stock soln*.—0.25 mg/mL. Weigh 25 mg nifursol (mp 224–226°) into 100 mL vol. flask. Add 5 mL DMF, mix until all material is dissolved, and dil. to vol. with MeOH. (*2*) *Working soln*.—6.25 μg/mL. Pipet 5.0 mL stock soln into 200 mL vol. flask, add 100 mL 95% DMF, and dil. to vol. with $H_2O$. (Nifursol solns are not stable for long periods of time; prep. fresh as needed.)

### D. Extraction

Weigh 5.00 g finely ground feed into 250 mL erlenmeyer. Add exactly 50 mL 95% DMF, stopper, and shake gently 5 min on mech. shaker. Place sample 30 min in 60° $H_2O$ bath, remove, and shake vigorously 30 min on mech. shaker. Filter thru rapid paper in buchner, using vac. Transfer 40.0 mL aliquot of filtrate to beaker, add 40.0 mL $H_2O$, stir, and let stand 30 min in dark.

### E. Chromatography

Add 7 cm specially prepd alumina to chromatgc tube contg glass wool plug. Tap column walls to settle alumina; then add 1.5 cm *Ottawa sand* (No. S-15195, Sargent-Welch Scientific Co.). Prewash column with 50 mL 50% DMF *just* before use. Add DMF sample soln to column, discard first 60 mL eluate, and collect next 12 mL. (Decrease in flow rate may occur with some feed exts due to accumulation of fine ppt that settles on surface of alumina. Increase flow rate by stirring top of alumina and sand to break up ppt layer or by applying gentle air pressure to top of column.)

### F. Determination

Pipet 5 mL aliquot into 20 mL test tube. Add 5 mL freshly prepd phenylhydrazine soln to tube, mix, and place 20 min in 40° $H_2O$ bath. Cool tube under cold tap $H_2O$ (<20°) 5 min, add exactly 5 mL toluene, stopper tube, and shake vigorously. (*Caution:* Do not use black rubber stoppers.) Centrf. 5 min to clear toluene layer and transfer 3.0 mL toluene to 1 cm cell. Add 0.1 mL methylbenzethonium hydroxide to cell and mix immediately. Read sample within 1 min at 555 nm on spectrophtr. Det. concn of nifursol from std curve.

### G. Preparation of Standard Curve

Pipet 0, 2, 3, 4, and 5 mL aliquots working soln (equiv. to 0.000, 0.0050, 0.0075, 0.0100, and 0.0125% in feed) into sep. 20 mL test tubes. Dil. to 5 mL with 50% DMF. Develop color as in **971.46F** and plot *A* against % drug in feed.

Ref.: JAOAC **54**, 66(1971).

CAS-16915-70-1 (nifursol)

## 961.25 Nihydrazone in Feeds

### Colorimetric Method

**First Action 1961**
**Final Action 1962**

### A. Reagents

(**a**) *95% Dimethylformamide* (*DMF*).—Dil. 95 parts DMF (Eastman Kodak Co. No. 5870, or equiv.) with 5 parts $H_2O$. (*Caution:* See safety notes on dimethylformamide.)

(**b**) *Nihydrazone std solns*.—(*1*) *Stock soln*.—Weigh 110 mg cryst. nihydrazone (available from Norwich Eaton Pharmaceuticals Inc., 17 Eaton Ave, Norwich, NY 13815) into 100 mL vol. flask, dissolve in DMF, and dil. to vol. with DMF. Protected from light, soln is stable several months. (*2*) *Working soln*.—For feeds contg 0.011% nihydrazone, pipet 1 mL aliquot stock soln into 100 mL vol. flask, add 50 mL DMF, and dil. to vol. with $H_2O$.

### B. Determination

Weigh 10 g sample into 125 mL erlenmeyer, add exactly 50 mL 95% DMF, stopper loosely, and place in boiling $H_2O$ bath 5 min (or until temp. of solv. reaches 90°). Shake mech. 10 min and filter thru rapid paper. To 25 mL filtrate add 25 mL $H_2O$ and mix. Let stand, protected from light, ≥30 min. (Some solids may sep.; standing for longer time is permissible.)

Prep. ca 20 mm diam. adsorption column contg adsorbent, **960.63A(e)**, to ht of 5 cm. (With highly colored feeds, use somewhat longer column.) Use plug of cotton or glass wool to support column, and similar plug or layer of washed sea sand on top. Pass sample soln thru column, collecting ca 15 mL eluate. Pipet 5 mL aliquots into each of 2 tubes. Protect 1 tube from light; to other add 3 drops *freshly prepd 2% Na hydrosulfite soln*, mix, and let stand 5 min. Treat 5 mL aliquots dild std soln similarly.

Pipet 5 mL phenylhydrazine soln, **960.63A(a)**, into all test tubes, mix, and heat 20 min in 40° $H_2O$ bath. Cool by placing tubes in 15° $H_2O$ bath 5 min. Add exactly 10 mL toluene to each tube, stopper, and shake vigorously 40 times. Sep. and centrf. toluene layer, and read $A$ at 440 nm.

$$\% \text{ Nihydrazone} = [(A_{\text{samp.}} - A_{\text{red.samp.}}) \times 0.011]/(A_{\text{std}} - A_{\text{red.std}})$$

Ref.: JAOAC **44,** 2(1961).

CAS-67-28-7 (nihydrazone)

## 970.86 Nitarsone in Feeds

### Spectrophotometric Method

**First Action 1970**
**Final Action 1973**

### A. Principle

Nitarsone is extd from feed with 50% dimethylsulfoxide (DMSO) and sepd from interferences by alumina chromatgy. Nitro group is reduced with $TiCl_3$ and resulting amine assayed colorimetrically at 530 nm with Bratton-Marshall reaction. Arsanilic acid and carbarsone interfere.

### B. Reagents

(**a**) *Nitarsone std solns*.—(*1*) *Stock soln*.—1 mg/mL. Weigh 100 mg nitarsone std (available from Salsbury Laboratories, Inc.) into 100 mL vol. flask and dil. to vol. with 4% NaOH. (*2*) *Working soln*.—50 μg/mL. Dil. 10 mL stock soln to 200 mL with 4% NaOH.

(**b**) *Activated alumina*.—Alcoa grade F-20, 80–200 mesh (available from Fisher Scientific Co. as Alumina, adsorption, Fisher No. A-540). To det. suitability of alumina, perform entire detn on 100 μg nitarsone. Recovery should be >95%.

(**c**) *Dimethylsulfoxide* (*DMSO*) *soln*.—50%. Dil. with equal vol. $H_2O$. (*Caution:* DMSO can be harmful. Avoid skin contact by wearing heavy rubber gloves. Use effective fume removal device.)

(**d**) *Titanous chloride soln*.—4% aq. Prep. fresh daily from 20% soln open ≤3 months and kept refrigerated, or from solid $TiCl_3$. If >1 min is required for color disappearance in detn, use fresh source of $TiCl_3$. (*Caution:* $TiCl_3$ is corrosive. Wear disposable plastic or rubber gloves. Avoid contact with eyes.)

(**e**) *Sodium nitrite soln*.—0.1% aq. Prep. weekly.

### C. Preparation of Standard Curve

Pipet 0, 2, 5, 10, 15, 20, and 25 mL working soln into sep. 100 mL vol. flasks and dil. to vol. with 4% NaOH. Pipet 10 mL from each flask into sep. 50 mL vol. flasks, add 15 mL 4% NaOH, and dil. to vol. with $H_2O$. Pipet 4 mL from each flask into sep. test tubes and develop color as in **970.86E**, beginning ". . . add 2 drops 4% $TiCl_3$ . . ." Std concns correspond to 0, 0.004, 0.010, 0.020, 0.030, 0.040, and 0.050% nitarsone in feeds. Plot std curve of $A$ against % drug in feed.

### D. Preparation of Sample

Accurately weigh 5 g finely ground feed into 100 mL vol. flask. Add 75 mL 50% DMSO, place sample on wrist action mech. shaker, and shake at room temp. 30 min. Dil. to vol. with 50% DMSO and mix. Transfer 30–40 mL to 50 mL centrf. tube and centrf. 10 min at 2000 rpm.

### E. Determination

(*Caution:* See safety notes on pipets.)

Add alumina to 20 × 400 mm chromatgc tube with fritted glass disk to depth of 7 cm. Tap tube wall to settle alumina; then add 1 cm layer of sand. Prewash column with 50 mL 50% DMSO before use.

Pipet 10 mL supernate from prepn of sample onto prewashed column. For feeds contg >0.04% nitarsone, use smaller aliquot. Let sample enter column and then wash into column with several 5 mL portions $H_2O$. Wash column with 75 mL $H_2O$ and discard eluate.

Elute nitarsone with 65 mL 4% NaOH, discarding first 15 mL. Collect remaining eluate in 100 mL vol. flask, letting column run dry. Nitarsone is eluted with ca 25–30 mL eluant. Dil. eluate to vol. with $H_2O$ and mix.

Pipet 4 mL dild eluate into 2 test tubes, add 2 drops 4% $TiCl_3$ to each with mixing, and shake or mix on Vortex mixer until black color disappears. Add 2 mL HCl to each and mix thoroly. Add 0.5 mL 0.1% $NaNO_2$, (**e**), and mix. After 5 min, add 0.5 mL 0.5% $NH_4$ sulfamate, **969.53A(c)**, and mix. After 2 min, add 0.5 mL 0.1% coupling reagent, **969.53A(d)**, to one tube and 0.5 mL $H_2O$ to second tube for blank. Let color develop 15 min; then read $A$ of sample and blank at 530 nm on spectrophtr. Correct sample $A$ for blank $A$ and det. amt nitarsone in sample from std curve.

Ref.: JAOAC **53,** 641(1970).

CAS-98-72-6 (nitarsone)

## 960.65★ Nithiazide in Feeds

### Spectrophotometric Method

**First Action 1960**
**Final Action 1961**
**Surplus 1989**

*See* **42.122–42.125**, 14th ed.

## 967.36★ Nitrodan in Feeds

**Colorimetric Method**

**First Action 1967**
**Final Action 1968**
**Surplus 1989**

(Not applicable in presence of interfering nitro compds)

*See* **42.126–42.129**, 14th ed.

## 959.18 Nitromide in Feeds

**Spectrophotometric Method**

**Final Action**

### *A. Reagents*

(**a**) *Diethylamine reagent* (*DEA*), *aged*.—(1 year or older.) Fresh DEA may be artifically aged as follows: Place 1 L DEA in dry 2 L flask with 40 g Na or K fluosilicate. Connect flask to 60 cm (24") bulb reflux condenser and reflux on sand bath 2–3 days in hood. When reagent is sufficiently "aged," 2 mL clear DEA added to 8 mL dimethylsulfoxide contg 50 μg 3,5-DNBA should develop max. color in ca 40 min. *A* as read on Beckman DU spectrophtr at 560 nm should be ca 0.375; on Klett-Summerson photoelec. colorimeter with No. 56 filter, ca 200. Reagent must be free from turbidity. Prep. new std curve for each batch of DEA.

(**b**) *Nitromide std solns*.—(*1*) *Stock soln*.—1 mg/mL. Weigh 100 mg nitromide (Salsbury Laboratories, Inc.) into 100 mL flask and dil. to vol. with MeOH. (*2*) *Working soln*.—20 μg/mL. Transfer 2.0 mL stock soln to 100 mL vol. flask and dil. to vol. with MeOH.

### *B. Preparation of Standard Curve*

Place 1.0, 2.0, 3.0, and 5.0 mL working soln contg 20, 40, 60, and 100 μg, resp., of nitromide in 4 colorimeter tubes. Evap. to dryness at 50° in air current. Dissolve residue in 8 mL dimethylsulfoxide at 70°, cool, and add 2 mL DEA reagent. Place in dark at 20–25° and read after 1 hr. Plot std curve, using *A* as ordinate and concn as abscissa.

### *C. Preparation of Sample*

Weigh 5.0 g feed, contg 0.025% nitromide, into 100 mL vol. flask and dil. to vol. with MeOH. Shake frequently 20 min and let stand 40 min to permit feed particles to settle.

If feed contains 0.075% nitromide, use 2 g finely ground feed; if 0.15%, use 1 g in 100 mL or 5 g in 500 mL MeOH. Prep. premixes by weighing appropriate sample and serially dilg MeOH ext.

### *D. Determination*

Pipet 4 mL aliquot of ext into g-s test tube. Place tube in 50° $H_2O$ bath and evap. to dryness with air current directed onto surface of MeOH. Add 8 mL dimethylsulfoxide and heat to 70° to hasten soln; cool, and add 2 mL DEA reagent. Place in dark 1 hr at 20– 25°. Det. *A* at 560 nm in Beckman DU spectrophtr, Klett-Summerson photoelec. colorimeter with No. 56 filter, or similar instrument, against dimethylsulfoxide as ref.

Det. amt of nitromide in tube from std curve.

% nitromide in feed
= μg nitromide in tube × 25 × 100/5,000,000

or μg nitromide in tube × 5 = μg nitromide/g feed or ppm.

Ref.: JAOAC **42,** 239(1959).

CAS-121-81-3 (nitromide)

## 952.29★ Nitrophenide in Feeds

**Spectrophotometric Method**

**Final Action**
**Surplus 1980**

(Applicable in presence of arsanilic acid)

*See* **42.120–42.122**, 13th ed.

## 967.37 Phenothiazine in Feeds

**Spectrophotometric Method**

**First Action 1967**
**Final Action 1970**

### *A. Reagent*

*Phenothiazine std soln*.—Dissolve 10 mg recrystd (from 10% soln in toluene) phenothiazine (ICI Americas, Inc., Eighty Four, PA 15330) in 50 mL alcohol and dil. to 100 mL with alcohol. For working stds, dil. with equal vol. of alcohol. (1 mL dild soln = 50 μg phenothiazine.) Use freshly prepd soln; alc. solns gradually develop rose tint within few hr.

### *B. Determination*

Place 1 g ground sample in 100 mL vol. flask, add 50 mL alcohol, and heat on steam bath 15 min. Cool, dil. to vol. with alcohol, mix, and let settle (ca 15 min) until supernate is clear.

Place 2 mL aliquot in 25 mL vol. flask and add 10 mL alcohol. To flask add, in order given, 1 mL *1% alc. p-aminobenzoic acid soln*, 1 mL *aq. 2%* $NaNO_2$ *soln*, and 1 mL HCl (1 + 3). Dil. to vol. with alcohol. Read *A* of green soln at 600 nm in spectrophtr against reagent blank. Det. amt of phenothiazine from std curve.

% Phenothiazine = μg/200

Prep. std curve, using 1, 2, and 3 mL dil. std soln, as above.

Refs.: JAOAC **41,** 338(1958); **42,** 254(1959).

CAS-92-84-2 (phenothiazine)

## 967.38 Piperazine in Feeds

**Spectrophotometric Method**

**Final Action**

### *A. Principle*

Piperazine or piperazine salt is quant. extd from feed into slightly acidic aq. soln. Dild filtrate is reacted with equal vol. benzoquinone soln at 80°. Colored complex formed is detd spectrophtric at 490 nm.

Applicable to detn of 0.05–0.5% piperazine, usually present as one of its salts, in animal feeds. Amines give similar color reaction. Alkalies produce increased color; pH adjustment in method overcomes interference of this kind.

### *B. Apparatus*

(**a**) *Water bath*.—Approx. 25 cm (10") diam. with 15–20 cm (6–8") depth $H_2O$. Thermostatically controlled at 80 ± 0.1°. (Viscosity bath is satisfactory.)

(**b**) *Test tubes*.—Pyrex, 15 × 125 mm, with rubber stoppers, and rack capable of supporting tubes when immersed in $H_2O$ bath.

### *C. Reagents*

(**a**) *Quinone soln*.—Dissolve 0.5 g *p*-benzoquinone in 2.5 mL HOAc and little alcohol in dry 100 mL vol. flask and dil. to vol. with alcohol. Keep soln in ice bath or refrigerator. Prep. fresh daily. (*Caution: p*-Benzoquinone is lachrymator; avoid breathing vapor and contact with skin and clothing.) If blanks are high or variable, purify $p$-benzoquinone by steam distn in hood.

(**b**) *Piperazine std solns.*—(*1*) *Stock soln.*—Dissolve exactly 185 mg pure piperazine.2HCl (Salsbury Laboratories, Inc.) (equiv. to 100 mg piperazine) in $H_2O$ and dil. to 250 mL. (*2*) *Working soln.*—20 μg/mL. Dil. 25.0 mL stock soln to vol. in 500 mL vol. flask.

### D. Preparation of Standard Curve

Using working soln, add, by microburet or pipets, 20, 40, 60, 80, and 100 μg piperazine equiv. and intermediate values, if required, into test tubes. Dil. to 5 mL in each test tube with $H_2O$. Include $H_2O$ blank with each detn.

Add 5 mL quinone reagent to each std and blank. Stopper tubes and mix by inverting. Remove stoppers and immerse in $H_2O$ bath at 80 ± 0.1° exactly 10 min. (Bath temp. can be varied; use same temp. for samples and stds.) Immediately immerse tubes in ice bath 3 min. Let stand at room temp. ≥20 min, but ≤40 min. Read $A$ at 490 nm in 1.0 cm cells, using reagent blank to zero instrument. Plot $A$ of each std against μg piperazine.

### E. Determination

Weigh 10.00 g well mixed feed (grind pellets in mortar) into 500 mL (16 oz) wide-mouth, screw-cap bottle. Add exactly 200 mL $H_2O$ from graduate and adjust to pH 4–5 (0.15 mL $H_2SO_4$ (1 + 2) is usually enough for 10 g feed). Cap or stopper bottle and place in wrist-action shaker 30 min. Add ca 5 g Celite (*Note:* Some grades may retain piperazine.) as slurry to buchner contg 9.0 cm Whatman No. 3 paper and pull down under full vac. Wash pad with small portion feed ext and discard washing. Rapidly filter remaining feed ext and reserve filtrate for color development. (Do not delay; turbidity may form.)

Pipet 25.0 mL filtrate into 250 mL vol. flask and dil. to vol. with $H_2O$. Pipet 5 mL aliquot into test tube and immediately proceed with color development as in **967.38D**. Include $H_2O$ blank with each detn.

Prep. sample color blank for each feed as follows: Pipet 5 mL aliquot dild ext into test tube and 5 mL $H_2O$ into another test tube as ref. To each, add 5 mL soln contg 2.5 mL HOAc dild to 100 mL with alcohol. Mix by inverting and omit heating. Read $A$ at same wavelength and subtract from sample reading. Include 1 or 2 stds with each detn to detect shift in std curve; adjust accordingly. Obtain μg piperazine from std curve.

$$\% \text{ Piperazine} = (\mu g \text{ piperazine} \times 10^{-4})/g \text{ sample in aliquot}$$

Ref.: JAOAC **50,** 268(1967).

CAS-110-85-0 (piperazine)

## 978.30 Pyrantel Tartrate in Feeds

**Spectrophotometric Method**

**First Action 1978**

**Final Action 1981**

(Applicable to range 0.0106–0.8811%. Pyrantel tartrate solns are light sensitive. Exts must be protected from direct sunlight or artificial light.)

### A. Apparatus

(**a**) *High-speed blender.*—Waring Blendor, or equiv.

(**b**) *Centrifuge.*—International Model EXD (International Equipment Co.), or equiv., equipped to hold 50 mL g-s centrf. tubes.

(**c**) *Filter aid.*—Celite 545, acid-washed (Manville Filtration and Minerals), or Millipore prefilter pad (No. AP2507500, Millipore Corp.), or equivs.

(**d**) *Filtrator.*—Fisher No. 9-788, low form (Fisher Scientific Co.), or equiv.

(**e**) *Mixer.*—Vortex Genie mixer (Scientific Products), or equiv.

(**f**) *Spectrophotometer.*—For use at 311 nm, with 1 cm cells.

### B. Reagents

(**a**) *Hydrochloric acid.*—0.1*N*. Dil. 8.33 mL HCl to 1 L with $H_2O$.

(**b**) *Leaching soln.*—Dissolve 100 g NaCl in 1 L $H_2O$, add 1 L MeOH, and mix vigorously. Prep. fresh daily.

(**c**) *Perchloric acid.*—0.24*M*. Dil. 20 mL $HClO_4$ to 1 L with $H_2O$.

(**d**) *Sodium hydroxide.*—0.1*N*. Dissolve 4 g NaOH in $H_2O$ and dil. to 1 L with $H_2O$.

(**e**) *Pyrantel tartrate std solns.*—(*1*) *Stock soln.*—0.80 mg/mL. Weigh 80.0 mg Pyrantel Tartrate Ref. Std (available from Pfizer, Inc., Quality Control, Agricultural Div., 1107 S Rt 291, Lee's Summit, MO 64081) into 100 mL vol. flask, dissolve in leaching soln, (**b**), and dil. to vol. with same solv. Use ultrasonic bath to speed soln. If std does not dissolve readily, remake. Prep. fresh daily. (*2*) *Working soln.*—0.16 mg/mL. Pipet 20 mL stock soln into 100 mL vol. flask, dil. to vol. with leaching soln, and mix well. Prep. fresh daily.

### C. Preparation of Samples

Weigh duplicate portions feed ground in high-speed blender, (**a**), into 125 mL erlenmeyers: 15.000 ± 0.010 g for 0.0106% pyrantel tartrate; 1.800 ± 0.010 g, 0.0881%; and 1.000 ± 0.010 g, 0.881%. Add 60 mL leaching soln to 1 portion; add 50 mL leaching soln and, by pipet, 10 mL working std soln, (**e**)(*2*), to other portion of 0.0106% or 0.0881% pairs or 10 mL stock std soln, (**e**)(*1*), to other of 0.881% pair. Cap with polypropylene stopper and shake mech. 1 hr.

Quant. transfer mixt. to buchner precoated with Celite or contg prefilter pad, (**c**), rinsing flask with small portions leaching soln, (**b**), and collecting filtrate under vac. with Filtrator, (**d**), contg 250 mL erlenmeyer, until vol. filtrate is 150 mL. Transfer filtrate to 200 mL vol. flask, and dil. to vol. with leaching soln. For 0.881% level, pipet 50 mL dild filtrate into 250 mL vol. flask, and dil. to vol. with leaching soln.

### D. Determination

Pipet 25 mL prepd sample soln into 250 mL separator contg 5 g finely ground KI, and shake to dissolve. Pipet in 100 mL $CHCl_3$ and shake 45 sec. Transfer lower $CHCl_3$ layer to another 250 mL separator contg 25 mL 0.24*M* $HClO_4$, and shake 10 sec. Transfer ca 35 mL lower $CHCl_3$ layer to 50 mL g-s centrf. tube contg 10.0 mL 0.1*N* NaOH. Stopper tube, and mix on vortex mixer, (**e**), 15 sec while shaking back and forth. Centrf. 10 min at 990 × $g$ (1800 rpm). Pipet 25 mL lower $CHCl_3$ layer from tube into another 50 mL g-s centrf. tube contg 10.0 mL 0.1*N* HCl. Stopper tube, mix, and centrf. as before. Rapidly record $A$ of upper aq. layer at 311 nm against $CHCl_3$-satd 0.1*N* HCl as ref. solv.

$$\% \text{ Pyrantel tartrate} = (A/W) \times [W'/(A' - A)] \times F \times 100$$

where $A$ and $A'$ refer to absorbance of sample and spiked sample, resp.; $W$ = g feed, supplement, or conc.; $W'$ = g pyrantel tartrate ref. std used to prep. std soln; $F$ = [(1/100 mL) × (20 mL/100 mL) × 10 mL] for 0.0106% and 0.0881% levels; and $F$ = [(1/100) × 10] for 0.881% level.

Ref.: JAOAC **61,** 296, 473(1978).

CAS-33401-94-4 (pyrantel tartrate)

## 966.27★ Racephenicol in Feeds

**Final Action 1970**
**Surplus 1975**

### A. Method I—First Action 1966

(Applicable to levels ≥0.002%)

*See* **42.128–42.131**, 12th ed.

### B. Method II—First Action 1968

(Applicable to levels ≥0.0005%)

*See* **42.133–42.136**, 12th ed.

## 960.66★ Reserpine in Feeds

**Photofluorometric Method**

**First Action 1960**
**Final Action 1964**
**Surplus 1989**

(Applicable at 0.2–2.0 ppm level)

*See* **42.148–42.151**, 14th ed.

## 969.56★ Ronnel in Feeds

**Gas Chromatographic Method**

**First Action 1969**
**Final Action 1970**
**Surplus 1989**

*See* **42.152–42.154**, 14th ed.

## 970.87★ Ronnel in Feeds

**Spectrophotometric Method**

**First Action 1970**
**Final Action 1972**
**Surplus 1989**

(For mineral feed mixts contg 1–40% ronnel)

*See* **42.155–42.159**, 14th ed.

## 971.47 Roxarsone in Feeds and Premixes

**Spectrophotometric Method**

**First Action 1971**
**Final Action 1974**

(Not applicable to pelleted feeds contg hemicellulose ext)

### A. Principle

Roxarsone is extd from feed with 2% $K_2HPO_4$. Proteins are pptd at isoelec. point and removed by centrfg. Ext is treated with activated C at pH 12 to remove interferences and roxarsone is detd spectrophtric at 410 nm.

### B. Apparatus

(**a**) *Centrifuge*.—International Model V, or equiv.
(**b**) *Mechanical shaker*.—Burrell wrist-action (Burrell Corp.), or equiv.

### C. Reagents

(**a**) *Potassium phosphate, dibasic, soln*.—2%. Dissolve 2 g $K_2HPO_4$ in 100 mL $H_2O$.
(**b**) *Dilute hydrochloric acid*.—Dil. 45 mL HCl to 200 mL with $H_2O$.
(**c**) *Sodium hydroxide soln*.—Dissolve 24 g NaOH in 100 mL $H_2O$.
(**d**) *Charcoal adsorbent*.—Activated (Darco G-60, or equiv.).
(**e**) *Roxarsone std soln*.—300 μg/mL. Accurately weigh 300 mg Roxarsone Ref. Std (Salsbury Laboratories) into 1 L vol. flask. Dissolve and dil. to vol. with 2% $K_2HPO_4$ soln.

### D. Preparation of Sample

Grind sample in high-speed blender to pass No. 20 sieve (ca 3 min) and mix thoroly. Weigh 15.0 g ground sample into 125 mL erlenmeyer, add 50.0 mL 2% $K_2HPO_4$ soln, place on mech. shaker, and shake vigorously 5 min at room temp. Immediately transfer to 100 mL centrf. tube and centrf. 10 min at 3000 rpm. Perform extn and centrfg with min. delay.

Decant 30 mL aliquot supernate into 50 mL graduated centrf. tube, pipet in 1 mL dil. HCl, stopper, and mix thoroly. Let sample stand until protein flocculates (ca 15 min) and centrf. 10 min at 3000 rpm.

### E. Purification

Pipet 25 mL clear supernate into 125 mL erlenmeyer. Pipet in 1 mL NaOH soln, and mix thoroly. Add 2.0 g activated C and swirl sample several times during 30 min standing. Filter thru Whatman No. 42 fluted paper into 50 mL erlenmeyer. Repeat C treatment with second 2.0 g portion.

### F. Determination

Place 3 mL filtrate in cell and det. *A* at 410 nm against $H_2O$ blank. Add 2 drops concd HCl from dropper (or 5 mL serological pipet), mix, and reread *A*. Difference in *A* of acidic and basic samples represents roxarsone present. Det. amt from std curve.

### G. Preparation of Standard Curve

Pipet 0, 1, 3, 5, 7, and 10 mL aliquots std roxarsone soln into 100 mL vol. flasks and dil. to vol. with 2% $K_2HPO_4$ soln. Pipet 30 mL aliquot from each flask into sep. erlenmeyers, pipet 1 mL dil. HCl into each flask, and mix. Pipet 25 mL aliquot this soln into sep. 50 mL erlenmeyers, pipet in 1 mL NaOH soln, and mix thoroly. Transfer 3 mL soln from erlenmeyers into sep. cells and det. *A* at 410 nm against $H_2O$ blank. Add 2 drops concd HCl from dropper (or 5 mL serological pipet), mix, and reread *A*. Plot difference in *A* of std solns against concn of solns expressed as 0, 0.001, 0.003, 0.005, 0.007, and 0.010% roxarsone in feed when 15 g sample is used.

### H. Determination in Premix

Weigh appropriate size sample of premix, place in 200 mL vol. flask, and add 50 mL 2% $K_2HPO_4$ soln and 4.0 mL NaOH soln. Let stand 20 min, shaking occasionally; then dil. to vol. with 2% $K_2HPO_4$ soln and let feed particles settle 30 min. Proceed as in **971.47D**, par. 2, using appropriate aliquots and dilns to give final concns in range of std curve.

(*Example:* 5% Premix.—Place 5 g thoroly mixed premix in 200 mL vol. flask and ext as above. Transfer 10 mL aliquot ext to 100 mL vol. flask, dil. to vol. with $H_2O$, and mix thoroly. Transfer 10 mL aliquot this soln to another 100 mL vol. flask and dil. to vol. with $H_2O$. Diln factor for this premix is 1200.)

After diln, transfer 30 mL aliquot to 125 mL erlenmeyer. Pipet in 1 mL dil. HCl and mix. Transfer 25 mL this soln to 125 mL erlenmeyer, pipet in 1 mL NaOH soln, mix thoroly, add 2.0 g activated C, shake several times during 30 min, and filter. Repeat C treatment. Read *A* of filtrate and det. concn of roxarsone as in **971.47F**, multiplying by diln factor.

Ref.: JAOAC **54,** 80(1971).

CAS-121-19-7 (roxarsone)

## 986.39 Roxarsone in Feeds

### Atomic Absorption Spectrophotometric Method

**First Action 1986**
**Final Action 1989**

(Method dets total As and is not specific for roxarsone. Applicable range is 0–50 ppm 4-hydroxy-3-nitrobenzene arsonic acid.)

#### *A. Principle*

Sample is extd with aq. ammonium carbonate soln and analyzed by furnace AAS for total As content, which is converted by factor to roxarsone concn in finished feed.

#### *B. Reagents*

(**a**) *Water.*—Super Quality from Millipore Super Q system.
(**b**) *Nitric acid.*—Mallinckrodt, ACS grade.
(**c**) *Argon.*—Linde purified.
(**d**) *Nickel nitrate.*—$Ni(NO_2)_2.6H_2O$ (Mallinckrodt AR).
(**e**) *Nickel nitrate soln.*—Approximately 2000 ppm Ni. Dissolve 10.0 g $Ni(NO_3)_2.6H_2O$ in $H_2O$ and dil. to 1 L with $H_2O$.
(**f**) *Ammonium carbonate.*—Powder, purified (EM Science No. AX1260).
(**g**) *Methanol.*—Anhyd., ACS (EM Science No. MX0485).
(**h**) *Diluting soln.*—Add 5 mL concd $HNO_3$ and 150 mL anhyd. MeOH to 1 L vol. flask, dil. to vol. with $H_2O$, and mix.
(**i**) *Tantalum pentoxide.*—99.99% $Ta_2O_5$ (Aldrich Chemical Co.).
(**j**) *Tantalum pentoxide soln.*—Suspend 2.0 g in 10 mL $H_2O$.
(**k**) *Roxarsone std soln.*—1250 ppm roxarsone (356 ppm As). Accurately weigh 625.0 mg roxarsone ref. std (Salsbury Laboratories, Inc., 2000 Rockford Rd, Charles City, IA 50616) into 500 mL vol. flask. Dissolve and dil. to vol. with 2% ammonium carbonate soln. *Caution:* Wear protective clothing and avoid breathing dust.
(**l**) *Dilute roxarsone std soln.*—12.5 ppm roxarsone (3.56 ppm As). Dil. 10.0 mL roxarsone std soln to 1 L with $H_2O$.
(**m**) *Control feed extract.*—Using typical nonmedicated poultry or swine ration, prep. feed ext as described under *Sample Preparation.* Test suitability of control feed ext by dilg 1 mL aliquot with dilg soln used in sample prepn. Set up AAS system and furnace conditions as described in procedure. It is not necessary to perform calibration for this test; absorbance reading is satisfactory. Zero spectrophtr on 20 μL injection of dilg soln and measure *A* on 20 μL control feed ext. Absorbance reading ≤0.010 indicates suitability.
(**n**) *Working std soln.*—Transfer 1.0 mL dil. roxarsone std soln to 10 mL vol. flask. Dil. to vol. with control feed ext. Transfer 1.0 mL aliquot of this soln to 25 mL erlenmeyer and add 9.0 mL dilg soln. Twenty μL working std soln = 50 ppm roxarsone in feed sample for wts and vols used in procedure.

#### *C. Apparatus*

(**a**) *Atomic absorption spectrophotometer.*—Perkin-Elmer Model 5000, or equiv., with heated graphite atomizer furnace, autosampler, and printer sequencer.
(**b**) *Mechanical shaker.*—Wrist-action.
(**c**) *Pipets.*—Eppendorf: 10, 20, 50, and 1000 μL.
(**d**) *Dispensing pipet.*—Repipet (Labindustries, 620 Hearst Ave, Berkeley, CA 94710), or equiv., 10 mL capacity set to deliver 9.0 mL dilg soln.

#### *D. Sample Preparation*

Grind sample in Wiley mill to pass 20 mesh sieve and thoroly mix. Weigh 5.0 g ground sample into 250 mL vol. flask or 300 mL erlenmeyer. Add 2.0 g ammonium carbonate powder and 200.0 mL $H_2O$, place on mech. shaker, and shake vigorously 5 min at room temp. Remove flask from shaker and let suspended feed particles settle 15–30 min. Transfer 1.0 mL aliquot of feed ext to 25 mL erlenmeyer and add 9.0 mL dilg soln. Mix thoroly. Repeat this step on reagent blank and on std-fortified control feed ext equiv. to 50 ppm roxarsone in feed. Samples and std are now ready for furnace AAS analysis.

#### *E. AAS Conditions*

Set up graphite furnace and spectrophtr according to following conditions and allow 30 min warm-up time. Operating conditions: lamp, As EDL operated at 8 watts, properly aligned; lamp current, 0 ma; wavelength, 193.7 nm; slit, 0.7 nm bandpass, low position; readtime, 5 s; mode, AA-BG; readout, concn; signal, peak ht on instrument display and *A* on recorder if used; std 1, S1 = 50.0 ppm (μg/g) roxarsone (use 3 digits), do not use S2 and S3.

Install furnace assembly in AAS system and align as in manufacturer's instructions.

#### *F. Furnace Tube Coating Procedure*

Prep. 20% $Ta_2O_5$ aq. suspension. Shake suspension vigorously, introduce 50 μL aliquot into pyrolytically coated graphite furnace tube, and perform following sequence of operations: $H_2O$ flow, 1–2 L/min to cool furnace; Ar pressure, 35 psi; on/off switch, on; gas control, on.

Step 1 (drying): temp., 100°; ramp time, 10 s; hold time, 90 s.

Step 2 (charring): temp., 1000°; ramp time, 10 s; hold time, 30 s.

Step 3 (atomizing): temp., 2700°; ramp time, 5 s; hold time, 10 s; stop flow, on.

Repeat coating procedure twice (3 applications). Tube is now ready for furnace AAS use.

(With initial coating, some material may flake after approx. 35 firings. If this happens, pass small brush or Kimwipe thru tube to remove loose tantalum and apply single recoating.)

#### *G. Furnace Conditions for Sample Assay*

Step 1 (drying): temp., 100°; ramp time, 10 s; hold time, 50 s.

Step 2 (charring): temp., 1000°; ramp time, 10 s; hold time, 30 s.

Step 3 (atomizing): temp., 2300°; ramp time, 0 s; hold time, 5 s; read, on; stop flow, on.

Step 4 (burnout): temp., 2400°; ramp time, 0 s; hold time, 5 s; read, off; flow, 300 mL/min (stop flow, off).

#### *H. Autosampler Conditions*

Install autosampler assembly in furnace and align as per manufacturer's instructions. Operating conditions: power switch, on, and let autosampler go thru count down; program sequence, press standby key to bring programmer into operating mode; method number, enter 1 and press method # key; recalibrate, for full tray recalibrate at 9A, 18B, and 27C; last sample, program number for last sample vol. key; alternate vol., enter 10 μL and press alternate vol. key ($Ni(NO_3)_2.6H_2O$ soln, 2000 ppm Ni); instrument program, enter 1 and press instrument program key; HGA program, enter 1 and press HGA program key.

Use sample tray 1 with method 1 in autosampler sequence. This method uses external std technic for instrument calibration. In this procedure, only position S1 is used because calibration is based on single point std with calibration std equiv. to 50.0 ppm roxarsone in finished feeds.

### I. Sample Analysis

Load sample tray 1 as follows: Place blank of reagents (dilg soln (**h**)) in AZ location of tray; place 50.0 ppm roxarsone feed std in S1 location and at position 1 (check sample), then load samples in sequence around tray, starting at position 2. Place 2000 ppm Ni soln in reagent container and place in appropriate location for alternate sample in autosampler. Cover sample tray with cover provided to minimize evapn; these solns contain MeOH.

Instrument and samples are now ready for calibration and sample analysis. Press start/stop key to start program in sampling cycle. AZ and S1 calibration should be done in duplicate. Observe *A* for duplicate S1 values. These values should be within reasonable agreement (± 5%) before program is allowed to proceed with samples. Instrument is recalibrated by autosampler in setup instructions which will monitor calibration for any changes and update calibration as time progresses.

### J. Calculations

Instrument is programmed to calc. sample ppm on basis of single point std equiv. to 50 ppm roxarsone. For wts or vols other than those specified, manual calcn is required.

Ref.: JAOAC **69,** 838(1986).

CAS-121-19-7 (roxarsone)

## 970.88 Sulfadimethoxine in Feeds
### Colorimetric Method
### First Action 1970

### A. Reagents and Apparatus

See **969.53A(b)**, (**c**), and (**d**), and in addn:

(**a**) *Ficin soln*.—0.2%. Disperse 500 mg ficin (Calbiochem, fig latex) in $H_2O$ (preheated to 40°) and dil. to 250 mL. Use 10 mL of this warm soln in detn. (*Caution:* Ficin is very potent proteolytic enzyme which attacks living tissues. Avoid contact with skin and eyes and breathing dust.) (Ficin product listed is no longer available. Method may be satisfactory without ficin treatment, but users should verify recovery.)

(**b**) *Petroleum ether*.—Bp 35–60°, purified on silica gel column.

(**c**) *Trichloroacetic acid soln*.—3%. Dissolve 30 g $CCl_3COOH$ in $H_2O$ and dil. to 1 L. (*Caution:* See safety notes on trichloroacetic acid.)

(**d**) *Sulfadimethoxine std soln*.—Accurately weigh 125 mg sulfadimethoxine USP Ref. Std and transfer quant. to 100 mL vol. flask. Add ca 70 mL acetone and shake until completely dissolved. Dil. to vol. with acetone and mix. Pipet 20 mL soln into 200 mL vol. flask, dil. to vol. with acetone, and mix. Pipet 10 mL (or 5 mL if working at 0.00625% level) of last diln into 200 mL vol. flask contg 10 mL 0.2% ficin soln. Add ca 120 mL acetone and 2 mL 40% NaOH soln, and mix. Dil. to vol. with acetone and mix. Pipet 25 mL final diln into 50 mL g-s centrf. tube, evap. almost to dryness under N stream in 50° $H_2O$ bath, and proceed as in **970.88B**, beginning "Pipet 15 mL pet ether into centrf. tube . . ." Resulting clear filtrate is std soln.

(**e**) *Reagent blank*.—Into 200 mL vol. flask pipet 10 mL 0.2% ficin soln and 2 mL 40% NaOH soln. Dil. to vol. with acetone and mix. Pipet 25 mL of this soln into 50 mL g-s centrf. tube, evap. almost to dryness under N stream in 50° $H_2O$ bath, and proceed as in **970.88B**, beginning "Pipet 15 mL pet ether into centrf. tube . . ." Resulting clear filtrate is blank soln.

(**f**) *Spectrophotometer*.—With 5 cm cells, or photoelec. colorimeter with 540 nm filter, or equiv.

### B. Preparation of Sample

(*Caution:* See safety notes on blenders, pipets, flammable solvents, and acetone.)

Pipet 10 mL 0.2% ficin soln into high-speed blender. Accurately weigh 10 g sample into blender, spreading carefully on surface of liq. Let sample soak 10 min.

Add ca 120 mL acetone. Blend 2 min, adjusting speed with variable transformer, so that acetone does not wet screw cap. (*Note:* To release pressure, stop blending after 3–4 sec and unscrew cap momentarily.) Blend 2 min and remove screw cap. Push down into acetone all solid particles adhering to container wall, using rubber policeman. Replace screw cap and continue blending 1 min. Remove screw cap, pipet 2 mL 40% NaOH soln into container, and continue blending 2 min. Push down into acetone all solid particles adhering to wall of container, using rubber policeman.

Quant. transfer blender contents to 250 mL g-s graduate, using small portions acetone to total vol. of 200 mL. Stopper, mix well, and let liq. and solids sep. Wrap tip of 50 mL pipet with glass wool and transfer ca 40 mL ext to 50 mL g-s centrf. tube. Centrf. 5 min at 2000 rpm. Pipet 25 mL clear acetone ext into another centrf. tube and evap. almost to dryness (only few drops of oily, sirupy liq. left) under N stream in 50° $H_2O$ bath. Pipet 15 mL pet ether into centrf. tube and dissolve or disperse residue in it.

Pipet 25 mL 0.2*N* NaOH into centrf. tube, stopper, and shake mech. 5 min. Centrf. 10 min at 2000 rpm. By pipet, transfer lower NaOH layer (ca 24 mL) into another centrf. tube and centrf. 10 min at 2000 rpm. Pipet 20 mL clear soln into 100 mL vol. flask. Dil. to vol. with 3% $CCl_3COOH$ soln. Mix and let stand 10 min. Filter entire soln thru Whatman No. 42 paper, discarding first 10 mL filtrate. If turbid, filter thru second paper. Clear filtrate is sample soln.

### C. Determination

(**a**) *Reading on spectrophotometer with 5 cm cells*.—Pipet following vols (mL) of indicated solns into 6 sep. labeled 25 mL vol. flasks:

| Soln | Sample 1 | Sample 2 | Sample Blank | Std 1 | Std 2 | Reagent Blank |
|---|---|---|---|---|---|---|
| Sample | 15 | 15 | 15 | — | — | — |
| Std | — | — | — | 15 | 15 | — |
| Blank | — | — | — | — | — | 15 |

Pipet 1 mL 0.1% $NaNO_2$ soln, **969.53A(b)**, into each, mix, and let stand 3 min. Pipet 1 mL 0.5% $NH_4$ sulfamate soln, **969.53A(c)**, into each, mix, and let stand 2 min. Pipet 1 mL 0.1% coupling reagent, **969.53A(d)**, into all except sample blank, and 1 mL $H_2O$ into sample blank. Mix and let stand 10 min in dark. Dil. each flask to vol. with $H_2O$ and mix. Measure *A* of each soln at 540 nm in 5 cm cell against reagent blank in ref. cell.

(**b**) *Reading on photoelectric colorimeter*.—Proceed as in (**a**) except do not dil. after standing, but read at existing vol.; 50 mL g-s centrf. tubes may be used in place of vol. flasks. Transfer solns from flasks or tubes to matched colorimeter tubes. Set instrument with 540 nm filter to 100% *T* (0 *A*) with tube contg reagent blank. Det. $A$ (= 2 − log (%$T$)) of each of other tubes contg samples, sample blank, and stds.

(**c**) *Calculation*.—Higher levels of sulfadimethoxine:

$$\% \text{ Sulfadimethoxine} = [(A - A_0) \times S]/(1000 \times A' \times W)$$

Lower levels of sulfadimethoxine:

$$\% \text{ Sulfadimethoxine} = [(A - A_0) \times S]/(2000 \times A' \times W)$$

where $A$, $A_0$, and $A'$ refer to sample, reagent blank, and std, resp.; $W$ = g original sample; and $S$ = mg std weighed.

Ref.: JAOAC **53,** 638(1970); **72,** 106(1989).

CAS-122-11-2 (sulfadimethoxine)

## 951.07★ Sulfaguanidine in Feeds

**Spectrophotometric Method**

**First Action**

**Surplus 1989**

*See* **42.171**, 14th ed.

## 969.57 Sulfamethazine in Feeds

**Spectrophotometric Method**

**First Action 1969**

(Applicable to feeds contg procaine penicillin. Not applicable to feeds made from granule-stabilized Tylan-Sulfa premixes.)

### A. Reagents

See **969.53A(b)**, (**c**), and (**d**) and in addn:

(**a**) *50% Methanol soln.*—50% (v/v) aq. soln of MeOH.

(**b**) *Sulfamethazine std solns.*—(*1*) *Stock soln.*—Accurately weigh 0.100 g pure sulfamethazine (available from American Cyanamid Co.) into 100 mL vol. flask. Add 50 mL 50% MeOH soln and shake until dissolved. Dil. to vol. with 50% MeOH soln. Soln is stable at least several weeks. (*2*) *Intermediate soln.*—Pipet 5 mL stock soln into 200 mL vol. flask, dil. to vol. with 50% MeOH, and mix well. Soln is also stable several weeks. (*3*) *Working soln.*—2.5 μg/mL. Pipet 10 mL intermediate soln into 100 mL vol. flask, add 1 mL HCl and 50 mL 50% MeOH, dil. to vol. with $H_2O$, and mix well. Soln is stable ca 2 weeks.

### B. Preparation of Sample

Weigh 5.00 g sample into 250 mL g-s erlenmeyer. Add 100.0 mL 50% MeOH soln, shake well on mech. shaker 1 hr, and centrf. Pipet aliquot supernate contg ca 250 μg sulfamethazine into 100 mL vol. flask, add 50% MeOH, if necessary, to vol. of ca 60 mL, followed by 1.0 mL HCl, and 10 mL *1% $ZnSO_4$ soln*. Let stand 10 min, dil. to vol. with $H_2O$, and mix.

### C. Determination

Filter portion of prepd soln thru Whatman No. 42 paper, or equiv., into 250 mL flask. Filtrate should be clear. Pipet two 10 mL aliquots filtrate and 10 mL working std soln into sep. 50 mL centrf. tubes. To each tube add 1.0 mL 0.1% $NaNO_2$ soln; mix and let stand 3 min. Add 1.0 mL 0.5% $NH_4$ sulfamate soln; mix and let stand 2 min. Add 1.0 mL 0.1% *N*-(1-naphthyl)ethylenediamine.2HCl soln to one of sample solns and to std soln. To second sample soln add 1.0 mL $H_2O$ (sample blank). Mix all solns well and let stand 10 min.

To sample, sample blank, and std soln add ca 10 mL $CHCl_3$, stopper, and shake *vigorously* 30 sec (30 sec is required to ensure complete removal of procaine dye). Add 0.8 mL 10*N* NaOH to sample, sample blank, and std soln. Stopper and shake vigorously ≥1 min to ensure complete removal of procaine dye. Centrf. solns at 2000 rpm 5 min or until aq. layer is completely clear. Remove 10.0 mL aq. phase with pipet and transfer to 50 mL erlenmeyer or 50 mL beaker. Add 1.0 mL HCl and remove fumes formed in flask with aspirator or air stream.

Read $A$ of sample, sample blank, and std ($A'$) at 540 nm in spectrophtr, against $H_2O$ blank. Correct $A$ of sample by subtracting that of sample blank.

$$\% \text{ Sulfamethazine} = (A/A') \times (2.5\ \mu g/mL) \times (100\ mL/mL \text{ ext aliquot taken}) \times (100\ mL/5\ g) \times (1\ g/10^6\ \mu g) \times 100$$

Refs.: JAOAC **51,** 1282(1968); **72,** 106(1989).

CAS-57-68-1 (sulfamethazine)

## 963.34 Sulfanitran in Feeds

**Spectrophotometric Method**

**Final Action**

### A. Reagents

*See* **969.53A(b)**, (**c**), and (**d**) and in addn:

*Sulfanitran std solns.*—(*1*) *Stock soln.*—100 μg/mL. Accurately weigh 100 mg pure sulfanitran (available from Salsbury Laboratories, Inc.) into 1 L vol. flask, add enough 1*N* NaOH for complete soln, and dil. to vol. with $H_2O$. (*2*) *Working soln.*—10 μg/mL. Dil. 10 mL stock soln to 100 mL with $H_2O$.

### B. Preparation of Standard Curve

Pipet 0, 4, 6, 8, and 10 mL aliquots working std soln into sep. 50 mL vol. flasks. Add 0.5 mL HCl and adjust vol. with $H_2O$ to ca 15 mL. Place flasks in boiling $H_2O$ bath 1 hr to deacetylate. Cool, and dil. to vol. Transfer 5 mL aliquot from each flask to sep. colorimeter tubes. Develop color by adding 0.5 mL 0.1% $NaNO_2$ soln (not >5 days old, stored in refrigerator), 0.5 mL 0.5% $NH_4$ sulfamate soln, and 0.5 mL coupling reagent. Det. $A$ at 540 nm against reagent blank (0 mL aliquot).

(To establish most reliable std curve, make detns on 3 sep. days and use av. values.)

### C. Extraction and Deacetylation

(For premixes, use proper dilns to give 5–10 μg sulfanitran in final aliquot, taking dilns into consideration in final calcn.)

Weigh 5.0 g sample into 100 mL vol. flask and add 80 mL MeOH. Place flask in 60° $H_2O$ bath until MeOH is hot. Repeatedly remove and immerse flask during 20 min, shaking frequently. Cool to room temp., and dil. to vol. with MeOH. Shake thoroly, and let stand 40 min to permit particles to settle.

Pipet 25 mL aliquot MeOH ext into 50 mL vol. flask. Add 10 mL $H_2O$, 5 mL *1.0% $ZnSO_4$ soln*, and ca 3 drops 1*N* NaOH to improve flocculation. (Keep near neutrality.) Place flask in boiling $H_2O$ bath 2 min to aid pptn; then cool to room temp., dil. to vol., mix thoroly, and filter thru Whatman No. 42 paper, or equiv. Discard first 5 mL filtrate.

Pipet 10 mL aliquot filtrate into 50 mL vol. flask contg 8.0 mL $H_2O$ and 0.5 mL HCl. Place flask in boiling $H_2O$ bath 1 hr to evap. MeOH and deacetylate sulfanitran, shaking frequently during first 15 min. Cool to room temp. and dil. to vol. with $H_2O$. Centrf. if turbidity appears.

### D. Determination

Place 5.0 mL aliquot in each of 2 colorimeter tubes. To 1 tube (blank) add 1.0 mL $H_2O$ and 0.5 mL coupling reagent. To other tube add 0.5 mL 0.1% $NaNO_2$ soln; after 3 min, add 0.5 mL 0.5% $NH_4$ sulfamate soln, wait 2 min, and add 0.5 mL coupling reagent. Close tube with thumb and invert immediately after adding each reagent. Let stand 10 min for color development and det. $A$ of sample and blank at 540 nm in spectrophtr or colorimeter against $H_2O$. Det. amt sulfanitran from std curve after subtracting $A$ of blank.

$$\% \text{ sulfanitran in sample} = \mu g \text{ sulfanitran in tube} \times 200 \times 100/(5{,}000{,}000 \times \mu g \text{ sample}) = \mu g \text{ sulfanitran} \times 0.004$$

Ref.: JAOAC **46,** 452(1963).

CAS-122-16-7 (sulfanitran)

## 963.35 Sulfaquinoxaline in Feeds

### Spectrophotometric Method
### First Action 1973
### Final Action 1988
### *Method I*

(Applicable only to nonpelleted feeds contg arsanilic acid. In absence of arsanilic acid, use **963.35F.**)

#### *A. Principle*

Sulfaquinoxaline is extd from feed with DMF and sepd from interfering substances by column chromatgy on alumina. Isolated sulfaquinoxaline is acidified, diazotized, and coupled in presence of Zr, and colored complex is extd with BuOH and measured at 550 nm. Arsanilic acid remains in final aq. soln and can be measured at 540 nm and compared with std treated similarly.

#### *B. Reagents*

*See* **969.53A(b)**, **(c)**, and **(d)** and in addn:

**(a)** *Alkaline salt soln.*—Dissolve 2.0 g NaOH and 100.0 g NaCl in 500 mL $H_2O$.

**(b)** *Zirconium soln.*—Dissolve 5.0 g zirconyl chloride, $ZrOCl_2.8H_2O$ (Fisher Scientific Co.), in 100 mL $H_2O$.

**(c)** *Sulfaquinoxaline std solns.*—(*1*) *Stock soln.*—Weigh 40.0 mg Sulfaquinoxaline Ref. Std (available from Merck & Co., Inc.) and dissolve in 50.0 mL DMF. Soln is stable at least 1 month if kept tightly stoppered and protected from light. (*2*) *Intermediate soln.*—80 μg/mL. Dil. 5 mL stock soln to 50 mL with DMF. (*3*) *Working soln.*—8 μg/mL. Dil. 5 mL intermediate soln to 50 mL with DMF. Prep. from freshly prepd intermediate soln just before use.

**(d)** *Butanol mixture.*—Mix 100 mL *n*-hexane with 400 mL *n*-BuOH.

#### *C. Preparation of Sample*

Weigh 4.00 g ground feed sample into 100 mL vol. flask. Add 50.0 mL DMF, stopper, and agitate by mag. stirrer or mech. shaker 60 min. Transfer mixt. to 50 mL centrf. tube and centrf. 5 min at 2500 rpm.

#### *D. Chromatography*

**(a)** *Preparation of column.*—Constrict end of 50–60 cm length of 9–11 mm id glass tubing by rotating in hot flame until opening is 4–5 mm. Insert small plug of Pyrex glass wool in lower end and compress with glass rod to thickness of 2–3 mm. Transfer 5.0 g alumina, **961.24B(b)**, to dry tube and pack by gentle tapping while applying vac.

**(b)** *Separation.*—Pipet 10 mL clear ext onto column and let pass thru by gravity. Do not let column run dry; keep 5 mm head of liq. Wash inner walls with two 5.0 mL portions $CHCl_3$. Let final washing drain until no further liq. appears at tip. Discard effluent and washings. Attach column tip to vac. and draw air thru until alumina is dry, indicated by tube returning to room temp. Elute column by gravity with 25 mL alk. salt soln, collecting eluate in 25 mL vol. flask. Add 1.0 mL HCl to eluate, dil. to vol. with $H_2O$, and mix well.

Prep. reagent blank by transferring 10 mL DMF onto fresh column and proceeding as for sample. Prep. std by transferring 10.0 mL sulfaquinoxaline working std soln onto fresh column and proceeding as for sample.

#### *E. Determination*

Transfer 10 mL aliquots of each eluate to sep. centrf. tubes. Add 2.0 mL Zr soln and mix. Add 1.0 mL *0.1% $NaNO_2$ soln,* mix, and let stand 2 min. Add 1.0 mL *0.5% $NH_4$ sulfamate soln,* mix, and let stand 2 min. Add 1.0 mL coupling reagent, **969.53A(d)**, mix, and let stand 10 min. Add 2.0 g NaCl and 10.0 mL BuOH mixt., stopper, and shake vigorously until NaCl dissolves. Centrf., carefully transfer portion of clear, colored top solv. layer to 1 cm cell, and read $A$ at 550 nm against BuOH mixt. Correct for reagent blank.

$$\% \text{ Sulfaquinoxaline} = 0.04 \times (A/A')/W$$

where $A$ and $A'$ refer to sample and std (blank corrected), resp., and $W$ = g sample.

### *Method II—Final Action 1960*

(Applicable in absence of arsanilic acid)

#### *F. Determination*

Weigh 5 g ground sample into 250 mL vol. flask, add 150 mL $H_2O$ and 5 mL 0.5*N* NaOH, and place in boiling $H_2O$ bath 15 min. Remove, cool, dil. to vol. with $H_2O$, mix, and let settle. Transfer 50 mL supernatant to 100 mL vol. flask, add 3 mL HCl, and dil. to vol. Mix, and filter thru 18.5 cm Whatman No. 2 paper (or equiv.), discarding first 15 mL filtrate if turbid.

To 10 mL filtrate in each of two 50 mL beakers add 2 mL *freshly prepd 0.1% $NaNO_2$ soln* and let stand 3 min. Add 2 mL *0.5% $NH_4$ sulfamate soln* and let stand 2 min. Add 1 mL coupling reagent, **969.53A(d)**, to first beaker and 1 mL $H_2O$ to second beaker. Mix thoroly after adding each reagent. After 10 min, read $A$ in spectrophtr at 545 nm. Subtract $A$ of feed blank from sample $A$ and det. amt of sulfaquinoxaline from std curve. Divide by 1000 to obtain % sulfaquinoxaline.

Prep. std curve as follows: Dissolve 0.250 g pure sulfaquinoxaline in 5 mL 0.5*N* NaOH and 50 mL $H_2O$ in 500 mL vol. flask, and dil. to vol. with $H_2O$. Pipet 5 mL aliquot of this soln into 100 mL vol. flask and dil. to vol. with $H_2O$. Pipet 2, 4, 6, 8, and 10 mL portions of this dild soln (equiv. to 50, 100, 150, 200, and 250 μg sulfaquinoxaline, resp.) into sep. 100 mL vol. flasks, add 3 mL HCl to each flask, and dil. to vol. with $H_2O$. Treat 10 mL aliquots of these final dilns as in second par. Det. $A$ at 545 nm against $H_2O$ blank, and plot $A$ against μg sulfaquinoxaline.

Refs.: JAOAC **33,** 156(1950); **38,** 229(1955); **39,** 307(1956); **56,** 758(1973); **59,** 399(1976); **62,** 423(1979).

CAS-59-40-5 (sulfaquinoxaline)

## 974.46 Sulfonamides in Feeds

### Spectrophotometric Method
### First Action 1974

(Applicable to premixes and concs)

#### *A. Determination of Absorptivities*

Prep. sep. std solns of sulfathiazole (SZ), sulfamerazine (SM), and sulfamethazine (SH) by accurately weighing ca 50 mg each compd and transferring to sep. 50 mL vol. flasks; add 5 mL alcohol and 2 mL $NH_4OH$, and swirl to dissolve. For sulfaquinoxaline (SQ), weigh 45 mg, warm on steam bath to dissolve, and cool. Dil. each flask to vol. with alcohol, and mix. Evap. 2 mL aliquots to dryness in sep. small beakers, transfer to sep. 200 mL vol. flasks (100 mL for SQ) with several small portions 0.1*N* NaOH, and dil. to vol. with 0.1*N* NaOH. Also dil. 25 mL aliquot SQ soln to 50 mL with 0.1*N* NaOH. Obtain spectrum of each soln in 1 cm cell against 0.1*N* NaOH from 400 to 220 nm.

$$\text{Calc. } a_{255}\ (1.00\ \text{mg}/100\ \text{mL}) = A \times 50/W$$

where *A* is reading at max., ca 255 nm, corrected for *A* at 400 nm, if any, and *W* is mg compd weighed. (For SQ, use *A* of dild soln.)

For SQ, also calc. $a_{358}$ (2.00 mg/100 mL) = $A \times 50/W$, where *A* is reading at max., ca 358 nm, corrected for *A* at 400 nm, if any, of more concd soln.

### B. Preparation of Sample

(**a**) *Solids*.—Transfer accurately weighed sample contg ca 50 mg sulfonamide with lowest concn to 50 mL vol. flask, add 5 mL alcohol and 2 mL $NH_4OH$, and warm 10 min on steam bath. Cool to room temp., dil. to vol. with alcohol, and mix.

(**b**) *Liquid concentrates*.—Pipet aliquot contg ca 200 mg sulfonamide with lowest concn into 200 mL vol. flask, dil. to vol. with alcohol, and mix.

For each sulfonamide declared, calc. an *R* value to 2 decimal places by dividing its labeled amt by that of sulfonamide with lowest labeled amt, whose *R* = 1.00. Calc. to 2 decimal places. Designate each ratio as $R_{SQ}$, $R_{SZ}$, $R_{SM}$, and $R_{SH}$, and their sum as $R_T$. (If 4 sulfonamides are present in equal amts, all *R* = 1.00 and $R_T$ = 4.00.)

### C. Determination of Sulfaquinoxaline

Pipet 5 mL sample soln into g-s flask and add accurately measured vol. alcohol so that total mL of final soln = 10 × $R_T$ (*Soln I*). Mix, pipet 10 mL into small beaker, and evap. to dryness on steam bath. Transfer residue to 100 mL vol. flask with several small portions 0.1*N* NaOH, dil. to vol. with 0.1*N* NaOH, and mix (*Soln II*). Obtain spectrum from 400 to 300 nm, and det. *A* at max., ca 358 nm.

$$\%\ \text{SQ} = [A_{358} \times 2 \times R_T \times (V/5) \times 100]/(a_{358} \times S)$$

where *V* = mL original sample soln (50 or 200), and *S* = mg original sample (for solids) or mL × 1000 (for liqs).

If Na salt declared, NaSQ = SQ/0.9318.

### D. Determination of Total Sulfonamides

Dil. 20.0 mL *Soln II* to 100 mL with 0.1*N* NaOH. Obtain spectrum from 400 to 230 nm and det. $A_T$ at max., ca 255 nm.

$$\%\ \text{Total sulfonamides} = [A_T \times V \times R_T^2 \times 100]/ [(R \times a)_{SQ} + (R \times a)_{SZ} + (R \times a)_{SM} + (R \times a)_{SH}] \times S$$

If Na salts are present, multiply each *a* by appropriate factor: NaSQ, 0.9318; NaSZ, 0.9207; NaSM, 0.9232; and NaSH, 0.9268.

Ref.: JAOAC **57**, 345(1974).

CAS-127-79-7 (sulfamerazine)
CAS-57-68-1 (sulfamethazine)
CAS-59-40-5 (sulfaquinoxaline)
CAS-72-14-0 (sulfathiazole)

## 974.47 Sulfonamides in Feeds
### Thin Layer Chromatographic Method
### First Action

### A. Preparation of Plates

(**a**) *Plates A*.—Weigh 30 g silica gel H or HF 254 (Brinkmann Instruments, Inc.) into 250 mL g-s flask. (Add 100 mg fluorescent indicator H 254 if silica gel H is used.) Add 70 mL $H_2O$ and shake well 1 min. Coat five 20×20 cm plates with 0.25 mm layer and air dry. Do not dry in oven and do not store in presence of drying agent.

(**b**) *Plates B*.—Proceed as in (**a**) but use 0.1*N* NaOH instead of $H_2O$ to slurry silica gel.

### B. Preparation of Blanks

Develop a plate A in $CHCl_3$-MeOH (97 + 3) to ht of ca 15 cm. Scrape 2 spots, each ca 2 sq cm, from developed section of plate, into centrf. tubes and ext with 10 mL 0.1*N* NaOH. Scrape 2 addnl spots, each ca 4 sq cm, from developed portion and ext with 50 mL 0.1*N* NaOH. Centrf. the 4 exts 5 min at high speed and decant most of soln into sep. small beakers, being careful not to disturb sediment.

Develop a plate B in $CHCl_3$-MeOH (90 + 10) and proceed as above.

Det. *A* at max., ca 255 nm, of all 8 exts. *A* should be ≤0.04. If readings are low and reproducible, average each set of 4 values for 2 and 4 sq cm exts, resp. If readings are high or not reproducible, recentrf. exts at higher speed, and be very careful to exclude sediment during decanting. Av. blank *A* may be used for all detns which use same batch of silica gel and same speed of centrfg.

### C. Thin Layer Chromatography

Spot 100 μL sample soln, **974.46B**, on plate A and plate B by repeated application of adjacent drops on line 4 cm long, drying each drop with gentle air current before applying another drop at same place. Develop plate A in $CHCl_3$-MeOH (93 + 7) and plate B in (90 + 10) until solv. front reaches top of plate.

View plates under shortwave UV light and delineate each spot with dissecting needle, including small margins whenever possible. Plate A should have 2 completely sepd spots, designated as *c* (lower: SZ) and *d* (upper: SM + SH + SQ); plate B should have 3 completely sepd spots designated as *f* (lower: SQ + SZ), *h* (middle: SM), and *p* (top: SH). If less than indicated no. of spots are sepd, spot another aliquot over slightly longer line and develop as before.

Use collection tube consisting of 7–8 cm of 8 mm od glass tubing with short constriction at one end and medium fritted glass disk fused near center. Clean tube with strong air current and attach wide tube to vac. Draw as much of spot as possible into tube, using narrow tube to loosen adsorbent layer. Without disconnecting vac., transfer tube to 10 mL vol. flask for spot *c* and 50 mL for spot *d*. Release vac., and transfer material to flask with repeated gentle tapping. Repeat until entire spot has been transferred; then blow out tube into flask with gentle air current to remove last of particles.

Fill each flask ca 1/2 full with 0.1*N* NaOH, swirl well 1 min, dil. to vol. with 0.1*N* NaOH, and mix. Centrf. all or ≥10 mL each soln at same speed used for blanks and carefully decant ca 8 mL into small beakers. Obtain spectrum of each soln from 400 to 230 nm and record $A_c$ of spot *c* ext and $A_d$ of spot *d* ext at max., ca 255 nm. Correct $A_c$ for av. blank of 2 sq cm exts and $A_d$ for av. blank of 4 sq cm exts.

Calc. recovery factor, $F = [(5 \times A_d) + A_c]/(R_T \times A_T)$

$$\% \text{ SZ} = (A_c \times V \times 100)/(a_{SZ,255} \times F \times S)$$

$$\% \text{ NaSZ} = \% \text{ SZ}/0.9207$$

From plate B, transfer spot $f$ to 50 mL vol. flask and spot $h$ and $p$ to 10 mL vol. flasks. Obtain blank corrected $A_f$, $A_h$, and $A_p$ as for exts of plate A.

Calc. recovery factor $F' = [(5 \times A_f) + A_h + A_p]/(R_T \times A_T)$

$$\% \text{ SM} = (A_h \times V \times 100)/(a_{SM} \times F' \times S)$$

$$\% \text{ SH} = (A_p \times V \times 100)/(a_{SH} \times F' \times S)$$

$$\% \text{ NaSM} = \% \text{ SM}/0.9232$$

$$\% \text{ NaSH} = \% \text{ SH}/0.9268$$

Ref.: JAOAC **57,** 345(1974).

CAS-127-79-7 (sulfamerazine)
CAS-57-68-1 (sulfamethazine)
CAS-59-40-5 (sulfaquinoxaline)
CAS-72-14-0 (sulfathiazole)

## 966.28 Thiabendazole in Feeds

### Spectrophotometric Method

**First Action 1966**
**Final Action 1967**

***Method I***

(Applicable to all feeds)

#### *A. Principle*

Thiabendazole is extd from feed with 0.1*N* HCl. Interferences are removed by adjusting ext to pH 5–6 with Na citrate and extg with $CHCl_3$. Thiabendazole is re-extd with 0.1*N* HCl and reduced with Zn slurry in 30% glycerol in presence of *p*-phenylenediamine. Oxidn with ferric iron yields blue complex which is extd with BuOH and measured at 605 nm.

#### *B. Reagents*

(**a**) *Zinc dust.*—Reagent grade. Crush fine lumps with spatula immediately before use.

(**b**) *Zinc slurry.*—Weigh 50 mg *p*-phenylenediamine.2HCl (*Caution: p*-Phenylenediamine may be harmful; *see* safety notes on toxic dusts.) and 2 g Zn dust into dry 100 mL g-s graduate. Add 100 mL 30% (v/v) glycerol soln, stopper, and shake ca 30 sec to suspend Zn dust uniformly. (There must be no agglomeration of Zn.) Prep. just before use and use immediately.

(**c**) *Ferric soln.*—Dissolve 15.0 g $FeNH_4(SO_4)_2.12H_2O$ in 75 mL $H_2O$, add 10.0 mL 1*N* $H_2SO_4$, dil. to 100 mL, and mix.

(**d**) *Thiabendazole std solns.*—(*1*) *Stock soln.*—0.5 mg/mL. Dissolve 50.0 mg Thiabendazole Ref. Std (available from Merck & Co.) in 0.1*N* HCl and dil. to 100 mL. Soln is stable ≥1 month. (*2*) *Intermediate soln.*—50 μg/mL. Dil. 10 mL stock soln to 100 mL with 0.1*N* HCl. Soln is stable ≥1 month. (*3*) *Working soln.*—5 μg/mL. Dil. 20.0 mL intermediate soln to 200 mL with 0.1*N* HCl. (Use same 0.1*N* HCl as in extn of feed.)

#### *C. Determination*

Grind ca 100 g well mixed sample to pass No. 30 sieve and mix. (3 min in high-speed blender should be enough.)

Weigh 2.000 g ground sample into 250 mL ₸ 24/40 flat-bottom extn flask. (For feeds contg <0.025% thiabendazole, weigh 5.000 g.)

Add 100.0 mL 0.1*N* HCl to sample and add mag. stirring bar. Connect flask to reflux condenser (Allihn, drip tip) and reflux gently on mag. hot plate, while stirring, 30 min. Cool, transfer mixt. to centrf. tube, and centrf. ca 5 min. Dil. measured aliquot of supernate to concn of 5 μg thiabendazole/mL (serial dilns may be necessary). Such dilns det. "dilution factor," *DF*:

| Declaration, % | Sample wt, g | Dilution(s) | DF |
|---|---|---|---|
| 0.01 | 5 | none | 1 |
| 0.025 | 2 | none | 1 |
| 0.1 | 2 | 25–100 | 4 |
| 1.0 | 2 | 10–100; 25–100 | 40 |
| 6.0 | 2 | 10–100; 10–250 | 250 |

Mark series of 50 mL centrf. tubes 1, 2, 3, 4, etc. Place 20.0 mL 0.1*N* HCl in tube 1 and 20.0 mL (100 μg) working std soln in tubes 2 and 3. Place 20.0 mL aliquots of sample solns in tubes 4, 5, etc. Add 3 g Na citrate, 3 g NaCl, and 20.0 mL $CHCl_3$ to each tube, stopper tightly with polyethylene stopper, and shake mech. 5 min. Centrf. ca 5 min and discard top layers. With pipet, transfer 10 mL $CHCl_3$ ext to dry, marked, centrf. tubes, add 25.0 mL 0.1*N* HCl to each, stopper, and shake 5 min. Centrf., and transfer, with pipet, 15 mL of top acid layer to another marked tube. (Because of timing, handle ≤10 tubes at one time.)

With rapid delivery pipet, add 5 mL freshly prepd Zn slurry, (**b**), to each tube. (5 mL pipet with tip cut off to give delivery in ca 5 sec is suitable. Hold pipet directly over center of soln.) Do not shake tube but *immediately stopper* tightly and let stand 4 min. Start timing after delivery of slurry to first tube.

After 4 min, add 5.0 mL ferric soln, (**c**), to each tube with rapid pipet, stopper, and mix by inverting tube. Let stand 5 min; then shake vigorously and centrf. ca 3 min. With pipet, transfer 15 mL clear, colored soln to marked, dry, centrf. tubes. Let stand 45 min from addn of ferric soln. Then add 5.00 mL *n*-BuOH and 3 g anhyd. $Na_2SO_4$ to each tube. Stopper, and immediately shake each tube ca 5 sec to avoid caking of $Na_2SO_4$; then shake all tubes ca 3 min or until $Na_2SO_4$ is completely dissolved, and centrf.

Transfer clear BuOH soln (top layer) to dry 1 cm cell and read *A* at 605 nm against *n*-BuOH as ref.

$$\% \text{ Thiabendazole in feed} = (A - A_0)(C)(DF)/360(A' - A_0)W$$

where $A$ refers to sample, $A_0$ to reagent blank (tube 1), $A'$ to std, $C$ = μg thiabendazole std in final 15.0 mL colored soln (18 μg), $DF$ = diln factor, and $W$ = g original sample.

Refs.: JAOAC **47,** 235(1964); **49,** 312(1966).

CAS-148-79-8 (thiabendazole)

## 966.29 Thiabendazole in Feeds, Supplements, and Premixes

### Spectrophotometric Method

**First Action 1966**
**Final Action 1967**

***Method II***

(Applicable to cattle supplements and premixes contg >1% thiabendazole. Principle is same as **966.28A**, except that single extn at room temp. with 0.1*N* HCl is used. Not applicable to feeds, premixes, or cattle supplements contg high levels of protein.)

***A. Reagents***

See **966.28B** except:

(**a**) *Thiabendazole working soln.*—2 μg/mL. Dil. 10.0 mL thiabendazole intermediate soln, **966.28B(d)**(*2*), to 250 mL with 0.1*N* HCl.

***B. Determination***

Prep. sample as in **966.28C**, except use ca 50 g representative sample.

Weigh 2.000 g ground sample into 1 L vol. flask and add 750 mL 0.1*N* HCl. Add mag. stirring bar, stopper, and mix vigorously on mag. stirrer 1 hr at room temp. (Mech. shaker providing vigorous agitation may be used.) Remove and rinse bar, and dil. to vol. with 0.1*N* HCl. Mix, centrf., and dil. aliquots of clear ext with 0.1*N* HCl to concn of 2 μg thiabendazole/mL. Diln factors, *DF*, are as follows (*see* **966.28C**):

| Declaration, % | Dilution | DF |
|---|---|---|
| 1.0 | 10–100 | 10 |
| 2.5 | 4.0–100 | 25 |
| 6.0 | 4.0–250 | 62.5 |

Develop color in exts as soon as possible after extn. (Acid exts of some feeds deteriorate upon standing.)

Mark series of 50 mL centrf. tubes ($\leq 10$) as in **966.28C**. Add 15.0 mL 0.1*N* HCl to tube 1, and 15.0 mL working soln, (**a**), to tubes 2 and 3. Add 15.0 mL sample exts to other tubes. Then with rapid delivery pipet, add 5.0 mL freshly prepd Zn slurry as in **966.28C**. Proceed as in **966.28C** with addn of ferric soln, observing same technics and time precautions. Read final clear BuOH ext, as above, in 1 cm cell at 605 nm.

$$\% \text{ Thiabendazole in feed} = (A - A_0)(C)(DF)/90(A' - A_0)W$$

where symbols are as defined in **966.28C**.

Refs.: JAOAC **47,** 235(1964); **49,** 312(1966).

CAS-148-79-8 (thiabendazole)

## 961.26 Zoalene in Feeds

### Spectrophotometric Method

**First Action 1961**
**Final Action 1962**

(Not applicable in presence of furazolidone, nitrofurazone, and nihydrazone)

***A. Principle***

Zoalene is extd from feeds, premixes, and concs contg 0.004–25% with 85% $CH_3CN$. For mixes contg <1%, alumina is added. After filtration and diln, zoalene is detd colorimetrically after reaction with ethylenediamine.

***B. Reagents***

(**a**) *Acetone.*—95%. Add 5 mL $H_2O$ to 95 mL acetone.

(**b**) *Acetonitrile.*—85%. Add 850 mL practical grade $CH_3CN$ to 150 mL $H_2O$ (deionized or distd).

(**c**) *Activated alumina.*—Alcoa grade F 20, 80–200 mesh. (Available from Fisher Scientific Co. "Alumina, Adsorption, Fisher.")

(**d**) *Dimethylformamide* (*DMF*).—95%. Add 5 mL $H_2O$ to 95 mL tech. DMF. Prep. fresh daily, since old solns may cause cloudiness. (*Caution:* see safety notes on dimethylformamide.)

(**e**) *Ethylenediamine.*—98–100%. (No. EX0510, EM Science). Reagent must be practically colorless.

(**f**) *Zoalene std soln.*—40 μg/mL. Weigh 40.0 mg Zoalene Ref. Std (available from Dow Chemical Co.) into 1 L vol. flask, dil. to vol. with 85% $CH_3CN$, and mix.

***C. Determination***

Weigh 10.0 g sample into 250 mL erlenmeyer and add 65 mL 85% $CH_3CN$. Warm on steam bath to 50 ± 5°, swirling occasionally. Let cool to room temp. (ca 30 min). Add 20 g alumina and swirl occasionally ca 3 min. (Addn of alumina is unnecessary for concs contg $\geq 1\%$ zoalene.) Filter with suction on medium or fine porosity 40 mm diam. fritted glass funnel, transferring as much solids as possible. Transfer remaining solids with min. vol. 85% $CH_3CN$, and suck dry. Suspend cake in funnel with min. vol. 85% $CH_3CN$ and slight stirring but without suction. Then filter with suction and repeat suspension and filtering, keeping total vol. <100 mL. Transfer combined filtrates to 100 mL vol. flask (or vol. flask may be used to collect filtrates directly), dil. to vol. with 85% $CH_3CN$, and mix.

Based on zoalene concn, make addnl dilns with 95% acetone and use aliquots indicated in Table **961.26**.

Pipet indicated aliquots into three 50 mL beakers, X, Y, and Z, for concns <0.25%; omit X for samples >0.25%. Pipet 1 mL std soln into beaker Z and evap. all solns to dryness with air current. (Heat may be used but temp. must not exceed 60°.) Pipet 10 mL 95% DMF into X and 2 mL each into Y and Z. Swirl intermittently during 5 min to dissolve zoalene. Pipet 8 mL ethylenediamine into Y and Z and mix. If turbidity persists after 2 min, filter thru small Reeve Angel No. 804, or equiv., paper. Read *A* of solns at 560 nm in stoppered 1 cm cells against 95% DMF 5 min after addn of ethylenediamine. Keep cell compartment of spectrophtr at <30° to avoid rapid fading of color. If *A* is >1, reanalyze, using greater diln or smaller aliquot.

$$\% \text{ Zoalene} = (A_Y - A_X) \times M/100\ (A_Z - A_Y)$$

*Caution:* $CH_3CN$ and ethylenediamine are toxic. Handle in hood and avoid contact with skin.

Refs.: JAOAC **44,** 18(1961); **45,** 294(1962); **51,** 501(1968).

CAS-148-01-6 (zoalene)

**Table 961.26 Dilution of Sample for Determination**

| % Zoalene in Sample | Addnl Diln | Aliquot Size, mL | Multiplication Factor *M* |
|---|---|---|---|
| 0.004– 0.012 | None | 4 | 1 |
| 0.012– 0.025 | None | 2 | 2 |
| 0.025– 0.050 | 10 to 100 | 10 | 4 |
| 0.050– 0.10 | 10 to 100 | 5 | 8 |
| 0.10 – 0.25 | 10 to 100 | 2 | 20 |
| 0.25 – 0.5 | 1 to 100 | 10 | 40 |
| 0.5 – 1.0 | 1 to 100 | 5 | 80 |
| 1.0 – 2.5 | 1 to 100 | 2 | 200 |
| 2.5 – 5.0 | 1 to 1000 | 10 | 400 |
| 5.0 –10.0 | 1 to 1000 | 5 | 800 |
| 10.0 –25.0 | 1 to 1000 | 2 | 2000 |

## MICROBIOLOGICAL METHODS FOR ANTIBIOTICS

## 957.23 Antibiotics in Feeds

### Microbiological Methods

***A. Culture Media***

(Deionized $H_2O$ may be used for prepn of media.)

(**a**) *Agar medium A.*—(Antibiotic Medium 1.) Dissolve 6.0 g pancreatic digest of gelatin, 4.0 g pancreatic digest of casein, 3.0 g yeast ext, 1.5 g beef ext, 1.0 g anhyd. glucose, and 15 g agar in $H_2O$, and dil. to 1 L. Adjust with 1*N* NaOH or HCl (1 + 9) so that after sterilization pH is 6.5–6.6. (Difco Penassay Seed Agar (antibiotic medium 1) and BBL Seed Agar have been found satisfactory.)

(**b**) *Agar medium B*.—(Antibiotic Medium 4.) Dissolve 6.0 g pancreatic digest of gelatin, 3.0 g yeast ext, 1.5 g beef ext, 1.0 g anhyd. glucose, and 15 g agar in $H_2O$, and dil. to 1 L. Adjust with 1*N* NaOH or HCl (1 + 9) so that after sterilization pH is 6.5–6.6. (Difco and BBL Yeast Beef Agar have been found satisfactory.)

(**c**) *Agar medium C*.—(Antibiotic Medium 2.) Dissolve 6.0 g pancreatic digest of gelatin, 3.0 g yeast ext, 1.5 g beef ext, and 15 g agar in $H_2O$, and dil. to 1 L. Adjust with 1*N* NaOH or HCl (1 +9) so that after sterilization pH is 6.5–6.6. (Difco Penassay Base Agar and BBL Base Agar have been found satisfactory.)

(**d**) *Agar medium D*.—(Antibiotic Medium 8.) Use agar medium C adjusted with 1*N* NaOH or HCl (1 + 9) so that final pH is 5.7–5.9. (Difco Antibiotic Medium 8 and BBL Base Agar with low pH have been found satisfactory.)

(**e**) *Agar medium E*.—(Antibiotic Medium 5.) Use agar medium C adjusted with 1*N* NaOH so that final pH is 7.8–8.0. (Difco Streptomycin Assay Agar (antibiotic medium 5) and BBL Streptomycin Assay Agar with Yeast Extract have been found satisfactory.)

(**f**) *Agar medium F*.—Adjust agar medium A with 3.5*N* NaOH (2.8–3.8 mL/L) so that after sterilization pH is 8.9–9.1.

(**g**) *Agar medium G*.—(Antibiotic Medium 32.) Use agar medium A to which is added 300 mg $MnSO_4.H_2O$ or 0.4 mL 1% $MnCl_2$ soln/L.

(**h**) *Agar medium H*.—Dil. 1 L agar medium A to 1.2 L and adjust to pH 8.1.

(**i**) *Agar medium I*.—Dissolve 9.4 g pancreatic digest of gelatin, 4.7 g yeast ext, 2.4 g beef ext, 10.0 g NaCl, 10.0 g anhyd. glucose, 13.0 g anhyd. $KH_2PO_4$, 1 g $Na_2HPO_4.7H_2O$, and 23.5 g agar in $H_2O$, and dil. to 1 L. After sterilization pH is 5.3–5.5. (BBL Nystatin Assay Agar supplemented with 13.0 g anhyd. $KH_2PO_4$ and 1 g $Na_2HPO_4.7H_2O$ has been found satisfactory.)

(**j**) *Agar medium J*.—(Antibiotic Medium 11.) Use agar medium A adjusted with 1*N* NaOH so that final pH is 7.9–8.0. (Difco and BBL Neomycin Assay Agar have been found satisfactory.)

(**k**) *Agar medium K*.—To each L agar medium J add 12.5 mL 2*M* $CaCl_2$ after autoclaving and just before pouring plates.

(**l**) *Agar medium L*.—Dissolve 0.69 g $K_2HPO_4$, 0.45 g $KH_2PO_4$, 2.5 g yeast ext, 10.0 g anhyd. glucose, and 15.0 g Difco Noble agar in $H_2O$ and dil. to 1 L. Adjust to pH 6.0 with HCl before use.

(**m**) *Agar medium M*.—Dissolve 2.5 g yeast ext, 10.0 g glucose, 0.69 g $K_2HPO_4$, 0.45 g $KH_2PO_4$, and 20.0 g agar in $H_2O$ and dil. to 1 L. Before adding inoculum, adjust liquified medium to pH 6.0 with 1*N* HCl (ca 2 mL/L).

(**n**) *Broth medium A*.—(Antibiotic Medium 3.) Dissolve 5.0 g pancreatic digest of gelatin, 1.5 g yeast ext, 1.5 g beef ext, 3.5 g NaCl, 1.0 g anhyd. glucose, 3.68 g anhyd. $K_2HPO_4$, and 1.32 g anhyd. $KH_2PO_4$ in $H_2O$, and dil. to 1 L. Adjust with 1*N* NaOH or HCl (1 + 9) so that after sterilization pH is 6.95–7.05. (Difco Penassay Broth (antibiotic medium 3) and BBL Antibiotic Assay Broth have been found satisfactory.)

(**o**) *Broth medium B*.—Dissolve 5.0 g pancreatic digest of casein, 5.0 g pancreatic digest of animal tissues, and 20 g anhyd. glucose in $H_2O$, and dil. to 1 L. Adjust with 1*N* NaOH or with HCl (1 +11) so that after sterilization pH is 5.6–5.7. (Difco Fluid Sabouraud Medium and BBL Sabouraud Liquid Broth Modified have been found satisfactory.)

### B. Reagents

(**a**) *Phosphate-bicarbonate buffer*.—pH 8. Dissolve 16.73 g anhyd. $K_2HPO_4$, 0.523 g anhyd. $KH_2PO_4$, and 20 g $NaHCO_3$ in $H_2O$ and dil. to 1 L.

(**b**) *Phosphate buffer*.—pH 8; 0.1*M*. Dissolve 16.73 g anhyd. $K_2HPO_4$ and 0.523 g anhyd. $KH_2PO_4$ in $H_2O$ and dil. to 1 L.

(**c**) *Phosphate buffer*.—pH 7.0; 0.1*M*. Dissolve 13.6 g anhyd. $K_2HPO_4$ and 4.0 g anhyd. $KH_2PO_4$ in $H_2O$ and dil. to 1 L.

(**d**) *5% Phosphate buffer*.—pH 6.5. Dissolve 22.15 g anhyd. $K_2HPO_4$ and 27.85 g anhyd. $KH_2PO_4$ in $H_2O$ and dil. to 1 L.

(**e**) *10% Phosphate buffer*.—pH 6. Dissolve 80 g anhyd. $KH_2PO_4$ and 20 g anhyd. $K_2HPO_4$ in $H_2O$ and dil. to 1 L.

(**f**) *1% Phosphate buffer*.—pH 6. Dissolve 8.0 g anhyd. $KH_2PO_4$ and 2.0 g anhyd. $K_2HPO_4$ in $H_2O$ and dil. to 1 L.

(**g**) *Phosphate buffer*.—pH 4.5; 0.1*M*. Dissolve 13.6 g anhyd. $KH_2PO_4$ in $H_2O$ and dil. to 1 L.

(**h**) *Acid-acetone*.—Mix 1 vol. 4*N* HCl, 13 vols acetone, and 6 vols $H_2O$.

(**i**) *Acid-methanol*.—Mix 1 vol. HCl and 50 vols MeOH.

(**j**) *Ethyl acetate*.—99% undenatured grade.

(**k**) *Buffer-acetone extractant*.—Mix equal vols pH 6 buffer, (**f**), and acetone.

(**l**) *Tris buffer*.—pH 8.0, 0.05*M*. Dissolve 6.05 g tris(hydroxymethyl)aminomethane (THAM, primary std, available from Fisher Scientific Co.) in 900 mL $H_2O$, adjust pH to 8.0 with HCl, and dil. to 1 L.

(**m**) *Calcium chloride soln*.—2*M*. Dissolve 294.04 g $CaCl_2.2H_2O$ in $H_2O$ and dil. to 1 L.

(**n**) *Sodium chloride-calcium chloride soln*.—Dissolve 200 g NaCL in $H_2O$, add 10 mL 2M $CaCl_2$, and dil. to 1 L.

(**o**) *Sodium hypochlorite soln*.—5.25%. Use freshly opened bottle com. soln. (Clorox has been found satisfactory.) Store in dark at 2–10°.

(**p**) *Sterile isotonic saline soln*.—Dissolve 9.0 g NaCl in $H_2O$ and dil. to 1 L. Autoclave 20 min at 121°.

(**q**) *Lead acetate soln*.—Dissolve 303 mg $Pb(OAc)_2.3H_2O$ in $H_2O$ and dil. to 1 L with $H_2O$.

### C. Apparatus

(High-speed blender jars, after disassembling, must be cleaned with great care to eliminate all traces of antibiotics. All app. that contacts sample and solns must be thoroly cleaned and be detergent-free.)

(**a**) *Cylinders*.—Polished open stainless steel cylinders, 8 ± 0.1 mm od, 6 ± 0.1 mm id, and 10 ± 0.1 mm high (obtainable from S & L Metal Products Corp., 58–29 57th Drive, Maspeth, NY 11378).

(**b**) *Petri dishes* (*plates*).—Glass or plastic; 100 mm wide × 20 mm deep. Porcelain covers glazed on outside or cover lids with filter pad inserts are satisfactory for absorbing $H_2O$ of syneresis. Glass or plastic covers may be used if they are raised slightly to allow escape of $H_2O$.

(**c**) *Cylinder dispenser*.—May be used to place cylinders on plates. Shaw Dispenser, available from Arthur E. Farmer, 47 Frazier St, PO Box 1785, Trenton, NJ 08618.

(**d**) *Agar cutter*—Used to prep. cups in agar, available from Biochem. Dept, Purdue Univ., W. Lafayette, IN 47907.

### D. Stock Cultures and Preparation of Test Organism Suspensions

For appropriate test organism designated below, prep. slant culture on ≥1 tube of agar medium A. Incubate overnight at indicated temp. held constant to ± 0.5°, and then store in dark at 2–10°. Do not use if >2 weeks old.

Prep. suspensions of test organisms as follows:

(**a**) *Micrococcus flavus*.—ATCC No. 10240. Incubate stock culture at 32–35°. Wash growth from stock culture with ca 3 mL broth medium A and transfer liq. to surface of 300 mL agar medium A in Roux bottle. Spread suspension evenly over entire surface, using sterile glass beads, and incubate overnight at 32–35°. Wash growth from agar surface with ca 25 mL sterile isotonic saline soln. Store bulk suspension at 2–10°. Use for bacitracin assay.

(**b**) *Sarcina subflava*.—ATCC No. 7468. Incubate stock culture at 32–35°. Prep. suspension as in (**a**) and use as alternative organism for bacitracin assay.

(**c**) *Bacillus cereus*.—ATCC No. 11778. Incubate stock culture at 30°. Wash growth from stock culture with ca 3 mL sterile $H_2O$, transfer to surface of 300 mL agar medium A, and incubate 7 days at 30°. Wash growth from agar surface with ca 25 mL sterile $H_2O$ and heat suspension 30 min at 65°. Centrf. and decant. Wash residual spores 3 times with sterile $H_2O$, centrfg and decanting each time. Discard wash $H_2O$. Heat residual spores 30 min at 65° and resuspend in sterile $H_2O$. Store this stock suspension at 2–10°. Use for chlortetracycline and oxytetracycline assays.

(**d**) *Bacillus subtilis*.—ATCC No. 6633. Incubate stock culture at 37°. Wash growth from stock culture with ca 3 mL sterile isotonic saline soln, transfer to surface of 300 mL agar medium G in Roux bottle, and incubate 7 days at 37°. Wash growth from agar surface with ca 50 mL sterile isotonic saline soln into centrf. bottle. Heat suspension 30 min in 65° $H_2O$ bath to destroy vegetative cells. Centrf., decant, and resuspend cells in ca 50 mL sterile isotonic saline soln. Repeat heating, centrfg, and suspending twice, or until supernate is clear. Final suspension is stock spore suspension. Store at 2–10°. Use for hygromycin B, monensin, and streptomycin assays.

(**e**) *Sarcina lutea*.—ATCC No. 9341. Incubate stock culture at 26–30°. Prep. organism suspension by one of following methods:

(*1*) *Roux bottle culture*.—Wash growth from 24 hr slant culture with ca 3 mL broth medium A, and transfer liq. to surface of 300 mL agar medium A in Roux bottle. Spread suspension evenly over entire surface, using sterile glass beads, and incubate 24 hr at 26–32°. Wash growth from agar surface with ca 15 mL sterile isotonic saline soln. Store bulk suspension ≤2 weeks at 2–10°.

(*2*) *Broth culture*.—Wash growth from stock culture with ca 3 mL broth medium A, and transfer liq. to 100 mL broth medium A. Incubate 48 hr at 26–32° with continuous mech. agitation. This 48 hr culture is inoculum. Store ≤2 weeks at 2–10°.

Use for erythromycin, lincomycin, novobiocin feed supplement, oleandomycin, penicillin, and tylosin assays.

(**f**) *Staphylococcus epidermidis*.—ATCC 12228. Incubate stock culture at 32°. Inoculate 30 mL broth medium A in 300 mL flask with 1 loop from stock culture, and incubate overnight at 26–32°. Prep. daily. Use for neomycin and for novobiocin final feed assays.

(**g**) *Saccharomyces cerevisiae*.—ATCC No. 9763. Incubate stock culture on agar medium I at 37°. Prep. inoculum by one of following methods:

(*1*) *Broth culture*.—Inoculate 100 mL broth medium B with 1 loop from stock culture and incubate overnight at 37°. This culture is inoculum. Store ≤2 weeks at 2–10°.

(*2*) *Roux bottle culture*.—Wash growth from stock culture with ca 3 mL sterile isotonic saline soln, and transfer liq. to surface of 300 mL agar medium I in Roux bottle. Spread suspension evenly over entire surface, using sterile glass beads, and incubate 24 hr at 37°. Wash growth from agar surface with ca 15 mL sterile isotonic saline soln. Store ≤2 weeks at 2–10°.

Use for nystatin assay.

(**h**) *Escherichia coli*.—UC 527 (available from The Upjohn Co.). Incubate at 36°. Inoculate 30 mL broth medium A in 250 mL flask from stock culture of *E. coli* and grow 18–24 hr at 36°. Prep. daily. Use for spectinomycin assay.

### E. Design and Plotting of Standard Response Line

Prep. concns of ref. std as described for each antibiotic. In general, it is preferable to use shorter 4-fold range between lowest and highest doses of std line. Use indicated concn as ref. concn. (Values of std or ref. concn could slightly vary from those indicated for each antibiotic without affecting validity of assay.)

Prep. plates with appropriate base agar layer and/or appropriate seed agar layer; one layer of media can be substituted for 2 layers of media if ref. concn gives adequate zone size as described for each antibiotic. Distribute agar evenly by tilting plates from side to side with circular motion and let harden. Use plates same day prepd.

Place 6 cylinders on each plate at ca 60° intervals on 2.8 cm radius. Fill 3 alternate cylinders with ref. concn and other 3 cylinders with one of other concns of std. Use 3 plates for each concn required for std response line, except ref. concn. Incubate plates overnight at appropriate temp., and measure diams of zones of inhibition as accurately as possible. (In most cases, it is possible to est. zone diams to nearest 0.1 mm.) Values given in each method for zones of inhibition to be obtained with ref. concns of antibiotics are for guidance only, but it is important that lowest concns on std response line give measurable zone and that slope of response line be adequate. In each set of 3 plates, average the 9 readings of ref. concn and the 9 readings of concn being tested. Av. of all 36 readings of ref. concn from 12 plates is correction point for response line. Correct av. value obtained for each concn to appropriate figure if ref. concn reading on that set of 3 plates was same as correction point.

For example, if in correcting second concn of std response line, av. of 36 readings of ref. concn is 20.0 mm, and av. of 9 readings of ref. concn of this set of 3 plates is 19.8 mm, correction is + 0.2 mm. If av. reading of second concn on same 3 plates is 17.0 mm, corrected value is 17.2 mm. Plot corrected values, including correction point, on semilog graph paper, using logarithmic scale for concn and arithmetic scale for av. zone diams. Manual plotting of std lines is possible but could be subject to large variation. Response lines would be more accurate if calcd. When std doses are equally spaced, i.e., interval between successive doses is the same, calc. $L$ and $H$ (calcd zone diams for low and high concns, resp., of std response line) as follows:

For method specifying 5 doses of std,

$$L = (3a + 2b + c - e)/5$$

$$H = (3e + 2d + c - a)/5$$

where $a$, $b$, $c$, $d$, and $e$ = corrected av. zone diams for each concn of std.

For methods specifying 4 doses of std,

$$L = (7a + 4b + c - 2d)/10$$

$$H = (7d + 4c + b - 2a)/10$$

For methods specifying 3 doses of std,

$$L = (5a + 2b - c)/6$$

$$H = (5c + 2b - a)/6$$

Plot values for $L$ and $H$ and connect with straight line. Ref. point is zone size intercept on arithmetic scale. This corrected ref. point is to be used for sample calcns (if corrected ref. point diam. varies significantly from av. ref. diam., error in prepn of std solns is indicated and validity of assay is in question.) For more accuracy in calcn, det. slope of std response line $B = (H - L)/(\log h - \log l)$, where $l$ and $h$ are high and low std concns, resp., and $B$ is increase in zone for each 10× increase in drug concn.

Computer or calculator can be used to calc. std lines whether std concns are equally spaced or not. Least square fitting using linear or polynomial equations may be performed based on best fit (polynomial fitting is most appropriate, especially for long range 8× or 16× range).

### *F. Determination of Potency*

Use 3 plates of each assay soln. On each plate, fill 3 alternate cylinders with ref. concn and fill other 3 alternate cylinders with assay soln. Incubate plates overnight at appropriate temp. and measure diam. of zones of inhibition. Average the 9 readings of ref. concn and the 9 readings of assay soln. If assay soln gives larger av. than ref. concn, add difference between them to ref. point on std response line. If assay soln gives smaller value than ref. concn, subtract difference between them from ref. point on std response line. Using corrected values of assay soln, det. amt of antibiotic by reading concn from std response line.

Alternatively, det. log relative potency, $M' = (Y_u - Y_s)/B$, where $Y_u$ and $Y_s$ are av. of 9 readings of assay soln and ref. concn, resp., and $B$ is slope of std response line. Antilog $M'$ = potency of assay soln relative to std; and (antilog $M'$) × 100 = potency of assay soln as % of std ref. concn.

For calcn of sample potency by computer or calculator, enter sample data and calc. antibiotic potency based on least square linear or polynomial lines.

For calcns, 1 ton = 908 000 g; 1 lb = 454 g.

Refs.: JAOAC **40,** 857(1957); **72,** 105(1989).

## 982.43 Bacitracin in Premix Feeds

**Cylinder Plate Method**

**First Action 1982**

(Applicable to premixes contg ≥10 g bacitracin/lb)

### *A. Principle*

Bacitracin is extd from feeds into acidified org. solv. system. Ext is centrfd, and supernate is dild in phosphate buffer and analyzed by cylinder plate assay with *M. flavus* as detection organism.

### *B. Reagents and Apparatus*

(**a**) *Microorganism.—Micrococcus flavus* ATCC 10240. Maintain culture as indicated in **957.23D(a)**.

(**b**) *Extracting solv.*—Mix, by vol., 27% $CH_3CN$, 27% MeOH, 3% pH 6.0 phosphate buffer (**957.23B(f)**), 41% $H_2O$, and 2% $H_3PO_4$ (85%); add 0.5 g EDTA/L. (Extg solv. is satd with EDTA.).

(**c**) *Phosphate buffer.*—5%, pH 6.5. See **957.23B(d)**.

(**d**) *Diluting solvent.*—Methanol–5% pH 6.5 phosphate buffer (12 + 88).

(**e**) *Dilute HCl.*—Carefully add 89 mL HCl to $H_2O$ and dil. to 1 L (1N). Further dil. soln 1:100 (0.01N).

(**f**) *Cylinders.*—See **957.23C(a)**.

(**g**) *Cylinder dispenser.*—Optional: see **957.23C(c)**.

### *C. Standard Solutions*

See **957.24★(a)** and (**b**). Also prep. 0.30 and 0.16 unit/mL solns to be plated as samples to monitor assay.

### *D. Preparation of Plates*

Use one layer (ca 15 mL) of agar antibiotic medium, 1, **957.23A(a)**. Det. by trial plates optimum concn (usually 0.02–0.05%) of *M. flavus* ATCC 10240 to be added to agar to obtain zones of inhibition 15–17 mm for 0.2 unit bacitracin/mL. Pour 4 plates for each point on std curve (i.e., 16 plates) and 4 plates for each sample soln. Std curve will be plated twice (i.e., 32 plates) as will check samples 0.30 and 0.16 unit/mL. Therefore, total of 48 plates will be needed for 2 curves and check samples, plus 4 addnl plates for each sample.

Let agar harden on level surface. Transfer to refrigerator and cool ≥1 h before dosing. Use plates same day prepd.

### *E. Extraction*

Accurately weigh amt feed contg ca 4600 units of bacitracin into 300 mL erlenmeyer flask, or equiv.

Add 100 mL extg solv. with 100 mL vol. pipet and ext feeds ≥5 min by shaking flask or mixing on mag. stirrer.

Transfer supernate to plastic centrif. tubes and centrif. 10 min at 2000 rpm. Filter supernate thru glass wool into graduate. Use vol. glassware and dilg solv. to prep. final diln 0.2 ± 0.05 unit/mL.

### *F. Plating*

Use 16 seeded plates for first curve. Use 0.20 unit/mL as plate ref. On each plate, fill 3 alternate stainless steel cylinders with plate ref. and the 3 remaining cylinders with 1 std. Be sure all cylinders are filled with const vol. (i.e., 0.25 mL). Preset Eppendorf pipet is best for this purpose. Use 4 plates for each sample, including 0.3 and 0.16 unit/mL check samples.

Use 16 seeded plates for second curve, to be plated after all samples are plated. Use 8 plates for second plating of 0.3 and 0.16 unit/mL check samples.

Incubate dosed plates 16–18 h at 37 ± 2°. Read zones of inhibition to nearest mm, using Fisher–Lily zone reader.

### *G. Determination*

Det. corrected av. zone diams for std ($Z'$) and sample ($Z$) solns according to **957.23E**. Det. response line as least squares linear regression of following equation:

$$Z' = m\log P' + b$$

where $P'$ = potency in unit/mL of std soln associated with $Z'$; $m$, $b$ = are least squares fitted slope and intercept parameters. Calc. potency of sample by following equation:

$$\text{g bacitracin/lb} = [\text{antilog}(Z - b/m) \times D \times 0.0108]/\text{sample wt}$$

where $D$ = total sample diln; 0.0108 = 453.6 (g/lb)/42 000 (units/g bacitracin).

Ref.: JAOAC **65,** 1168(1982).

CAS-1405-87-4 (bacitracin)

## 957.24★ Bacitracin in Feed Supplements

**Microbiological Method**

**First Action 1957**
**Final Action 1960**
**Surplus 1981**

(Applicable to supplements contg ≥6 g/lb)

*See* **42.223–42.226**, 14th ed.

## 965.48★ Bacitracin in Mixed Feeds

**Microbiological Method**
**First Action 1965**
**Surplus 1981**

(Applicable to feeds contg ≥20 g bacitracin/ton)

*See* **42.227–42.231**, 14th ed.

## 967.39 Chlortetracycline HCl in Feeds

**Microbiological Method**
**First Action 1967**
**Final Action 1968**

(Applicable to feeds contg ≥10 ppm)

### *A. Standard Solutions*

(**a**) *Chlortetracycline (CTC) stock soln.*—Accurately weigh ca 40 mg CTC.HCl USP Ref. Std and dissolve in enough 0.01*N* HCl to give concn of exactly 1000 μg/mL. Store in dark ≤5 days at 2–10°.

(**b**) *Std solns and response line for samples containing more than 50 ppm chlortetracycline.HCl.*—Dil. appropriate aliquots of stock soln, (**a**), with enough pH 4.5 buffer, **957.23B(g)**, to obtain concns of 0.01, 0.02, 0.04, 0.08, and 0.16 μg/mL. Ref. concn is 0.04 μg/mL.

(**c**) *Std solns and response line for samples containing 10–50 ppm chlortetracycline.HCl.*—Prep. as in (**b**), but dil. with inactivated diluent, (**d**), instead of buffer soln and include concns of 0, 0.005, and 0.32 μg CTC.HCl/mL. Draw best line of fit by inspection.

(**d**) *Inactivated diluent.*—To 10 mL acid-acetone feed ext (prepd from feed under test as in **967.39C(b)**) in 600 mL beaker, add 90 mL pH 4.5 phosphate buffer, **957.23B(g)**, and adjust to pH 4.5–4.7 with 1*N* NaOH. Add 1.0 mL fresh 5.25% NaOCl soln, **957.23B(o)**, and stir 1–2 min, rinsing sides of beaker. *Heat, stirring thoroly at 10 min intervals, in uncovered beaker in boiling $H_2O$ bath 30 min.* Cool to room temp. under tap $H_2O$ stream and transfer quant. to 100 mL vol. flask. Rinse beaker with 6 mL acetone, add rinsings to vol. flask, and dil. to vol. with pH 4.5 buffer. Transfer quant. to another flask and dil. with enough pH 4.5 buffer so that final concn of feed ext is same as that in assay soln.

### *B. Plates*

(**a**) *Base layer.*—Add 6.0 mL melted agar medium D to sterile petri dishes, distribute evenly, and let harden on *perfectly level surface.*

(**b**) *Seed layer.*—Before assay, det. by prepn of trial plates optimum concn (usually 0.03–0.10%) of organism suspension of *B. cereus*, **957.23D(c)**, to be added to agar medium D to obtain zones of inhibition with as little as 0.01 μg CTC.HCl/mL for assaying samples contg >50 ppm and 0.005 μg/mL for samples contg ≤50 ppm CTC.HCl. Zone of 20 mm ± 10% should be obtained with 0.04 μg/mL. For actual assay add appropriate amt of suspension to agar medium D previously melted and cooled to 48°. Mix thoroly and add 4.0 mL to each of plates contg base layer. Alternatively, use 10–12 μL agar medium D (single layer).

### *C. Assay Solution*

(Solv. losses may occur from evapn of volatile solvs in open containers. Carefully measure and record vols of solvs, and make appropriate mathematical corrections for any losses in vol.)

(**a**) *Samples containing more than 50 ppm chlortetracycline.HCl.*—Obtain and prep. sample as in **965.16** and **950.02**. Place 2, 10, or 20 g sample, resp., contg CTC.HCl ≥10 g/lb (>2%), >400 ppm to 2%, or 50–400 ppm in 150 mL beaker and pipet in 40 mL acid-acetone soln, **957.23B(h)**. Stir ca 2 min with glass rod, let stand 2 min, and stir. Adjust pH to 1.0–1.2 with HCl, if necessary, and note vol. HCl added. Transfer to 1 qt (1 L) high-speed blender jar, using addnl 20 mL (minus vol. equiv. to HCl added in adjusting pH) acid-acetone to rinse beaker and pH meter electrodes. Cover jar and blend 3 min at high speed. Transfer mixt. to 100 mL centrf. tubes. Wash blender jar with 40 mL acid-acetone and combine washings with ext in centrf. tubes. Shake well 5 min. Centrf. ca 15 min at 2000 rpm. Combine and mix clarified exts. Adjust 10 mL aliquot to pH 4.5 with 1*N* NaOH. Dil. adjusted soln with enough pH 4.5 buffer, **957.23B(g)**, to obtain estd concn of 0.04 μg/mL. Designate soln as assay soln.

(**b**) *Samples containing 10–50 ppm chlortetracycline.HCl.*—Obtain and prep. sample as in **956.16** and **950.02**. Place 50 g sample in 250 mL beaker and pipet in 100 mL acid-acetone soln, **957.23B(h)**. Stir, adjust pH, and blend as in (**a**), using 50 mL (less vol. equiv. to HCl added in adjusting pH) acid-acetone to transfer to blender jar. After blending, transfer quant. to 250 mL centrf. bottle, rinsing jar with 50 mL acid-acetone soln. Shake thoroly and centrf. ca 15 min at 2000 rpm. Pipet 5 mL clear supernate into 50 mL beaker, add ca 40 mL pH 4.5 buffer, **957.23B(g)**, mix, and adjust pH to 4.5–4.7 with 1*N* NaOH. Transfer quant. to flask, rinse beaker and pH meter electrodes with pH 4.5 buffer, and add rinsings to flask. Add enough pH 4.5 buffer to obtain estd concn of 0.04 μg/mL. Designate as assay soln.

### *D. Assay*

Using CTC.HCl std response line, assay soln, and plates, proceed as in **957.23E–F**, incubating at 30°.

Refs.: JAOAC **40,** 857(1957); **50,** 446(1967).

CAS-64-72-2 (chlortetracycline.HCl)

## 977.37 Chlortetracycline HCl in Feeds

**Turbidimetric Method**
**First Action 1977**

(Applicable to feed supplements contg ≥20 g/lb)

### *A. Apparatus and Reagents*

(**a**) *Assay broth.*—Prep. as in **957.23A(n)**, but in 1.7× quantity.

(**b**) *Homogenizer.*—Omni-Mixer (Du Pont Instrument Co., Sorvall Operations, Peck's Ln, Newtown, CT 06470), or equiv.

(**c**) *For manual assay.*—(*1*) *Spectrophotometer.*—Sequoia-Turner Model 330 (replacement Model 340, Sequoia-Turner Corp., 850 Maude Ave, Mountain View, CA 94043), or equiv. Response time must be rapid, <4 sec. (*2*) *Flowcell.*—10 mm light path and 0.25 mL vol. (No. 8495-L10, Thomas Scientific, or equiv.) and adapter (No. 8475-F10, Thomas Scientific, or equiv.) to hold cell assembly in spectrophtr. Polyethylene tubing, 0.055″ (1.4 mm) id, is used as inlet and outlet. Fit inlet tube with short length of stainless steel tube and connect outlet to vac. thru solenoid valve (AU-0034, Elanco Products Co., or equiv.). Adjust vac. to obtain flow rate of 1.0 mL/sec. Flowcell must be rigidly held in its holder and holder rigidly fixed in adapter. (*3*) *Water bath and heater.*—Part of AUTOTURB System, (**d**), or equiv. (*4*) *Constant voltage transformer.*—Sola transformer, wave form corrected, for stabilizing current voltage (EU-0020, Elanco Products Co., or equiv.); connected to spectrophtr. (5) *Filling unit.*—Filamatic single nozzle liquid filler (National Instrument Co., Inc., 4119 Fordleigh Rd, Baltimore, MD 21215), or equiv. Use with Teflon tubing, 0.063" (1.6 mm) id, to fill assay tubes. (6) *Digital voltmeter.*—$3^1/_2$ or $4^1/_2$ digit. Newport Model 400AS3 (Newport Electronics, Inc., 630 E Young St, Santa Ana, CA 92705), or equiv. Connect to spectrophtr output to measure % *T*.

(**d**) *For automated assay.*—Autoturb System (Elanco Products Co.).

### B. Standard Solutions

(**a**) *For manual assay.*—Dil. aliquots of stock soln, **967.39A(a)**, in enough pH 4.5 buffer, **957.23B(g)**, to give concns of 0.02, 0.04, 0.06, 0.08, and 0.10 μg CTC.HCl/mL.

(**b**) *For automated system.*—Prep. concns of 0.2, 0.4, 0.6, and 0.8 μg CTC.HCl/mL as in (**a**).

### C. Preparation of Inoculum

Inoculate 200 mL broth medium A, **957.23A(n)**, with 1 loop from 24 hr stock culture of *Staphylococcus aureus*, ATCC 9144, and incubate overnight at 37° on rotary shaker. Store ≤2 weeks at 2–10°.

### D. Preparation of Samples

Weigh 2 g sample into 250 mL glass or plastic centrf. bottle. Add 50 mL acid-acetone, **957.23B(h)**. Stopper or cap immediately, agitate intermittently 5 min, and adjust to pH 1.0–1.2 with HCl, if necessary, using pH meter. Note vol. HCl used. Add 50 mL (minus vol. HCl used) of acid-acetone. Insert blades of homogenizer into centrf. bottle and blend 3 min at high speed while keeping bottle covered. Rinse blades into bottle with 100 mL acid-acetone. Tightly cap bottle and centrf. ca 15 min at 2000 rpm. Filter thru Whatman No. 2V paper, or equiv. Make further dilns with pH 4.5 buffer, **957.23B(g)**, to ca 0.09 and 0.06 μg CTC.HCl/mL for manual assay, and 0.6 for automated assay.

### E. Assay

(**a**) *Manual method.*—Inoculate assay broth, **957.23A(n)**, with 0.5–1.0 mL inoculum, **977.37C**/100 mL. Incubate at 37° (20–30 min) until *A* is ca 0.05 at 600 nm in 10 mm flowcell, using uninoculated broth as blank.

Completely fill test tube carrier with 18 × 150 mm test tubes contg medium even tho assay may require only portion of these tubes. These tubes are included only to maintain uniform $H_2O$ flow in bath.

Pipet 1 mL pH 4.5 buffer, **957.23B(g)**, into each of 4 blank tubes and into each of 4 zero level std tubes. Pipet into each of 4 tubes 1 mL of each std and each sample soln. Add 9.0 mL inoculated assay broth to all tubes. Refrigerate blank tubes. Incubate all other tubes at 37° until % *T* of zero level tubes is ca 30 at 600 nm (3–4 hr). Do not remove tubes from bath during incubation to observe growth. Use extra tubes for this purpose and after inspection, replace in bath but do not measure. Stop growth in all tubes by heating 1–2 min at 80°; then cool rapidly in cold $H_2O$. Shake each tube by placing thumb over tube and inverting once. Do not shake mech. Measure turbidity at 600 nm in static suspension. Let culture flow ca 4 sec, stop flow ca 2 sec to dislodge air bubbles, and let flow again ca 3 sec. Stop flow and read % *T*. Average the 4 readings for each std and sample.

(**b**) *Automated method.*—System pipets two 0.10 and two 0.15 mL portions of sample soln and std solns into assay tubes, dils with inoculated broth, and reads % *T* at 600 nm. Average the 2 readings.

### F. Calculations

(**a**) *Manual assay.*—Convert av. % *T* to *A* and plot log *A* against μg CTC.HCl/mL on semi-log paper. Draw std response line. Read μg CTC.HCl/mL in sample from line.

(**b**) *Automated assay.*—Read μg CTC.HCl/mL from graph made as in (**a**) for 0.10 mL vols and for 0.15 mL vols. Average results.

$$g/lb = \mu g \text{ CTC.HCl (from curve)} \times D \times 454 \times 10^{-6}/g \text{ sample}$$

where $D$ = diln factor, $454 \times 10^{-6}$ = conversion of μg/g to g/lb.

Ref.: JAOAC **60**, 1119(1977).

CAS-64-72-2 (chlortetracycline.HCl)

## 971.48 Erythromycin in Feeds
### Microbiological Method
### First Action 1971

(Applicable to feeds contg 9.25 and 92.5 g/ton without pelleting adjuvants and ≥ 92.5 g/ton with bentonite or Masonex)

### A. Reagent

*Dimethoxymethane (methylal).*—Tech., $CH_2(OCH_3)_2$ (Aldrich Chemical Co., Inc.; or Eastman Kodak No. 525).

### B. Standard Solution

(**a**) *Erythromycin stock soln.*—Accurately weigh amt USP Erythromycin Ref. Std and dissolve in enough methylal-MeOH (4 + 1) to give concn of 1000 μg erythromycin base/mL. (1 μg base is equiv. to 1.08 μg of the thiocyanate.) Dil. further with pH 8 buffer, **957.23B(b)**, to final concn of 100 μg/mL. Store in refrigerator ≤1 week.

(**b**) *Std response line.*—Dil. appropriate aliquots of stock soln, (**a**), with enough pH 8 buffer, **957.23B(b)**, to obtain concns of 0.05, 0.1, 0.2, 0.4, and 0.8 μg erythromycin base/mL. Ref. concn is 0.2 μg/mL.

### C. Plates

Before assay, det. by prepn of trial plates optimum concn (usually 0.05–0.2%) of organism suspension of *Sarcina lutea*, **957.23D(e)**(*1*), to be added to agar medium J, **957.23A(j)**, to obtain zones of inhibition of adequate size (17.5 mm ± 10% with ref. concn) and sharpness. For actual assay add appropriate amt suspension to agar medium J previously melted and cooled to 48°. Place 10 mL inoculated medium in each of required number of plates, let harden, and refrigerate in inverted position until just before use.

### D. Assay Solution

Accurately weigh ca 10 g sample contg equiv. of ≥92.5 g erythromycin base/ton or 40 g contg equiv. of 9.25 g erythromycin base/ton and transfer to 250 mL g-s erlenmeyer.

For feeds contg no pelleting adjuvants add 20.0 mL $H_2O$ and 80.0 mL methylal-MeOH (4 + 1) soln. For feeds contg bentonite or Masonex, add 20.0 mL 5% phosphate buffer, **957.23B(b)**, and 15.0 mL MeOH. Mix and let feed slurry stand 10 min before adding 65.0 mL methylal. Stopper and mix 1 hr on mag. stirrer or mech. shaker (add glass beads for adequate mixing on shaker).

Let feed settle and dil. to 0.203 μg erythromycin base/mL as in (**a**) or (**b**) below:

(**a**) *Feeds containing equivalent of, or more than 92.5 g erythromycin base/ton.*—Dil. 2.0 mL ext to 100 mL with pH 8 buffer, **957.23B(b)**.

(**b**) *Feeds containing equivalent of 9.25 g erythromycin base/ton.*—Dil. 5 mL ext to 100 mL with pH 8 buffer.

### E. Assay

Using erythromycin std response line, **971.48B(b)**, assay soln, **971.48D**, and plates, **971.48C**, proceed as in **957.23E–F**, except use 4 plates for each concn required for std response line (total of 16 plates) and for each assay soln. Incubate at 30°.

Refs.: JAOAC **54,** 940, 944(1971); **60,** 176(1977).

CAS-114-07-8 (erythromycin)

## 960.67 Hygromycin B in Feeds

**Microbiological Method**

**Final Action 1965**

(Applicable to feeds contg ≥6000 units/lb)

### A. Standard Solutions

(**a**) *Hygromycin B stock soln.*—Accurately weigh amt of Hygromycin B Ref. Std (available from Elanco Products Co.) contg 50,000 units, transfer to 50 mL vol. flask, and dil. to vol. with pH 7 phosphate buffer, **957.23B(c)**. Store in refrigerator ≤2 weeks.

(**b**) *Std response line.*—Dil. appropriate aliquots of stock soln daily with enough pH 7 buffer, **957.23B(c)**, to obtain concns of 15, 25, 50, and 75 units/mL. Ref. concn is 25 units/mL.

### B. Plates

(**a**) *Base layer.*—Add 10 mL melted agar medium E to sterile petri dishes, distribute evenly, and let harden *on perfectly level surface*.

(**b**) *Seed layer.*—Before assay, det. by prepn of trial plates optimum concn (usually 0.2% of 1:10 diln) of spore suspension of *B. subtilis,* **957.23D(d)**, to be added to agar medium E. Zone of 16 mm ± 10% should be obtained with 25 units/mL. For actual assay add appropriate amt of spore suspension to agar medium E which has been melted and cooled to 48°. Mix thoroly and add 4.0 mL to each plate contg base layer. Store plates at 2–10° until just before use.

### C. Assay Solution

(**a**) *Preparation of ion exchange resin column.*—Slurry ca 1 lb (450 g) Amberlite IRC-50 ion exchange resin with 2 L 1*N* $H_2SO_4$ 3 hr. Wash until neut. with $H_2O$ and gradually add solid LiOH with stirring until pH remains at 7–8. Let stand overnight and wash with $H_2O$ ≥5 times. Neutze to pH 7.0 with 1*N* $H_3PO_4$. Store under $H_2O$ in glass container.

Place glass wool plug at bottom of 6 mm id × 140 mm long tube fitted with valve to control flow and 50 mL reservoir at top. Fill tube with $H_2O$ and add wet resin to within 20 mm of top of tube. Drain $H_2O$ to within 5 mm of resin surface. Wash with 25 mL sterile $H_2O$ immediately before use.

(**b**) *Preparation of assay soln.*—Obtain and prep. sample as in **965.16** and **950.02**. Weigh 50 g sample contg 6000–12,000 units/lb (30 g for 18,000–24,000 units/lb, 20 g for >24,000) into jar of high-speed blender. Add 300 mL (500 for the higher potency feeds) pH 7 phosphate buffer, **957.23B(c)**, and blend 5 min, operating blender from variable transformer set at 70. Centrf. 10 min at 2600 rpm. Adjust 125 mL supernate to pH 5.0 with HCl (ca 0.5 mL). Add 50 mL $CHCl_3$ previously washed with pH 7.0 buffer, stopper, and shake thoroly. Centrf. mixt. 10 min at 2600 rpm. Remove aq. phase, adjust to pH 7.0 with 40% NaOH soln (ca 0.7 mL), and centrf.

Transfer 100 mL neutzd soln (75 mL if feed contains ≥42,000 units/lb) to ion exchange column and adjust flow rate to 40 drops/min. Wash column with four 20 mL portions sterile $H_2O$. Elute hygromycin B with 50 mL $NH_4OH$ (1 + 9) into 100 mL Pyrex beaker. Evap. to 3–5 mL and adjust to pH 7.0 with 1*N* HCl. Transfer to 10 mL vol. flask, dil. to vol. with pH 7.0 phosphate buffer, and designate as assay soln. (Final concn should be ca 25 units/mL.)

### D. Assay

Using hygromycin std response line, assay soln, and plates, proceed as in **957.23E–F**, except use 6 plates for each concn required for std response line (total of 18 plates) and for each assay soln. Equations for *L* and *H* cannot be used. Incubate at 37°.

### E. Calculation

Units/lb = [1.1 × (units/mL assay soln) × 454 × mL pH 7 buffer (300 or 500) × 10 × (125 + mL HCl + mL 40% NaOH)]/[125 mL × g sample × mL neutzd soln put on column].

Ref.: JAOAC **43,** 213(1960).

CAS-31282-04-9 (hygromycin)

## 975.60 Lasalocid in Feeds

**Microbiological Method**

**First Action 1975**

### A. Reagents and Apparatus

*See* **957.23C(a)–(c)**, **975.61C(a)**, and following:

(**a**) *Ethyl acetate.*—Purify by passage over silica gel and distil.

(**b**) *Methanol.*—75% and 19.4% by vol. in $H_2O$.

(**c**) *Automatic pipetting machine.*—Brewer (available from Scientific Equipment Products (SEPCO), or equiv.

### B. Standard Solutions

(**a**) *Lasalocid sodium stock soln.*—100 μg/mL. Accurately weigh suitable amt Lasalocid Na Ref. Std (available from Hoffmann-La Roche Inc.) and dil. to appropriate vol. with anhyd. MeOH.

(**b**) *Std response line.*—Dil. aliquots stock soln, (**a**), using anhyd. MeOH and $H_2O$, to obtain concns of 0.25, 0.5, 1.0, 2.0, and 4.0 μg/mL in 25% MeOH (v/v). Ref. concn is 1 μg/mL. Solns are stable ≤1 month at room temp.

### C. Stock Culture and Preparation of Inoculum

Prep. slant culture of *Bacillus subtilis,* ATCC 6633, on ≥1 tube of agar medium A, **957.23A(a)**. Incubate 16–24 hr at 37°. Wash growth from stock culture with ca 3 mL sterile distd $H_2O$, transfer liq. to surface of 300 mL agar medium G, **957.23A(g)**, in Roux bottle, and incubate 7 days at 37°. Wash growth from agar surface with ca 25 mL sterile distd $H_2O$ into centrf. bottle. Heat suspension 30 min in 65° $H_2O$ bath. Centrf., decant, and resuspend cells in ca 25 mL sterile distd $H_2O$. Repeat centrfg and suspending 3 times, discarding $H_2O$ washings. Heat residual spores 30 min in 65° $H_2O$ bath. Resuspend spores in ca 35 mL sterile distd $H_2O$. Suspension may be kept 1 yr at ca 5°. Before use, dil. suspension with sterile distd $H_2O$ (usually 1 + 50) to read 20% *T* on spectrophtr at 530 nm; store ≤1 week at 5°.

### D. Plates

*Seed layer.*—Use single inoculated agar layer. Before assay, det. by prepn of trial plates optimum concn (usually 5 mL for each 100 mL seed agar) of dild suspension of *B. subtilis*, **975.60C**, to be added to agar medium M to obtain zones of inhibition of adequate size (17.5 ± 2.5 mm with 1.0 μg/mL) and sharpness. For actual assay, add appropriate amt of suspension to agar medium M previously melted, adjusted to pH 6.0, and cooled to 60°. Mix thoroly and add 6.0 mL to each plate. Distribute evenly and let harden *on perfectly level surface*. Prep plates 2.5–3 hr before use.

### E. Assay Solution

(**a**) *Premixes, 15%.*—Accurately weigh 1.0 g premix, transfer to 200 mL vol. flask, add 100 mL MeOH, shake vigorously 3 min, and dil. to vol. with MeOH. Dil. 4.0 mL of this diln to 100 mL with MeOH. Further dil. 3.0 mL of last diln with 22 mL MeOH and $H_2O$ to 100 mL (1 mL = ca 1 μg lasalocid Na/mL 25% MeOH).

(**b**) *Final feed, 0.0075%.*—Weigh 20 g mash feed or pellets ground to pass No. 20 sieve and transfer to 500 mL vol. flask. Add 12 mL pH 4.7 buffer (**975.61C(a)**) and wet feed thoroly. Immerse flask 5 min in 70° $H_2O$ bath. Cool to room temp. Add 200 mL EtOAc, stopper, and shake mech. 10 min. Centrf. ca 100 mL EtOAc ext 10 min at 2000 rpm. Pipet 60 mL clear EtOAc ext into 200 mL vol. flask, add 8 mL 1.5*N* HCl, and shake 10 min. Let layers sep., transfer EtOAc layer to 100 mL g-s centrf. tube, and centrf. 10 min at 2000 rpm. Pipet 40 mL clear EtOAc ext into another 100 mL g-s centrf. tube and add 2 mL 40% NaOH soln. Stopper and shake briefly by hand, add 8 g anhyd. $Na_2SO_4$, and shake again. Centrf. 10 min at 2000 rpm and decant 25 mL clear supernate into 50 mL g-s graduate. Evap. all EtOAc under stream of N with graduate immersed in 60° $H_2O$ bath. Dissolve residue in 5 mL hexane, add exactly 25 mL 75% MeOH (v/v), stopper, and shake vigorously 1 min. Transfer to 125 mL separator and let stand ca 1 hr. Withdraw lower (MeOH) layer into 25 mL beaker, pipet 5 mL into 50 mL vol. flask, and dil. to vol. with 19.4% (v/v) MeOH.

### F. Assay

Using lasalocid Na std response line, assay soln, and plates, proceed as in **957.23E–F**, incubating at 35 ± 1°. Calc. L and H and fit straight line by simplified least square method, **957.23E**.

Refs.: JAOAC **57**, 978(1974); **58**, 941(1975); **59**, 398, 1286(1976).

CAS-25999-31-9 (lasalocid)

## 967.40 Lincomycin in Feeds
**Microbiological Method**
**First Action 1967**
***Method I***

(Applicable to feeds contg ≥3.63 g/ton)

### A. Standard Solutions

(**a**) *Lincomycin stock soln.*—Accurately weigh ca 40 mg USP Lincomycin.HCl Ref. Std and dissolve in enough pH 8 buffer, **957.23B(b)**, to give concn of exactly 100 μg lincomycin base/mL. Store ≤30 days at 2–10°.

(**b**) *Std response line.*—Dil. aliquots stock soln, (**a**), with enough pH 8 phosphate buffer, **957.23B(b)**, to obtain concns of 0.2, 0.4, 0.8, 1.6, and 3.2 μg lincomycin base/mL. Ref. concn is 0.8 μg/mL.

### B. Plates

(**a**) *Base layer.*—Add 10 mL melted agar medium E to sterile petri dishes, distribute evenly, and let harden *on perfectly level surface*.

(**b**) *Seed layer.*—Before assay, det. by prepn of trial plates optimum concn of organism suspension of *S. lutea* (usually 0.02–0.05% of suspension prepd as in **957.23D(e)**(*1*) or 0.2–1% as in **957.23D(e)**(*2*)) to be added to agar medium J to obtain zones of inhibition of adequate size (16 mm ± 10% with 0.8 μg/mL) and sharpness. For assay, add appropriate amt of organism suspension to agar medium J previously melted and cooled to 48°. Mix thoroly and add 4.0 mL to each plate contg base layer.

### C. Assay Solution

Obtain and prep. sample as in **965.16** and **950.02**. Accurately weigh ca 10 g ground sample and transfer to 250 mL g-s, r-b flask, add 20 mL $H_2O$, and shake 10 min on wrist-action shaker. Add 50 mL *0.1N HCl-MeOH* (*1 + 4*) and shake 10 min. Filter thru Whatman No. 4 paper, using 42 mm buchner and 500 mL flask. Repeat extn twice, using 50 mL HCl-MeOH each time. (Do not add more $H_2O$.) Alternatively, conduct extns in 250 mL centrf. bottle and centrf. to clarify.

Transfer combined filtrates to 500 mL r-b flask and evap. to 15–20 mL, using rotary evaporator. (Do not heat >60°.) Transfer aq. ext to 125 mL separator. Rinse flask successively with 10 mL Skellysolve B, **951.01A(o)**, 7–8 mL phosphate buffer, **957.23B(b)**, and 10 mL Skellysolve B. Add all rinsings to separator, shake, and let sep. Drain aq. phase, ext Skellysolve B twice with 7–8 mL buffer, and adjust combined exts to pH 8.0 with dil. NaOH soln. Adjust vol. with pH 8 buffer to 0.6–1.0 μg lincomycin base/mL.

### D. Assay

Using lincomycin std response line and assay soln, proceed as in **957.23E–F**, incubating at 32°.

Refs.: JAOAC **50**, 442(1967); **61**, 1107(1978).

CAS-154-21-2 (lincomycin)

## 978.31 Lincomycin in Feeds
**Microbiological Method**
**First Action 1978**
***Method II***

(Applicable to feeds, feed supplements, and vitamin-mineral premixes contg 20-2600 g/ton)

### A. Standard Solutions

*Std response line (for monolayer plates).*—Dil. aliquots stock soln, **967.40A(a)**, with enough pH 8 phosphate buffer, **957.23B(b)**, to obtain concns of 0.15, 0.3, 0.6, 1.2, and 2.4 μg lincomycin base/mL. Ref. concn is 0.6 μg/mL.

### B. Plates

*Monolayer.*—Proceed as in **967.40B(b)**, except zones of inhibition should be 18.5 mm ± 10% with 0.6 μg/mL; add 7.5 mL final mixt. to each plate, distribute evenly, and let harden *on perfectly level surface*.

### C. Assay Solution

Obtain and prep. sample as in **965.16** and **950.02**.

(**a**) *For 20–80 g/ton.*—Accurately weigh ca 10–20 g ground sample (*see* Table **978.31**), and transfer quant. to 250 mL centrf. bottle. Add 75.0 mL 0.1N HCl-MeOH (1 + 4), and shake 20 min on mech. shaker. Centrf. 5 min at ca 2500 rpm to clarify.

Decant supernate into 250 mL separator, add 75 mL hexane, and shake moderately 1 min. Let layers sep. ≥15 min. Drain lower aq. layer into 100 mL beaker, and pipet 2.5–5 mL aliquot into 50 mL mixing cylinder. Adjust vol. to ca 40 mL with pH 8 phosphate buffer, **957.23B(b)**, and add 1 drop 4*N* NaOH. Stopper and shake vigorously. If necessary, adjust to pH 8 and dil. to 50 mL with pH 8 buffer. Final concn should be 0.5–0.8 μg lincomycin base/mL.

(**b**) *For 80–2600 g/ton.*—Accurately weigh 4–6 g ground sample (*see* Table **978.31**), and transfer quant. to 250 mL centrf. bottle. Add 50.0 mL 0.1*N* HCl-MeOH (1 + 4), shake 20 min on mech. shaker, and add 50 mL pH 8 buffer. Shake mech. 5 min and centrf. 5 min at ca 2500 rpm to clarify. Dil. 1.0 mL aliquot with pH 8 buffer to 0.4–0.8 μg lincomycin base/mL.

### D. Assay

Using lincomycin std response line and assay soln, proceed as in **957.23E–F**, incubating at 32°, except use 2 plates instead of 3 and 6 readings instead of 9. Av. of all 24 readings of ref. concn from 8 plates is correction point for response line.

Refs.: JAOAC **50,** 442(1967); **61,** 1107(1978).

CAS-154-21-2 (lincomycin)

## 972.56 Monensin in Feeds

**Microbiological Method**

**First Action 1972**

(Applicable to feeds contg ≥90 g/ton)

### A. Standard Solutions

(**a**) *Monensin std solns.*—(*1*) *Stock soln.*—1 mg/mL. Accurately weigh enough Monensin Na Salt Ref. Std (Elanco Products Co.) into 100 mL vol. flask to give concn of 1 mg free acid/mL. Dil. to vol. with anhyd. MeOH. Store at 5°; discard after 2 weeks. (*2*) *Working soln.*—100 μg/ mL. Dil. 1 mL stock soln to 10 mL with aq. MeOH (1 + 1). Prep. fresh daily.

(**b**) *Std response line.*—Dil. aliquots of working soln, (**a**)(*2*), with enough aq. MeOH (1 + 1) to obtain concns of 0.25, 0.5, 1.0, and 2.0 μg monensin/mL. Ref. concn is 0.5 μg/mL.

### B. Plates

*Seed layer.*—Use single inoculated agar layer. Before assay, det. by prepn of trial plates optimum concn (usually 0.5 mL for each 100 mL seed agar) of suspension of *B. subtilis*, **957.23D(d)**, to be added to agar medium L to obtain zones of inhibition of adequate size (17.5 ± 2.5 mm with 0.5 μg/mL) and sharpness. Before use, dil. suspension with sterile $H_2O$ to read 20% *T* on spectrophtr at 530 nm; prep. dild suspension daily. For actual assay, add appropriate amt of suspension to agar medium L previously melted and cooled to 48–50°. Mix thoroly and add 6.0 mL to each plate. Cover and refrigerate ≥1 hr before use.

### C. Assay Solution

Place glass wool plug at bottom of 19 mm id × 500 mm chromatgc tube and add ca 75 mm alumina, **961.23C(a)**, with gentle tapping. Accurately weigh sample (20 g finished feed, 5 g premix) and add to column. Elute column with MeOH-$H_2O$ (9 + 1), using 200 mL vol. flask as receiver. Do not restrict flow. Collect 200 mL eluate, mix, and dil. with aq. MeOH (1 + 1) to 0.5 μg monensin/mL. Designate soln obtained as assay soln.

### D. Assay

Use 10 seeded agar plates for std curve. Place 4 stainless steel cylinders, **957.23C(a)**, on each plate at 90° intervals. Fill 1 cylinder on each plate with different concn of std soln, (**b**), and incubate all plates 16–18 hr at 35–37°. Measure diams of zones of inhibition. Calc. av. zone diam. at each std concn and fit straight line by simplified least squares method, **957.23E**.

### E. Determination

Use 5 plates for each assay soln and align cylinders at 90° intervals. On each plate fill alternate cylinders with ref. concn and fill other cylinders with assay soln. Incubate plates 16–18 hr at 35–37° and measure diams of zones of inhibition to nearest 0.1 mm. Average 10 readings of ref. concn and 10 readings of assay soln. Proceed as in **957.23F**.

Ref.: JAOAC **55,** 718(1972).

CAS-17090-79-8 (monensin)

## 976.37 Monensin in Feeds

**Turbidimetric Method**

**First Action 1976**

### A. Apparatus and Reagents

(**a**) *Assay broth.*—In 500 mL erlenmeyer, dissolve 9.0 g low K ion medium (N-Z Case, Humko-Sheffield Chemical, PO Box 398, Memphis TN 38101), 3.0 g yeast ext, and 8.0 g glucose in 100 mL $H_2O$ while heating to bp. Cool immediately, and add to bottle contg 1500 mL sterile $H_2O$, 4.5 mL 10% soln of polysorbate 80, and 12.5 mL citrate buffer soln, (**c**). pH should be 5.2 ± 0.1; adjust if necessary.

(**b**) *Automated turbidimetric system.*—Autoturb® (Elanco Products Co.), or equiv. System pipets samples into assay tubes, dils with inoculated broth, incubates, and measures turbidity.

(**c**) *Citrate buffer soln.*—pH 4.0. Dissolve 105 g citric acid.$H_2O$, 142 g Na citrate.$2H_2O$, and 1.9 g KCl in $H_2O$ and dil. to 1 L with $H_2O$.

(**d**) *Monensin std solns.*—(*1*) *Stock soln.*—1 mg free acid/mL. Accurately weigh enough Monensin Na Salt Ref. Std (Elanco Products Co., Department MC757) into 100 mL vol.

**Table 978.31 Examples of Sample Size and Dilution of Extract**

| Assay Soln. 978.31C | Feed Level of Lincomycin, g/ton | Feed Level of Lincomycin, μg/g | Sample Size, g | Total Extn Vol., mL | Aliquot Vol., mL | Final Vol., mL | Final Concn, μg/mL |
|---|---|---|---|---|---|---|---|
| (**a**) | 20 | 22 | 20.0 | 75 | 5.0 | 50 | 0.59 |
| (**a**) | 40 | 44 | 10.0 | 75 | 5.0 | 50 | 0.59 |
| (**a**) | 80 | 88 | 10.0 | 75 | 2.5 | 50 | 0.59 |
| (**b**) | 80 | 88 | 6.0 | 100 | 1.0 | 10 | 0.53 |
| (**b**) | 140 | 154 | 6.0 | 100 | 1.0 | 15 | 0.62 |
| (**b**) | 200 | 220 | 6.0 | 100 | 1.0 | 25 | 0.53 |
| (**b**) | 400 | 440 | 6.0 | 100 | 1.0 | 50 | 0.53 |
| (**b**) | 1000 | 1100 | 4.0 | 100 | 1.0 | 100 | 0.44 |
| (**b**) | 2600 | 2860 | 4.0 | 100 | 1.0 | 200 | 0.57 |

flask to give concn of 1 mg free acid/mL. Add MeOH to dissolve salt, dil. to vol. with MeOH, and stopper tightly. (Soln is stable at room or refrigerator temp. 1 month, except for gain in potency from loss of MeOH. Warm refrigerated soln to room temp. before pipetting.) (*2*) *Intermediate soln.*—10 μg/mL. Dil. 1.0 mL stock soln to 100 mL with MeOH. (*3*) *Autoturb working solns.*—Using pipets and vol. flasks, prep. solns contg 0.25, 0.50, 0.75, and 1.0 μg/mL MeOH. (*4*) *Manual working solns.*—Dil. stock soln to 10.0 μg/mL with MeOH. Prep. std solns of 0.0, 0.05, 0.10, 0.15, and 0.20 μg/mL by adding appropriate vols of 10.0 μg/mL soln to vol. flasks, adding enough MeOH so that final MeOH concn is 20% in each flask, and adjusting to vol. with $H_2O$.

### B. Preparation of Inoculum

Use *Streptococcus faecalis* (ATCC 8043) as assay organism in either frozen suspension or freshly grown inoculum. Prep. latter by inoculating flask of broth medium A, **957.23A(n)** (Difco No. 3 Broth), in afternoon and leaving flask overnight at room temp. Use 10–20 mL inoculum/L assay medium. Let assay medium inoculated with frozen suspension stand at room temp. 45 min before using. (This treatment prevents drift within test.)

### C. Preparation of Samples

Grind ≥100 g feed sample, and mix. Accurately weigh 10.0 g representative well ground sample, and transfer to 4 oz (125 mL) jar fitted with Al foil-lined cap. Add 100.0 mL MeOH and tightly cap jar. Shake well and let stand overnight or ≥8 hr. Approx. 30 min before dilg, shake once more and let solids settle.

(**a**) *Autoturb system measurement.*—Dil. 1.0 mL ext to 20.0 mL with MeOH to prep. soln to be dild by system.

(**b**) *Manual measurement.*—Dil. ext 100-fold (1 + 99) and 66-fold (3 + 197) to obtain approx. assay concns of 0.10 and 0.15 μg/mL by adding aliquots of sample to sep. vol. flasks, adding MeOH to 20% final concn, and dilg to vol. with $H_2O$. These solns are stable ≥1 week at room temp.

### D. Assay

(**a**) *Autoturb system measurement.*—Place assay tube carrier in diluter unit. Place total of 20 sample tubes in sample turntable (including 5 tubes contg the 4 std levels and 1 tube with MeOH) in middle of test series. Fill remaining places in turntable with samples dild to estd concn of 0.6 μg/mL. When diluter unit has processed tubes, place carrier in 37.5° $H_2O$ bath 3–4 hr or until turbidity of 0 tube measures 40% *T* or slightly less. Stop growth by heating carrier with tubes in 80° $H_2O$ bath 1–2 min. Cool and read at 650 nm.

(**b**) *Manual measurement.*—(*1*) *Std curve.*—Prep. series of culture tubes, in triplicate, by adding 0.5 mL of each working std soln to sep. tubes. Add 9.5 mL inoculated assay broth to each tube. (*2*) *Samples.*—Prep. 2 dilns for each sample to contain ca 0.1 and 0.15 μg monensin activity/mL soln. Add 0.5 mL of each diln to 3 sep. tubes, and add 9.5 mL inoculated assay broth to each tube. Sample levels are ca 0.05 and 0.075 μg/tube.

Incubate std and sample tubes 4–5 hr at 37.5° or until turbidity of control std tubes (0.0 monensin) measures ca 30% *T* at 650 nm. Terminate growth by heating tubes ≥2 min at 80°. Cool tubes, and measure turbidity of each tube at 650 nm.

### E. Preparation of Standard Curve

(**a**) *Autoturb system measurement.*—Prep. 2 dose-response curves, 1 for 0.1 mL loop and other for 0.15 mL loop. Prep. graph of log % *T* against concn. Use av. turbidity of each pair of tubes in prepg std curves. Assay each sample in duplicate at 2 concns, 0.10 mL and 0.15 mL sample. Average readings from 0.10 mL loop for sample and obtain monensin equiv. by interpolation from 0.10 mL std curve. Repeat for 0.15 mL loop and use 0.15 mL std curve.

(**b**) *Manual measurement.*—Prep. only single std curve. Prep. graph of log % *T* against concn. Average 3 readings obtained for each diln of sample. Obtain monensin equiv. for each sample diln by interpolation from std curve.

### F. Calculations

(**a**) *Autoturb system measurement.*—Average 2 readings of 0.10 mL vols for feed sample and obtain monensin equiv. by interpolation from 0.10 mL std curve ($P_1$). Repeat for 0.15 mL sample vols to obtain $P_2$.

$$\mu g \text{ Monensin activity/mL} = (P_1 + P_2)/2 = P_A$$

$$\mu g \text{ Monensin/g feed} = P_A \times 200$$

$$\text{g Monensin activity (free acid/ton feed)} = P_A \times 200 \times 0.908$$

(**b**) *Manual measurement.*—Multiply av. monensin equiv. for each of the 2 sample dilns ($M_1$ = 0.10 mL diln, $M_2$ = 0.15 mL diln) by its respective diln factor (e.g., 100 and 66). Average the 2 values to obtain potency of original material ($P_A$).

$$\mu g \text{ Monensin activity/mL} = (M_1 + M_2)/2 = P_A$$

$$\mu g \text{ Monensin/g feed} = P_A \times 10$$

$$\text{g Monensin activity (free acid/ton feed)} = P_A \times 10 \times 0.908$$

Refs.: JAOAC **55,** 114(1972); **60,** 179(1977).

CAS-17090-79-8 (monensin)

## 970.89 Neomycin in Feeds
### Microbiological Method
### First Action 1970

(Applicable to feeds contg ≥28 g neomycin base/ton; soybean content >40% reduces accuracy of method.)

### A. Standard Solutions

(**a**) *Stock soln.*—Dry USP Neomycin Sulfate Ref. Std 3 hr in vac. oven at ≤5 mm (0.66 kPa). Accurately weigh enough dried std (10–50 mg) and dissolve in Tris buffer, **957.23B(l)**, to give concn of 100 μg neomycin base/mL. (Neomycin sulfate equiv. to neomycin base is given on container.) Store ≤4 weeks at 2–10°.

(**b**) *Std response line.*—Dil. aliquots of stock soln (**a**) with enough inactivated feed ext, **970.89C(b)**, to obtain 0.50, 0.75, 1.13, 1.69, and 2.53 μg neomycin base/mL. Prep. std response line for each feed sample.

### B. Plates

(**a**) *Base layer.*—Add 10 mL melted agar medium K to petri dishes, distribute evenly, and let harden *on perfectly level surface.*

(**b**) *Seed layer.*—Add appropriate amt (usually 0.5– 2%) broth culture of *S. epidermidis,* **957.23D(f)**, to agar medium K previously melted and cooled to 48°. Mix thoroly and add 4.0 mL to each plate contg base layer. Distribute agar evenly by tilting plates from side to side with circular motion and let harden. *Use plates same day prepd.* Zone of inhibition of 18 mm ± 15% should be obtained with 1.13 μg/mL.

### C. Assay Solution

(**a**) *Preparation of sample.*—Obtain and prep. sample as in **965.16** and **950.02**. Weigh 20 g sample into 500 mL r-b flask.

**Table 970.89 Dilution of Extract**

| Neomycin Base, g/ton | Sample Extract, Diln I | Neomycin Base, Final Concn, μg/mL | Inactivated Extract, Diln II |
|---|---|---|---|
| 140 | 1 to 20 | 1.54 | 10 to 200 |
| 70 | 1 to 10 | 1.54 | 20 to 200 |
| 35 | 1 to 10 | 0.77 | 20 to 200 |

Add 100.0 mL NaCl-$CaCl_2$ soln, **957.23B(n)**, and shake 15 min on wrist-action shaker. Transfer to 250 mL centrf. bottle (*do not rinse*), centrf. 15 min at 1800–2000 rpm, and decant supernate into beaker. Transfer 20 mL aliquot to 100 mL beaker and set aside to prep. std response line diluent. Using pH meter, adjust remaining portion with HCl to pH 2.0. Wait ≥5 min; then readjust to pH 8.0 with 10*N* NaOH. (High concns of acid and base are used to avoid significant changes in vol.) Centrf. 30–35 mL 15 min at 1800–2000 rpm. Dil. ext soln with Tris buffer according to neomycin content in feed as in Table **970.89** (diln I).

(**b**) *Preparation of std response line diluent.*—Inactivate the 20 mL aliquot from (**a**) by adjusting to pH 4.5–4.7 with 2*N* HCl. Add 1.5 mL fresh 5.25% NaOCl soln, **957.23B(o)**, and heat 45 min in boiling $H_2O$ bath, stirring thoroly at least every 10 min during heating period. Cool to room temp., adjust to pH 8.0 with 3.5*N* NaOH, and dil. to 20 mL with $H_2O$. Dil. inactivated ext with Tris buffer, **957.23B(l)**, according to neomycin content in feed as in diln II column of Table **970.89**.

Use dild soln to prep. std response line solns.

### D. Assay

Using neomycin std response line, assay soln, and plates, proceed as in **957.23E–F**, incubating at 32–35°. Result may be calcd as neomycin sulfate by multiplying neomycin base by 1.428 (1.0 mg neomycin sulfate is equiv. to 0.7 mg neomycin base).

Ref.: JAOAC **53,** 60(1970).

CAS-1404-04-2 (neomycin)

## 962.25 Novobiocin in Feeds
### Microbiological Method
**First Action 1962**
**Final Action 1965**

(Applicable to feeds contg ≥350 ppm)

### A. Standard Solutions

(**a**) *Novobiocin stock soln.*—Dry USP Novobiocin Ref. Std 3 hr at 60° in vac. oven at ≤5 mm (0.66 kPa). Accurately weigh ca 30 mg dried std, dissolve in 10 mL absolute alcohol, and dil. with enough pH 8 phosphate buffer, **957.23B(b)**, to give concn of 1 mg/mL. Store ≤3 weeks at 2–10°.

(**b**) *Std response line for feed supplements.*—Dil. aliquots of stock soln, (**a**), with enough pH 6 buffer, **957.23B(f)**, to obtain concns of 1.9, 2.4, 3.0, 3.8, and 4.7 μg/mL. Ref. concn is 3.0 μg/mL.

(**c**) *Std response line for finished feeds.*—Dil. aliquots of stock soln, (**a**), with enough pH 6 buffer, **957.23B(f)**, to obtain concns of 0.128, 0.16, 0.20, 0.25, and 0.312 μg/mL. Ref. concn is 0.20 μg/mL.

### B. Plates

(**a**) *For feed supplements.*—(*1*) *Base layer.*—Add 21 mL melted agar medium C to sterile petri dishes, distribute evenly, and let harden *on perfectly level surface.*

(*2*) *Seed layer.*—Before assay, det. by prepn of trial plates optimum concn of organism suspension of *S. lutea* (usually 0.2–0.5% of suspension prepd as in **957.23D(e)**(*1*) or 2–5% as in **957.23D(e)**(*2*)) to be added to agar medium C to obtain zones of inhibition of adequate size (14 mm ± 10% with 3.0 μg/mL) and sharpness. For actual assay, add appropriate amt of organism suspension to agar medium C, previously melted and cooled to 48°. Mix thoroly and add 5.0 mL to each plate contg base layer.

(**b**) *For final feed.*—(*1*) *Base layer.*—Prep. as in (**a**) (*1*), using 15 mL melted agar medium C.

(*2*) *Seed layer.*—Add appropriate amt (usually 0.5– 2%) broth culture of *S. epidermidis*, **957.23D(f)**, to agar medium C previously melted and cooled to 48°. Mix thoroly and add 5 mL to each plate contg base layer. Zone of 14 mm ± 10% should be obtained with 0.20 μg/mL.

### C. Assay Solution

(**a**) *For feed supplements containing 50 mg/g.*— Obtain and prep. sample as in **965.16** and **950.02**. Accurately weigh suitable size sample and add enough absolute alcohol to give estd concn of 2 mg/mL. Let stand 30 min, shaking occasionally. Add equal vol. pH 8 phosphate buffer, **957.23B(b)**, and mix. Dil. to estd concn of 3 μg/mL with pH 6 buffer, **957.23B(f)**, and use as assay soln.

(**b**) *For final feed containing not less than 350 μg/g.*—Obtain and prep. sample as in **965.16** and **950.02**. Weigh 1 g sample into 50 mL g-s graduate; ext twice with 20 mL EtOAc, shaking vigorously 2 min. Decant supernate into second 50 mL g-s graduate. Dil. to 50 mL with EtOAc. Transfer 2.0–4.0 mL aliquot to 100 mL vol. flask, add 5.0 mL pH 8 phosphate buffer, **957.23B(b)**, and mix thoroly. Dil. to vol. with pH 6 buffer, **957.23B(f)**, and shake vigorously to dissolve all EtOAc. Final concn of novobiocin should be 0.15–0.30 μg/mL.

### D. Assay

Using proper novobiocin std response line, assay soln, and plates, proceed as in **957.23E–F**, incubating at 32–35°.

Ref.: JAOAC **45,** 310(1962).

CAS-303-81-1 (novobiocin)

## 974.48 Nystatin in Feeds
### Microbiological Method
**First Action 1974**

(For feeds contg ≥50 g/ton)

### A. Standard Solutions

(**a**) *Nystatin stock soln.*—Dry ca 25 mg USP Nystatin Ref. Std 2 hr at 40° in vac. oven at ≤5 mm (0.66 kPa). Accurately det. dry wt and add enough MeOH to give concn of exactly 500 units/mL. Dissolve by shaking on mech. shaker 0.5 hr (soln may be slightly hazy). Prep. fresh daily. (1 g nystatin = 2,800,000 units.)

(**b**) *Std response line.*—Dil. aliquots stock soln, (**a**), with enough inactivated feed ext, **974.48C(b)**, to obtain concns of 10, 20, and 40 units/mL. Prep. std response line for each feed sample. Ref. concn is 20 units/mL.

### B. Plates

*Seed agar.*—Use single inoculated agar layer. Before assay, det. by prepn of trial plates optimum concn of organism suspension of *Sacch. cerevisiae* (usually 2.5% of suspension prepd as in **957.23D(g)**(*1*)), to be added to agar medium I, **957.23A(i)**, to obtain zones of inhibition of adequate size (18 mm ± 10%

for 20 units/mL) and sharpness. For actual assay, add appropriate amt of organism suspension to agar medium I, previously melted and cooled to 48°. Mix thoroly and add 10 mL to each sterile petri dish.

### C. Assay Solution

(**a**) *Preparation of sample.*—Obtain and prep. sample as in **965.16** and **950.02**. Weigh amt sample contg ca 50 g nystatin/ton (ca 7700 units total), into 500 mL erlenmeyer. Add 150 mL anhyd. MeOH, mix thoroly by hand, and then shake 1 hr on rotary shaker. Centrf. briefly. Dil. 4 parts ext with 6 parts 10% phosphate buffer, **957.23B(e)**. Set aside 10 mL as sample test soln and use remainder to prep. response line diluent.

(**b**) *Preparation of std response line diluent.*—Measure vol. of remaining dild ext soln from (**a**). Place soln in cotton-plugged erlenmeyer having twice capacity of vol. to be contained, and autoclave 15 min at 121° (slow exhaust). Cool to room temp. and restore to original vol. by adding anhyd. MeOH. Mix thoroly and use dild soln to prep. std response line solns.

### D. Assay

Use 10 plates for each sample. On each plate, fill alternate cylinders, each with 1 of std response line solns, and fill other 3 cylinders with sample test soln. Incubate plates overnight at 30–37°; then measure diams of zones of inhibition to nearest 0.1 mm. Calc. av. zone diam. at each std concn; plot values on semilog graph paper and draw line as in **957.23E**. Calc. av. zone diam. of sample test soln. Using this value, det. potency of antibiotic from std response line.

Ref.: JAOAC **57,** 536(1974).

CAS-1400-61-9 (nystatin)

## 974.49 Oleandomycin in Feeds

**Microbiological Method**
**First Action 1974**
**Final Action 1975**

(For feeds contg ≥2 g/ton)

### A. Standard Solutions

(**a**) *Oleandomycin stock soln.*—250 μg/mL. Accurately weigh Oleandomycin Chloroform Adduct Ref. Std (available from Pfizer, Inc., 235 E. 42nd St, New York, NY 10017) or USP Ref. Std. Dissolve in ca 5 mL MeOH and dil. with enough pH 8.0 phosphate-bicarbonate buffer, **957.23B(a)**, to give concn of 250 μg/mL. Prep. fresh daily.

(**b**) *Intermediate std soln.*—5 μg/mL. Dil. 5.0 mL stock soln to 250 mL in vol. flask with pH 8.0 phosphate-bicarbonate buffer, **957.23B(a)**.

(**c**) *Std response line.*—Dil. intermediate std soln with pH 8.0 buffer, **957.23B(a)**, to obtain concns of 0.025, 0.05, 0.10, 0.20, and 0.40 μg/mL. Ref. concn is 0.10 μg/mL.

### B. Assay Solution

Obtain and prep. sample as in **965.16** and **950.02**. Accurately weigh 20 g sample contg ≥2 g oleandomycin/ ton into 500 mL boiling flask, add 200 mL pH 8.0 phosphate-bicarbonate buffer, **957.23B(a)**, and shake mech. 45 min. Let settle, decant into 50 mL centrf. tube, and centrf. 5 min at 2000 rpm. Dil. supernate with addnl pH 8.0 buffer to approx. ref. concn of 0.1 μg/mL. Use sample ext same day prepd.

### C. Plates

Use single inoculated agar layer. Before assay, det. by prepn of trial plates optimum concn of organism suspension of *S. lutea* (usually 0.05–0.1% of suspension prepd as in **957.23D(e)**(*1*)) to be added to agar medium J to obtain zones of inhibition of adequate size (16 mm ± 10% with 0.1 μg/mL) and sharpness. For actual assay, add appropriate amt of organism suspension to agar medium J previously melted and cooled to 48°. Mix thoroly and add 10.0 mL to each petri dish.

### D. Assay

Using oleandomycin std response line, assay soln, and plates, proceed as in **957.23E–F**, incubating plates at 37°.

Refs.: JAOAC **56,** 1149(1973); **57,** 823(1974).

CAS-3922-90-5 (oleandomycin)

## 968.50 Oxytetracycline in Feeds

**First Action 1968**
**Final Action 1970**

(Applicable to feeds contg ≥10 g/ton)

### A. Standard Solutions

(**a**) *Oxytetracycline stock soln.*—Accurately weigh ca 40 mg Oxytetracycline USP Ref. Std and dissolve in enough 0.1*N* HCl to give exact concn of 100 μg oxytetracycline/mL. (1 μg base is equiv. to 1.08 μg of the hydrochloride.) Store in dark ≤5 days at 2–10°.

(**b**) *Std response line.*—Dil. appropriate aliquots stock soln, (**a**), with enough pH 4.5 buffer, **957.23B(g)**, to obtain concns of 0.05, 0.10, 0.20, 0.40, and 0.80 μg oxytetracycline/mL. Ref. concn is 0.20 μg/mL.

### *Microbiological Method I*

(Applicable to >220 mg/lb)

### B. Assay Solution

(Solv. losses may occur from evapn of volatile solvs in open containers. Carefully measure and record vols. of solvs, and make appropriate mathematical corrections for any losses in vol.)

Obtain and prep. sample as in **965.16** and **950.02**. Using mortar and pestle or high-speed blender, grind 2 g sample with 50 mL acid-MeOH, **957.23B(i)**, and transfer mixt. to 100 mL centrf. tube. Wash mortar and pestle or blender jar with 50 mL acid-MeOH and combine washings with ext in centrf. tube. Shake well 5 min. Centrf. ca 15 min at 2000 rpm. Remove 10 mL clear soln and adjust to pH 4.5 with 1*N* NaOH. Dil. adjusted soln with enough pH 4.5 buffer, **957.23B(g)**, to obtain estd concn of 0.20 μg/mL. Designate as assay soln.

### C. Assay

Using oxytetracycline std response line and assay soln, and chlortetracycline plates, **967.39B(b)**, proceed as in **957.23E–F**, incubating at 30°.

### *Microbiological Method II*

(Applicable to ≤220 mg/lb)

### D. Plates

Use single inoculated agar layer. Before assay, det. by prepn of trial plates optimum concn (usually 0.03–0.10%) of stock suspension of *B. cereus*, **957.23D(c)**, to be added to agar medium D, **957.23A(d)**, to obtain zones of inhibition with as little as 0.05 μg oxytetracycline/mL. Zone of 18 mm ± 10%

should be obtained with 0.20 μg/mL. For actual assay add appropriate amt of inoculum to agar medium D previously melted and cooled to 48°. Mix thoroly, and add 9.0 mL to each plate.

***E. Assay Solution***

(Solv. losses may occur from evapn of volatile solvs in open containers. Carefully measure and record vols of solvs, and make appropriate mathematical corrections for any losses in vol.)

Accurately weigh 20 g ground finished feed into 250 mL extn flask, add 100 mL acid-MeOH, **957.23B(i)**, stopper, and shake mech. 5 min. Centrf. ca 5 min at 2000 rpm. Remove 20 mL supernate and adjust to pH 4.5 with 1*N* NaOH. Dil. adjusted soln with enough pH 4.5 buffer, **957.23B(g)**, to obtain estd concn of 0.20 μg/mL and filter thru Whatman No. 2V paper, or equiv. Designate as assay soln.

***F. Assay***

Using oxytetracycline std response line, assay soln, **968.50E**, and plates, proceed as in **957.23E–F**, incubating at 28–30°. Calc. result as oxytetracycline.HCl by multiplying oxytetracycline base by 1.08.

Ref.: JAOAC **51,** 548(1968).

CAS-79-57-2 (oxytetracycline)

## 967.41 Procaine Penicillin in Feeds

### Microbiological Method

**First Action 1967**
**Final Action 1968**

(Applicable to feeds contg ≥1.5 g/ton)

***A. Standard Solutions***

(**a**) *Penicillin stock soln.*—Accurately weigh, in atm. of ≤50% relative humidity, ca 30 mg USP Potassium or Sodium Penicillin G Ref. Std. Dissolve in enough pH 6 buffer, **957.23B(f)**, to give known concn of 100–1000 units/mL. Store in dark ≤2 days at 2–10°.

(**b**) *Std response line.*—Dil. appropriate aliquots of stock soln, (**a**), with enough pH 6 buffer, **957.23B(f)**, to obtain concns of 0.0125, 0.025, 0.05, 0.10, and 0.20 unit/mL. Ref. concn is 0.05 unit/mL.

***B. Plates***

(**a**) *Base layer.*—Add 10 mL melted agar medium A to sterile petri dishes, distribute evenly, and let harden on *perfectly level surface*.

(**b**) *Seed layer.*—Before assay, det. by prepn of trial plates optimum concn of organism suspension of *S. lutea* (usually 0.2–0.5% of suspension prepd as in **957.23D(e)**(*1*) or 2–5% as in **957.23D(e)**(*2*)) to be added to agar medium B to obtain zones of inhibition of adequate size (19 mm ± 10% with 0.05 unit/mL) and sharpness.

For actual assay, add appropriate amt of organism suspension to agar medium B previously melted and cooled to 48°. Mix thoroly and add 4.0 mL to each plate contg base layer.

***C. Assay Solution***

(Solv. losses may occur from evapn of volatile solvs in open containers. Carefully measure and record vols of solvs, and make appropriate mathematical corrections for any losses in vol.)

Obtain and prep. sample as in **965.16** and **950.02**. Vary amts of sample and extractant according to penicillin content of feed as follows:

| Penicillin Content | Sample Size, g | Vol. Extractant, mL |
|---|---|---|
| ≥100 g/lb | 1 | 100 |
| 1–100 g/lb | 3 | 100 |
| 0.1–1 g/lb | 10 | 100 |
| <0.1 g/lb (200 g/ton) | 50 | 200 |

Ext appropriate amt of sample with pH 6 buffer-acetone extractant, **957.23B(k)**, in suitable container, using either wrist-action or reciprocating mech. shaker 30 min or high-speed blender 2 min; let settle and decant supernate. Centrf. if more than slightly turbid. Dil. aliquot of supernate with enough pH 6 buffer, **957.23B(f)**, to obtain estd concn of 0.05 unit/mL.

***D. Assay***

Using penicillin std response line, assay soln, and plates, proceed as in **957.23E–F**, incubating at 26–32°. Calc. result in terms of units or wt (1 mg Na penicillin G = 1667 units; 1 mg K salt = 1595 units; 1 mg procaine salt = 1009 units).

***E. Identity***

To aliquot of supernate assay soln, **967.41C**, add enough *penicillinase soln* to inactivate penicillin by incubating mixt. 1 hr at 37°. Further dil. with enough pH 6 buffer, **957.23B(f)**, to give same diln factor as in **967.41C**. Assay as in **967.41D**. Absence of zone of inhibition indicates that activity in sample is due to penicillin. (*See* **962.14E**.)

Ref.: JAOAC **50,** 450(1967).

CAS-54-35-3 (penicillin G procaine)

## 973.81 Spectinomycin in Feeds

### Microbiological Method

**First Action 1973**

(Applicable in presence of lincomycin to feeds contg ≥18 g/ton)

***A. Standard Solutions***

(**a**) *Spectinomycin stock soln.*—1 mg spectinomycin base/mL. Accurately weigh amt of Spectinomycin.HCl.$5H_2O$ Ref. Std (available from Agricultural Division, The Upjohn Co.) and dissolve in enough $H_2O$ to give concn of exactly 1.0 mg spectinomycin base/mL. Store in dark ≤30 days at 2–10°.

(**b**) *Std response line.*—Dil. stock soln with Tris buffer, **957.23B(l)**, to obtain concn of 100 μg/mL. Dil. this soln with Tris buffer to obtain 2.8, 4.4, 7.0, 11.0, and 17.4 μg spectinomycin base/mL. Prep. daily. Ref. concn is 7.0 μg/mL.

***B. Plates***

Before assay, det. by prepn of trial plates optimum concn of culture of *E. coli*, **957.23D(h)** (usually ca 0.04%) to be added to agar medium F to obtain zones of inhibition of adequate size and sharpness. Zones of 13 ± 1 and 23 ± 1 mm should be obtained for 2.8 and 17.4 μg/mL, resp.

For actual assay, add appropriate amt of culture to agar medium F, previously melted and cooled to 48°. Mix thoroly and add 7.0 mL to each plate.

***C. Assay Solution***

Weigh 20 g feed into 250 mL centrf. tube and add 100 mL acid-MeOH (20 mL 1*N* HCl dild to 1 L with MeOH). Shake

15 min on wrist-action shaker, or equiv., centrf. 5 min at 2000 rpm, and decant into 500 mL r-b flask. Repeat extn twice, combining exts in r-b flask. Evap. ext under vac. until all MeOH and most of $H_2O$ have been removed. Do not exceed 60° and do not evap. to complete dryness. Add 30 mL $Pb(OAc)_2$ soln, **957.23B(q)**, to flask, shake vigorously 2 min, and transfer quant. to 100 mL beaker. Rinse flask with 10–15 mL Tris buffer and add rinse to beaker. Adjust to pH 8.0 with 3.5*N* NaOH. Transfer quant. to 100 mL graduate, rinsing beaker with buffer, and dil. to 100 mL with buffer. Mix thoroly, and let sample stand 30 min. Centrf. 30–50 mL 10 min at 2000 rpm. Decant supernate into test vials for storage (≤2 days) until assay. Designate soln obtained as assay soln.

### D. Assay

Using spectinomycin std response line, assay soln, and plates, proceed as in **957.23E–F**, incubating at 26°.

g Spectinomycin/ton
= (μg spectinomycin base/mL assay soln) × 4.54

If zone size is plotted against g/ton instead of μg base/mL working stds as follows, calcns are not necessary.

| μg Base/mL | g/ton |
|---|---|
| 2.8 | 12.7 |
| 4.4 | 20.0 |
| 7.0 | 31.8 |
| 11.0 | 50.0 |
| 17.4 | 79.0 |

Ref.: JAOAC **56,** 834(1973).

CAS-1695-77-8 (spectinomycin)

## 971.49 Streptomycin in Feeds

**Microbiological Method**
**First Action 1971**
**Final Action 1973**
***Method I***

(Applicable to feeds contg ≥30 g/ton)

### A. Standard Solutions

**(a)** *Streptomycin stock soln.*—Dry ca 40 mg USP Streptomycin Sulfate Ref. Std 3 hr at 60° in vac. oven at ≤5 mm (0.66 kPa). Det. accurate dry wt and dissolve in enough $H_2O$ to give concn of exactly 100 μg streptomycin base/mL. Store ≤30 days at 2–10°.

**(b)** *Std response line.*—Dil. aliquots of stock soln, **(a)**, with enough pH 8 buffer, **957.23B(b)**, to obtain concns of 0.64, 0.80, 1.0, 1.25, and 1.56 μg streptomycin base/mL. Ref. concn is 1.0 μg/mL.

### B. Plates

**(a)** *Base layer.*—Add 12 mL melted agar medium E to sterile petri dishes. Distribute agar evenly and let harden on *perfectly level surface.*

**(b)** *Seed layer.*—Before assay, det. by prepn of trial plates optimum concn (usually 0.05–0.2%) of spore suspension of *B. subtilis,* **957.23D(d)**, to be added to agar medium E. For actual assay, sharp zones of inhibition (14 mm ± 10%) should be obtained with 0.64 μg streptomycin base/mL. Add appropriate amt of spore suspension to agar medium E which has been melted and cooled to 48°. Mix thoroly and add 4.0 mL to each plate contg base layer.

### C. Assay Solution

Obtain and prep. sample as in **965.16** and **950.02**. Using 10.0 g sample and 200 mL 0.5*N* HCl, shake mech. 30 min or blend 2 min in high-speed blender. Centrf. ca 15 min at 2000 rpm. Transfer aliquot of supernate to beaker, add ca 25 mL pH 8 buffer, **957.23B(b)**, and adjust to pH 8 ± 0.1 with 5*N* and 1*N* NaOH. Transfer quant. to suitable vol. flask, dil. to vol. with pH 8 buffer, **957.23B(b)**, and mix. Dil. aliquot with enough pH 8 phosphate buffer to obtain estd concn of 1.0 μg streptomycin base/mL. Filter dild soln without suction thru Whatman No. 2V paper, or equiv. Designate as assay soln.

### D. Assay

Using streptomycin std response line, assay soln, and plates, proceed as in **957.23E–F**, incubating at 37°.

Refs.: JAOAC **54,** 116(1971); **55,** 714(1972).

CAS-57-92-1 (streptomycin)

## 972.57 Streptomycin in Feeds

**Microbiological Method**
**First Action 1972**
**Final Action 1973**
***Method II***

(Applicable to feeds contg 5–30 g/ton)

### A. Standard Solutions

*Std response line.*—Dil. aliquots of stock soln, **971.49A(a)**, with enough pH 8 buffer, **957.23B(b)**, to obtain concns of 0.19, 0.24, 0.30, 0.38, and 0.47 μg streptomycin base/mL. Ref. concn is 0.30 μg/mL.

### B. Plates

**(a)** *Base layer.*—Prep. as in **971.49B(a)**, except use 10 mL agar medium E.

**(b)** *Seed layer.*—Prep. as in **971.49B(b)**, except that sharp zones of inhibition (11 mm ± 10%) should be obtained with 0.19 μg/mL std.

### C. Assay Solution

Proceed as in **971.49C**, using 40 g sample and dilg to estd final concn of 0.3 μg streptomycin base/mL. Let soln stand 1 hr before filtering.

*Note:* Feeds may produce considerable gas during acid extn. When using mech. shaker, let each sample stand in acid extractant ca 1 hr with occasional swirling before placing on shaker. When using high-speed blender, use jar with lid cover rather than sealed jar, and hold lid down by hand at start of blending.

### D. Assay

Using streptomycin std response line, assay soln, and plates, proceed as in **957.23E–F**, incubating at 22–25°.

Refs.: JAOAC **54,** 116(1971); **55,** 714(1972).

CAS-57-92-1 (streptomycin)

## 962.26 Tylosin in Feeds

**Microbiological Method**
**First Action 1962**
**Final Action 1965**

(Applicable to feeds containing ≥11 g/ton)

### A. Standard Solutions

**(a)** *Tylosin stock soln.*—Dry Tylosin base Ref. Std (available from Elanco Products Co.) 4 hr at 70° and store in desiccator over fresh $P_2O_5$. Accurately weigh suitable amt (10–15 mg) of dried std and dissolve in 5 mL MeOH. Adjust vol. with pH 7 phosphate buffer, **957.23B(c)**, to give concn of 1000 μg/mL. Store in refrigerator ≤2 weeks.

(**b**) *Std response line*.—Dil. appropriate aliquots of stock soln with mixt. of MeOH and pH 8 phosphate buffer, **957.23B(b)**, (4 + 6), to obtain concns of 0.125, 0.25, 0.50, 1.0, and 2.0 μg/mL. Ref. concn is 0.50 μg/mL.

### B. Plates

(**a**) *Base layer*.—Add 10 mL melted agar medium H to petri dishes, distribute evenly, and let harden on *perfectly level surface*.

(**b**) *Seed layer*.—Before assay, det. by prepn of trial plates optimum concn of organism suspension of *S. lutea* (usually 0.05–0.2% of suspension prepd as in **957.23D(e)**(*1*) or 0.5–2% as in **957.23D(e)**(*2*)) to be added to agar medium H to obtain zones of inhibition of adequate size (15 mm ± 10% with 0.50 μg/mL) and sharpness. For actual assay, add appropriate amt of organism suspension to agar medium H melted and cooled to 48°. Mix thoroly and add 5.0 mL to each plate contg base layer. Refrigerate plates until just before application of assay solns.

### C. Assay Solution

Obtain and prep. sample as in **965.16** and **950.02**. Accurately weigh 10 g feed premix or 20 g final feed into 250 mL homogenizer cup or blender jar. Add 90 mL hot (70–80°) pH 8 phosphate buffer, **957.23B(b)**, and place on steam bath 10 min. Blend 5 min, add 60 mL MeOH, and blend addnl 5 min. Centrf. or filter thru Whatman No. 1 paper and dil., if necessary, with mixt. of MeOH and pH 8 phosphate buffer, **957.23B(b)**, (4 + 6), to concn of 0.5 μg tylosin/mL.

### D. Assay

Prep. 10 plates for std response line and 5 for each sample. Place 5 cylinders on each std response line plate at 72° intervals on 2.8 cm radius. Place 4 cylinders on each sample plate at 90° intervals. Fill cylinders on each of 10 std plates with each concn of std response line. On each sample plate fill 2 diagonally opposite cylinders with ref. concn and remaining 2 cylinders with assay soln. Incubate plates overnight at 30°. Measure zones of inhibition to nearest 0.1 mm. Record av. zone diam. for each concn of std on std plates and proceed as in **957.23E**.

Average the 10 readings of ref. concn on sample plates and the 10 readings of assay soln. Proceed as in **957.23F**.

Ref.: JAOAC **45**, 317(1962).

CAS-1401-69-0 (tylosin)

## CHEMICAL METHODS FOR ANTIBIOTICS

## 982.44 Bacitracin in Premix Feeds

**Liquid Chromatographic Method**

**First Action 1982**

### A. Principle

Bacitracin is extd from feed into acidified org. solv. system. Ext is centrfgd, and supernate is analyzed by ion-suppressed reverse phase LC with photometric detection at 254 nm.

### B. Reagents and Apparatus

(**a**) *Liquid chromatograph*.—Hewlett-Packard Model 1084-A, equipped with UV photometric detector. Operating condition: flow rate 2.0 mL/min; detector wavelength 254 nm; 20 μL loop injection valve (Valco Instruments Co., Inc., PO Box 55603, Houston, TX 77255); ambient temperature.

(**b**) *Chromatographic column*.—15 cm × 4.6 mm id, containing 5 μm Supelcosil LC-8 reverse phase packing. Use column for bacitracin analysis only.

(**c**) *Phosphate–EDTA buffer*.—pH 4.5. Dissolve 13.6 g $KH_2PO_4$ and 2.5 g EDTA in 1 L $H_2O$.

(**d**) *Phosphate buffer*.—pH 6.0. Dissolve 1.5 g $K_2HPO_4$ and 8.5 g $KH_2PO_4$ in 1 L $H_2O$.

(**e**) *Solvent systems*.—Measure vol. indicated below with graduate (except where noted otherwise) into 100 mL vol. flask and dil. to vol. with $H_2O$:

| Solvent | Vol.% A Solv. | B. Solv. | Extg Solv. |
|---|---|---|---|
| $CH_3CN$ | 0 | 40 | 28 |
| MeOH | 0 | 12 | 28 |
| Phosphate-EDTA buffer | 20 | 20 | 0 |
| Phosphate buffer[a] | 0 | 0 | 3 |
| Concd phosphoric acid[a] | 0 | 0 | 1.2 |

[a] Use vol. pipet

(**f**) *Mobile phase*.—Mix 59% (v/v) B solv. with 41% (v/v) A solv. Mix, and adjust pH to 6.8 with NaOH. Slight adjustment to % vol. of B solv. may be required to obtain desired sepn.

### C. Preparation of Standard

(**a**) *Drying of std*.—*Caution:* Bacitracin is very hydroscopic. Dry std day before use and store in desiccator overnight. Accurately weigh 130–140 mg bacitracin ref. std (IMC, Pitman-Moore Inc., 1331 First St, PO Box 207, Terre Haute, IN 47808; 56.3 units/mg) into tared (= A) 50 mL vol. flask. Dry std 3 h at 60° under vac. at <5 mm pressure. Remove from oven and place in desiccator to cool. Reweigh (= B). Amt bacitracin std = B − A.

(**b**) *Preparation of std soln*.—*Note:* Store stds under refrigeration if not analyzed within 3 h of prepn. Preferably, prep. std, store in refrigerator >30 min before analysis, and remove from refrigerator just before analysis. *Std soln 1:* Dissolve bacitracin std in 50 mL vol. flask with ca 20 mL extg solv. and dil. to vol. Prep. following dilns from this soln. *Std soln 2:* Pipet 20 mL std soln 1 into 25 mL vol. flask; dil. to vol. with extg solv. *Std soln 3:* Pipet 15 mL std soln 1 into 25 mL vol. flask; dil. to vol. with extg solv.

### D. Extraction

Accurately weigh amt of feed contg ca 6000 units bacitracin activity into 125 mL erlenmeyer. Add 50 mL extg solv. with vol. pipet and ext with wrist-action shaking >5 min. Centrifuge 10 mL portion of ext 2–3 min at 2000–3000 rpm. Use clear supernate for assay. *Note:* Store extd sample soln under refrigeration if not analyzed within 3 h. Preferably, prep. sample solns, store in refrigerator >30 min before analysis, and remove from refrigerator just before analysis.

### E. Determination

Inject clear supernate from centrfgd feed and std solns into chromatograph, starting with std soln, then 2 sample solns, and then another std soln, until all samples and stds have been injected. Measure and total peak hts of the 3 active component peaks (Fig. **982.44**) for sample (*PH*) and std (*PH'*) solns.

Calc. response line for stds, using least squares linear fitting of following equation:

$$PH' = m(P') + b$$

where $PH'$ = peak hts of std solns 1, 2, and 3; $P'$ = potency of std soln in units/50 mL for std solns 1, 2, and 3; $m$, $b$ = least squares detd slope and intercept.

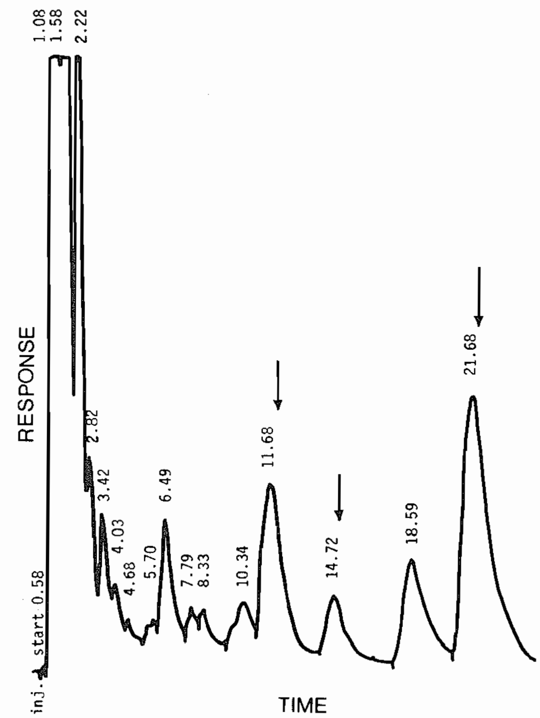


**FIG. 982.44—Liquid chromatogram of bacitracin active components (indicated by arrows)**

Det. bacitracin content of feed from:

$$\text{g bacitracin/lb} = \frac{0.01080\,(PH - b)}{m \times \text{sample wt}}$$

where 0.01080 = 453.6 (g/lb)/42 000 (units/g bacitracin).

Ref.: JAOAC **65,** 1178(1982).

CAS-1405-87-4 (bacitracin)

## 968.49 Chlortetracycline in Feeds

### Microscopic Test
### First Action 1968
### Final Action 1969

#### A. Apparatus

*Microscopes.*— *See* **964.07A(a)** and **(b)**.

#### B. Reagent

*Modified Sakaguchi reagent.*—Dissolve 5 g $H_3BO_3$ in 150 mL $H_2O$ and add 350 mL $H_2SO_4$. Store in g-s bottle in refrigerator. Use cold.

#### C. Determination

Grind sample to pass sieve with circular openings 1 mm (1/25 in.) diam. and mix thoroly. If sample cannot be ground, reduce to as fine condition as possible. Do not grind molasses feeds. Pipet ca 10 mL Sakaguchi reagent into 9 cm petri dish. Place No. 60 sieve over petri dish. With top of spatula, sprinkle ca 0.5 g sample on sieve, and gently tap it to obtain good distribution of particles over liq. surface. Place under stereoscopic microscope and examine with transmitted light at ca 15×. If substage illumination is not available, place petri dish on white surface and illuminate with blue light.

As particles of antibiotic slowly dissolve, diffusing chlortetracycline turns intense purple. Color fades in 5–10 min.

Refs.: JAOAC **51,** 750(1968); **59,** 357(1976).

CAS-57-62-5 (chlortetracycline)

## 966.30 Griseofulvin in Feeds

### Spectrophotometric Method
### First Action 1966
### Final Action 1975

(Applicable to concns ≥10 mg/oz.)

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

#### A. Reagents

(**a**) *Activated alumina.*—Alcoa grade F-20.

(**b**) *Solvent mixture.*—Pet ether-$CHCl_3$ (65+35).

(**c**) *Griseofulvin std soln.*—10 μg/mL. Accurately weigh ca 25 mg USP Griseofulvin Ref. Std into 250 mL vol. flask; dissolve and dil. to vol. with solv. mixt. Dil. 10 mL of this soln to 100 mL in vol. flask.

#### B. Apparatus

(**a**) *Chromatographic tube.*—20 × 400 mm, with fritted disk and stopcock.

(**b**) *High-speed blender.*—Waring-type, or equiv., 1 L capacity.

(**c**) *Spectrophotometer.*—Capable of accurate readings at 290 and 320 nm.

#### C. Preparation of Sample

Grind 250 g feed pellets or mash in high-speed blender 5 min. Accurately weigh ca 14 g finely powd feed into fat-free thimble and ext in Soxhlet app. 2 hr with 100 mL $CHCl_3$. Evap. ext to 10 mL on steam bath, dil. with 100 mL pet ether, and chromatograph.

#### D. Preparation of Chromatographic Column

Place 50 mL solv. mixt., (**b**), in tube and add 45 mL activated alumina portionwise, with tapping to ensure uniform packing. Place small glass wool pad on top of alumina and drain solv. to just below top of pad.

#### E. Determination

Add pet ether-$CHCl_3$ sample ext to column. As last of ext passes thru glass wool pad, rinse sample flask with solv. mixt., add to column, and begin elution with solv. mixt. Adjust liq. head to give flow rate of 15–20 mL/min. Start collecting 25 mL fractions when green eluate first appears (discard yellow and almost colorless eluates which precede). When $A$ of fractions at 290 nm exceeds $A$ at 320 nm, stop fractionating, and collect next 700 mL eluate. Dil. eluate to vol. in 1 L vol. flask with solv. mixt. Det. $A$ of this soln and of griseofulvin std soln at 290 and 320 nm against solv. mixt. blank.

$$\text{mg Griseofulvin/oz} = (A_{290} - A_{320})(W')$$
$$(10)\,(28.35)/(A'_{290} - A'_{320})\,(25)\,(\text{g sample})$$

where $A$ refers to sample eluate, $A'$ to std soln, and $W'$ = mg ref. std griseofulvin used to prep. std soln.

Ref.: JAOAC **49,** 494(1966).

CAS-126-07-8 (griseofulvin)

## 975.61 Lasalocid in Feeds

### Spectrofluorometric Method

**First Action 1975**
**Final Action 1977**

### *A. Principle*

Compd is extd from pH 4.7 soln with EtOAc, fluorescent impurities are removed by acid and alkali treatments, and compd is detd fluorometrically, correcting for nonspecific fluorescence by complexing with $H_3BO_3$. Monensin and ethoxyquin do not interfere.

### *B. Apparatus*

*Spectrofluorometer.*—With 10 mm fused quartz cells. Excitation and emission wavelengths, ca 310 and 419 nm, resp. Accurately det. peak excitation and emission wavelengths using std soln I, following manufacturer's directions. Do not change wavelength settings between readings.

For routine setting and checking of instrument, use std soln I.

Adjust settings to compensate for decreased intensity with age from dulled reflecting surfaces and lamp.

### *C. Reagents*

(**a**) *Acetate buffer soln.*—pH 4.7. Dissolve 5.0 g NaOAc in ca 50 mL $H_2O$, adjust to pH 4.7 with HOAc, and dil. to 100 mL with $H_2O$.

(**b**) *Ethyl acetate.*—Must have ca 0 fluorescence. If necessary, purify as follows: Elute 1 gal. EtOAc thru 9–10 cm (od) column packed with ca 100 cm silica gel (activated desiccant, 100–200 mesh, Grade H, W. R. Grace & Co., Davison Chemical Div., 10 E. Baltimore St, PO Box 2117, Baltimore, MD) topped with 5 cm layer of $NaHCO_3$. Redistil eluate from all-glass app. with 60 cm jacketed distg column, discarding first and last 10%. (*Caution:* See safety notes on distillation and ethyl acetate.) To redistd EtOAc add 40% aq. NaOH and mix briefly. Follow with anhyd. $Na_2SO_4$ and shake again. (Ratio of EtOAc:40% NaOH:$Na_2SO_4$ is 1000:50:200 or multiple thereof.)

(**c**) *Methanolic boric acid soln.*—Dissolve 20.0 g $H_3BO_3$ in MeOH and dil. to 500 mL with MeOH. Prep. fresh daily.

(**d**) *Lasalocid std solns.*—(*1*) *Std soln I.*—Dissolve 30.0 mg Lasalocid Ref. Std (available from Hoffmann-La Roche Inc.) in EtOAc and dil. to 100 mL with EtOAc. Pipet 4 mL into 100 mL vol. flask, dil. to vol. with EtOAc, and mix. Pipet 25 mL final diln into 50 mL g-s centrf. tube contg 2.4 mL pH 4.7 buffer. Shake mech. 25–30 min and centrf. 10 min at 2000 rpm. Pipet 2 mL clear EtOAc ext into 100 mL vol. flask, dil. to vol. with EtOAc, and mix. (*2*) *Std soln II.*—Pipet addnl 2 mL clear EtOAc ext into another 100 mL vol. flask contg 10 mL methanolic $H_3BO_3$ soln, dil. to vol. with EtOAc, and mix.

### *D. Preparation of Sample*

(**a**) *Feeds.*—Grind 200 g sample (mash, pellets, or crumbles) to pass No. 30 sieve, and mix thoroly. Accurately weigh ca 4 g sample into 50 mL g-s centrf. tube contg 2.4 mL pH 4.7 buffer. Turn and shake tube by hand to wet uniformly. Immerse tube 4–5 min in 70° $H_2O$ bath. Cool to room temp. and add 25 mL EtOAc by pipet. Stopper tube and shake briefly but vigorously by hand to disperse sample. If necessary, break up lumps with narrow-tip spatula or glass rod. Stopper tube and shake mech. 25–30 min. Centrf. 10 min at 2000 rpm. Pipet 15 mL clear EtOAc ext into 50 mL centrf. tube. Add 2 mL 1.5*N* HCl and shake 10 min. Centrf. 10 min at 2000 rpm. Pipet 10 mL clear EtOAc ext into another 50 mL g-s centrf. tube and add 0.5 mL 40% NaOH soln. Shake briefly by hand, add 2 g anhyd. $Na_2SO_4$, and shake again. Centrf. 10 min at 2000 rpm. If EtOAc soln is not clear or fine particles are present at surface, swirl tube gently by hand and recentrf. Pipet 2 mL EtOAc layer into 100 mL vol. flask without disturbing aq. alk. soln, dil. to vol. with EtOAc, and mix. Designate as *Sample soln I*. Pipet another 2 mL aliquot EtOAc layer into second 100 mL vol. flask contg 10 mL methanolic $H_3BO_3$ soln, dil. to vol. with EtOAc, and mix. Designate as *Sample soln II*.

Pipet 25 mL EtOAc into 50 mL g-s centrf. tube contg 2.4 mL pH 4.7 buffer and proceed as above, beginning "Stopper tube and shake briefly . . ." Designate final solns as *Reagent blank soln I* and *Reagent blank soln II*.

(**b**) *Premixes.*—Accurately weigh 2.00 g 15% premix and transfer into 500 mL vol. flask. Add exactly 250 mL EtOAc and shake 25 min on mech. shaker. Centrf. aliquot, and dil. with EtOAc as in (**a**) to obtain *Sample soln I* and *Sample soln II* (complex) contg ca 0.24 μg lasalocid/mL EtOAc. Proceed with fluorescence measurements as in **975.61E**. (*Note:* Omit treatment with pH 4.7 buffer for both premix and std.)

### *E. Determination*

Set excitation and emission wavelengths of app. at max. Adjust instrument with std. soln I, (**d**)(*1*), in cell to microammeter reading of 0.400. Check this ref. point before and after each reading, using same cell for all ref. readings. Because of decomposition in UV, discard and replace std soln I after every second reading. Measure fluorescence at 419 nm in order: *Sample soln I* ($U_1$), *Std soln I* ($S_1$), *Reagent blank soln I* ($R_1$), *Sample soln II* ($U_2$), *Std soln II* ($S_2$), and *Reagent blank soln II* ($R_2$). If reading of std soln I drifts, adjust gain to initial setting. If drift is beyond 0.393–0.407, recheck readings of all solns.

Altho fluorescence response of std is linear from 0.12 to 0.48 μg/mL, concn of lasalocid in sample soln should be ± 25% of that of std soln.

### *F. Calculations*

(**a**)
$$\% \text{ Lasalocid Na in feed} = [(U_1 - R_1) - (U_2 - R_2)] \times D \times S/[(S_1 - R_1) - (S_2 - R_2)] \times W \times 96 \times 100$$

where $U$, $S_1$, $S_2$, and $R$ are defined in **975.61E**; $D$ = diln factor ($25 \times 100/2 = 1250$); $S$ = concn of lasalocid in *Std soln I* (= 0.24 μg/mL); $W$ = g sample; and 96 = % recovery. When $S$ = 0.24 μg/mL, $W$ = 4.00 g, and $R_1$ and $R_2 = 0$,

$$\% \text{ Lasalocid Na in feed} = (U_1 - U_2) \times 0.00781/(S_1 - S_2)$$

(**b**)
$$\% \text{ Lasalocid Na in premix} = [(U_1 - R_1) - (U_2 - R_2)] \times D \times S/[(S_1 - R_1) - (S_2 - R_2)] \times W \times 10{,}000$$

where $U$, $S_1$, $S_2$, and $R$ are defined in **975.61E**; $D$ = diln factor ($250 \times 50 \times 50 \times 100/5 \times 5 \times 2 = 1{,}250{,}000$); $S$ = concn of lasalocid in *Std soln I* (= 0.24 μg/mL); $W$ = g sample. When $S$ = 0.24 μg/mL, $W$ = 2.00 g and $R_1$ and $R_2 = 0$,

$$\% \text{ Lasalocid Na in 15\% premix} = (U_1 - U_2) \times 15/(S_1 - S_2)$$

Ref.: JAOAC **58,** 507(1975).

CAS-25999-31-9 (lasalocid)

**Common and Chemical Names of Drugs in this Chapter**

| Common Name | Chemical Name |
|---|---|
| Aklomide | 2-Chloro-4-nitrobenzamide |
| Amprolium | 1-[(4-Amino-2-propyl-5-pyrimidinyl)methyl]-2-methylpyridinium chloride |
| Arprinocid | 9-[2-Chloro-6-fluorophenyl)methyl]-9*H*-purin-6-amine |
| Arsanilic acid | (4-Aminophenyl)arsonic acid |
| Bithionol | 2,2′-Thiobis(4,6-dichlorophenol) |
| Buquinolate | 4-Hydroxy-6,7-bis(2-methylpropoxy)-3-quinolinecarboxylic acid ethyl ester |
| Carbadox | 2-(2-Quinoxalinylmethylene)hydrazinecarboxylic acid methyl ester $N^1,N^4$-dioxide |
| Chlortetracycline hydrochloride | (4α,4aα,5aα,6β,12aα)-7-Chloro-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide monohydrochloride |
| Decoquinate | 6-(Decyloxy)-7-ethoxy-4-hydroxy-3-quinolinecarboxylic acid ethyl ester |
| Dibutyltin dilaurate | Dibutylbis[(1-oxododecyl)oxy]stannane |
| Diethylstilbestrol | 4,4′-(1,2-Diethyl-1,2-ethenediyl)bisphenol |
| Dimetridazole | 1,2-Dimethyl-5-nitro-1*H*-imidazole |
| Ethopabate | 4-(Acetamido)-2-ethoxybenzoic acid methyl ester |
| Furazolidone | 3-[[(5-Nitro-2-furanyl)methylene]amino]-2-oxazolidinone |
| Glycarbylamide | 1*H*-Imidazole-4,5-dicarboxamide |
| Griseofulvin | 7-Chloro-2′,4,6-trimethoxy-6′-methyl-spiro[benzofuran-2(3*H*),1′-[2]cyclohexene]-3,4′-dione |
| Hygromycin B | *O*-6-Amino-6-deoxy-L-glycero-D-galacto-heptopyranosylidene-(1→2-3)-*O*-β-D-talopyranosyl-(1→5)-2-deoxy-$N^3$-methyl-D-streptamine |
| Ipronidazole | 1-Methyl-2-(1-methylethyl)-5-nitro-1*H*-imdazole |
| Lasalocid | 6-[7(*R*)-[5(*S*)-Ethyl-5-(5(*R*)-ethyltetrahydro-5-hydroxy-6(*S*)-methyl-2*H*-pyran-2(*R*)-yl)tetrahydro-3(*S*)-methyl-2(*S*)-furanyl]-4-(*S*)-hydroxy-3-(*R*),5(*S*)-dimethyl-6-oxononyl]-2-hydroxy-3-methylbenzoic acid |
| Lincomycin | Methyl-6,8-dideoxy-6[[1-methyl-4-propyl-2-pyrrolidinyl)carbonyl]amino]-1-thio-D-*erythro*-α-D-*galacto*-octopyranoside |
| Melengestrol (acetate) | 17-Hydroxy-6-methyl-16-methylenepregna-4,6-diene-3,20-dione |
| Nequinate | 6-Butyl-1,4-dihydro-4-oxo-7-(phenylmethoxy)-3-quinolinecarboxylic acid |
| Nicarbazin | *N,N*′-Bis(4-nitrophenyl)urea compd with 4,6-dimethyl-2(1*H*)-pyrimidinone (1:1) |
| Nicotine | 3-(1-Methyl-2-pyrrolidinyl)pyridine |
| Nifursol | [(5-Nitro-2-furanyl)methylene]hydrazide-2-hydroxy-3,5-dinitrobenzoic acid |
| Nihydrazone | 5-Nitrofurfurylidenehydrazide acetic acid |
| Nitarsone | (4-Nitrophenyl)-arsonic acid |
| Nithiazide | *N*-Ethyl-*N*′-(5-nitro-2-thiazolyl)urea |
| Nitrodan | 3-Methyl-5-[(4-nitrophenyl)azo]-2-thioxo-4-thiazolidinone |
| Nitrofurazone | 2-[(5-Nitro-2-furanyl)methylene]hydrazinecarboxamide |
| Nitromide | 3,5-Dinitrobenzamide |
| Nitrophenide | Bis(*m*-nitrophenyl)disulfide |
| Oxytetracycline | (4α,4aα,5α,5aα,6β,12aα)-4-(Dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide dihydrate |
| Penicillin G procaine | (2α,5a,6β)-3,3-Dimethyl-7-oxo-6-[(phenylacetyl)amino]-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid |
| Phenothiazine | Thiodiphenylamine |
| Pyrantel tartrate | E-1,4,5,6-Tetrahydro-1-methyl-2-[2-(2-thienyl)vin]pyrimidine tartrate (1:1) |
| Racephenicol | 2,2-Dichloro-*N*-[2-hydroxy-1-(hydroxymethyl)-2-[4-(methylsulfonyl)phenyl]ethyl]-acetamide |
| Reserpine | (3β,16β,17α,18β,20α)-11-17-Dimethoxy-18-[(3,4,5-trimethoxybenzoyl)oxy]yohimban-16-carboxylic acid methyl ester |
| Ronnel | *O,O*-Dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioic acid ester |
| Roxarsone | (4-Hydroxy-3-nitrophenyl)-arsonic acid |
| Spectinomycin | Decahydro-4a,7,9-trihydroxy-2-methyl-6,8-bis(methylamino)-4*H*-pyrano[2,3-*b*][1,4]benzodioxin-4-one |
| Streptomycin | *O*-2-Deoxy-2-(methylamino)-α-L-glucopyranosyl-(1→2)-*O*-5-deoxy-3-*C*-formyl-α-L-lyxofuranosyl-(1→4)-*N,N*′-bis(aminoiminomethyl)-D-streptamine |
| Sulfadimethoxine | 4-Amino-*N*-(2,6-dimethoxy-4-pyrimidinyl)-benzenesulfonamide |
| Sulfaguanidine | 4-Amino-*N*-(diaminomethylene)-benzenesulfonamide |
| Sulfamerazine | 4-Amino-*N*-(4-methyl-2-pyrimidinyl)-benzenesulfonamide |
| Sulfamethazine | 4-Amino-*N*-(4,6-dimethyl-2-pyrimidinyl)-benzenesulfonamide |
| Sulfanitran | *N*-[4-[[(4-Nitrophenyl)amino]sulfonyl]phenyl]-acetamide |
| Sulfaquinoxaline | 4-Amino-*N*-2-quinoxalinyl-benzenesulfonamide |
| Sulfathiazole | 4-Amino-*N*-2-thiazolyl-benzenesulfonamide |
| Thiabendazole | 2-(4-Thiazolyl)-1*H*-benzimidazole |
| Zoalene | 2-Methyl-3,5-dinitrobenzamide |

Sources: *USAN and the USP Dictionary of Drug Names* (1989). U.S. Pharmacopeial Convention, Rockville, MD; *The Merck Index* (1989) 10th ed., Merck & Co., Inc., Rahway, NJ.

# 6. Disinfectants

**Aram Beloian, *Associate Chapter Editor***
*Environmental Protection Agency*

## PHENOL COEFFICIENT METHODS

### 955.11 Testing Disinfectants against *Salmonella typhi*

**Phenol Coefficient Method**

**Final Action 1964**

(Applicable to testing disinfectants miscible with $H_2O$ that do not exert bacteriostatic effects that cannot be neutzd by one of subculture media specified, or overcome by suitable subtransfer procedures. The 95% confidence limits are ±12%.)

#### *A. Culture Media*

(**a**) *Nutrient broth.*—Boil 5 g beef ext (Difco), 5 g NaCl, and 10 g peptone (Anatone, peptic hydrolysate of pork tissues, manufactured by American Laboratories, Inc., 4410 S 102nd St, Omaha, NE 68127) in 1 L $H_2O$ 20 min, and dil. to vol. with $H_2O$; adjust to pH 6.8. (If colorimetric method is used, adjust broth to give dark green with bromothymol blue.) Filter thru paper, place 10 mL portions in 20 × 150 mm test tubes, and autoclave 20 min at 121°. Use this broth for daily transfers of test cultures.

(**b**) *Synthetic broth.*—*Soln A:* Dissolve 0.05 g L-cystine, 0.37 g DL-methionine, 0.4 g L-arginine.HCl, 0.3 g DL-histidine.HCl, 0.85 g L-lysine.HCl, 0.21 g L-tyrosine, 0.5 g DL-threonine, 1.0 g DL-valine, 0.8 g L-leucine, 0.44 g DL-isoleucine, 0.06 g glycine, 0.61 g DL-serine, 0.43 g DL-alanine, 1.3 g L-glutamic acid.HCl, 0.45 g L-aspartic acid, 0.26 g DL-phenylalanine, 0.05 g DL-tryptophan, and 0.05 g L-proline in 500 mL $H_2O$ contg 18 mL 1*N* NaOH.

*Soln B:* Dissolve 3.0 g NaCl, 0.2 g KCl, 0.1 g $MgSO_4.7H_2O$, 1.5 g $KH_2PO_4$, 4.0 g $Na_2HPO_4$, 0.01 g thiamine.HCl, and 0.01 g niacinamide in 500 mL $H_2O$.

Mix *Solns A* and *B*, dispense in 10 mL portions in 20 × 150 mm tubes, and autoclave 20 min at 121°. Before using for daily transfers of test cultures, aseptically add 0.1 mL sterile 10% glucose soln per tube. Grow cultures with tube slanted 8° from horizontal.

(**c**) *Nutrient agar.*—Dissolve 1.5% Bacto agar (Difco) in nutrient broth and adjust to pH 7.2–7.4 (blue-green with bromothymol blue) or in synthetic broth, tube, autoclave, and slant.

(**d**) *Subculture media.*—Use (*1*), (*2*), or (*3*), whichever gives lowest result. (Com. dehydrated brands made to conform with preceding specifications may be used.) With oxidizing products and products formulated with toxic compds contg certain heavy metals like Hg, (*2*) will usually give lowest result. With products contg cationic surface active materials, (*3*) will usually give lowest result. *See also* **955.14C**, par. 5

(*1*) *Nutrient broth* described in (**a**).

(*2*) *Fluid thioglycolate medium USP XX:* Mix 0.5 g L-cystine, 0.75 g agar, 2.5 g NaCl, 5.5 g glucose.$H_2O$, 5.0 g $H_2O$-sol. yeast ext, and 15.0 g pancreatic digest of casein with 1 L $H_2O$. Heat on $H_2O$ bath to dissolve, add 0.5 g Na thioglycolate or 0.3 g thioglycolic acid, and adjust with 1*N* NaOH to pH 7.1 ± 0.2. If filtration is necessary, reheat without boiling and filter hot thru moistened filter paper. Add 1.0 mL freshly prepd 0.1% Na resazurin soln, transfer 10 mL portions to 20 × 150 mm tubes, and autoclave 20 min at 121°. Cool at once to 25° and store at 20–30°, protected from light.

(*3*) *"Letheen broth":* Dissolve 0.7 g lecithin (Asolectin, Associated Concentrates, 32–34 61st St, Woodside, NY 11377) and 5.0 g polysorbate 80 (Tween 80, or equiv.) in 400 mL hot $H_2O$ and boil until clear. Add 600 mL soln of 5.0 g beef ext (Difco), 10.0 g peptone (Anatone, (**a**)), and 5 g NaCl in $H_2O$, and boil 10 min. Adjust with 1*N* NaOH and/or 1*N* HCl to pH 7.0 ± 0.2 and filter thru coarse paper; transfer 10 mL portions to 20 × 150 mm tubes, and autoclave 20 min at 121°.

(*4*) *Cystine trypticase agar* (*BBL* Microbiology Systems): Suspend 29.5 g in 1 L $H_2O$. Heat gently with frequent agitation and boil ca 1 min or until soln is complete. Transfer 10 mL portions to 20 × 150 mm tubes, and autoclave 15 min at 12 lb pressure. Cool in upright position and store ≤25 days at 20–30°. Use for monthly transfer of stab stock cultures of *Ps. aeruginosa* PRD 10 (ATCC 15442).

(*5*) *Other subculture media:* Use (**d**)(*2*) with 0.7 g lecithin (Asolectin, Associated Concentrates, Inc.) and 5.0 g polysorbate 80 (Tween 80, or equiv.) added; or suspend 29.8 g prepd fluid thioglycolate medium (Difco), 0.7 g lecithin, and 5.0 g polysorbate 80 in 1 L $H_2O$, and boil until soln is clear. Cool, dispense in 10 mL portions in 20 × 150 mm tubes, and autoclave 20 min at 121°. Store at 20–30°. Protect from light.

#### *B. Apparatus and Reagents*

(**a**) *Glassware.*—1, 5, and 10 mL vol. pipets; 1, 5, and 10 mL Mohr pipets graduated to 0.1 mL or less; 100 mL g-s cylinders graduated in 1 mL divisions; Pyrex lipped test tubes, 25 × 150 mm (medication tubes) reusable or disposable borosilicate; bacteriological culture tubes, 20 × 150 mm (test culture and subculture tubes). Plug medication tubes with cotton wrapped in 1 layer of cheesecloth (tubes capped with Morton closures are an acceptable alternative). Sterilize all glassware 2 hr in hot air oven at 180°. Loosely plug pipets with cotton at mouth and place in closed metal containers before sterilizing.

(**b**) *Water bath.*—Const temp. relatively deep $H_2O$ bath capable of maintaining 20 ± 0.2°, with cover having ≥10 well-spaced holes which admit medication tubes but not their lips.

(**c**) *Racks.*—Any convenient style. Blocks of wood (size depending on space in incubator) with deep holes are satisfactory. Have holes well spaced to ensure quick manipulation of tubes. It is convenient to have them large enough to admit medication tubes while dilns are being made.

(**d**) *Transfer loop.*—Make 4 mm id single loop at end of 50–75 mm (2–3″) Pt or Pt alloy wire No. 23 B&S gage or 4 mm loop fused on 75 mm (3″) shaft (available from Matthey-Bishop, Inc., 1401 King Rd, West Chester, PA 19380). Fit other end in suitable holder (glass or Al rod). Bend loop at 30° angle with stem, Fig. **955.11**.

(**e**) *Test organism.*—Hopkins strain 26 of *Salmonella typhi* (Schroeter) Warren and Scott, ATCC No. 6539 (formerly called *Bac. typhosus* and *Eberthella typhosa*). Maintain stock culture on nutrient agar slants by monthly transfers. Incubate new stock transfer 2 days at 37°; then store at 2–5°. From stock culture inoculate tube of nutrient broth and make at least 4 consecutive

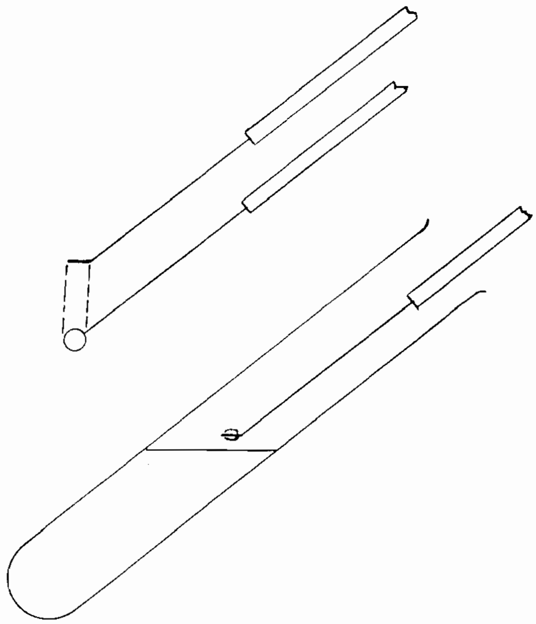

**FIG. 955.11—Transfer loop and manner of using in phenol coefficient technic**

daily transfers (≤30) in nutrient broth, incubating at 37°, before using culture for testing. (If only 1 daily transfer has been missed, it is not necessary to repeat the 4 consecutive transfers.) Use 22–26 hr culture of organism grown in nutrient broth at 37° in test. Shake, and let settle 15 min before using.

With *Ps. aeruginosa* PRD 10, proceed as in **964.02**.

(**f**) *Phenol stock soln.*—5% (w/v). Weigh 50 g USP phenol, which congeals at ≥40°, in beaker. Dissolve in $H_2O$, rinse soln into 1 L vol. flask, and dil. to vol. Stdze with 0.1*N* KBr-$KBrO_3$ soln, (**g**), as follows: Transfer 25 mL stock soln to 500 mL vol. flask and dil. to vol. with $H_2O$. Transfer 15 mL aliquot of dild soln to 500 mL I flask and add 30 mL std KBr-$KBrO_3$ soln. Add 5 mL HCl and immediately insert stopper. Shake frequently during 30 min and let stand 15 min. Remove stopper just enough to quickly add 5 mL 20% KI soln, taking care that no Br vapors escape, and immediately stopper flask. Shake thoroly, remove stopper, and rinse it and neck of flask with little $H_2O$ so that washings flow into flask. Titr. with 0.1*N* $Na_2S_2O_3$, using starch indicator: Mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring constantly, and immediately discontinue heating. Add ca 1 mL Hg, shake, and let stand over the Hg. 1 mL 0.1*N* KBr-$KBrO_3$ = 0.001569 g phenol.

$$\% \text{ phenol in stock soln} = (30 - \text{mL } 0.1N\ Na_2S_2O_3 \text{ soln from titrn}) \times 0.001569 \times 1333 \times 100/1000$$

where 30 = mL 0.1*N* KBr-$KBrO_3$ soln added, 0.001569 = g phenol equiv. to 1 mL 0.1*N* KBr-$KBrO_3$ soln, 1333 = diln factor, and 1000 = original vol. phenol stock soln.

If necessary, adjust stock soln to 5.00 ± 0.05% phenol by adding $H_2O$ or phenol. Keep in well stoppered amber bottles in cool place, protected from light.

(**g**) *Potassium bromide-bromate soln.*—0.1*N*. Prep. as in **947.13A**. Stdze as follows: Transfer 30 mL to I flask, and add 25 mL $H_2O$, 5 mL 20% KI soln, and 5 mL HCl. Shake thoroly and titr. with 0.1*N* $Na_2S_2O_3$, using starch indicator.

### *C. Operating Technic*

Make 1% stock diln of substance to be tested (or any other convenient diln, depending on anticipated concn) in g-s cylinder. Make final dilns, from 1% stock diln, directly into medication tubes and remove all excess >5 mL. (Range of dilns should cover killing limits of disinfectant in 5–15 min and should at same time be close enough for accuracy.) From 5% stock phenol soln (1–20) dil. further to make 1–90 and 1–100 dilns, and place in medication tubes. Place these tubes, contg 5 mL each of final dilns of disinfectant and of phenol, and tube contg test culture in $H_2O$ bath at 20° and leave 5 min. Add 0.5 mL test culture to each of dilns at time intervals corresponding to intervals at which transfers are to be made. (Thus, by time 10 tubes have been seeded at 30 sec intervals, 4.5 min has elapsed, and 30 sec interval intervenes before transference to subculture begins.) Add culture from graduated pipet large enough to seed all tubes in any one set. In using *Ps. aeruginosa* PRD 10 (ATCC 15442), proceed as in **964.02**.

In inoculating medication tubes, hold them in slanting position after removal from bath, insert pipet to just above surface of disinfectant, and run in culture without letting tip touch disinfectant. After adding culture, agitate tubes gently but thoroly to ensure even distribution of bacteria, and replace in bath; 5 min after seeding first medication tube, transfer 1 loopful of mixt. of culture and dild disinfectant from medication tube to corresponding subculture tube. To facilitate transfer of uniform drops of medication mixt., hold tube at 60° angle, and withdraw loop so that plane of loop is parallel with surface of liq. (Fig. **955.11**). After 30 sec, transfer loopful from second medication tube to second subculture tube and continue process for each successive diln; 5 min after making first transfer, begin second set of transfers for 10 min period, and finally repeat for 15 min. period.

Gently agitate medication tubes before taking each interval loop subsample for transfer to subculture medium. Before each transfer, heat loop to redness in flame and flame mouth of every tube. Sterilize loop immediately after each transfer (before replugging tubes) to allow time for cooling. Use care in transferring and seeding to prevent pipet or needle from touching sides or mouth of medication tube, and see that no cotton threads adhere to inner sides or mouths of tubes. Incubate subculture 48 hr at 37° and read results. Thoroly agitate individual subculture tubes before incubation. Macroscopic examination is usually sufficient. Occasionally 3-day incubation period, agar streak, microscopic examination, or agglutination with antityphoid serum may be necessary to det. feeble growth or suspected contamination.

### *D. Calculation*

Express results in terms of phenol coefficient number, or highest diln killing test organism in 10 min but not in 5 min, whichever most accurately reflects germicidal value of disinfectant. Phenol coefficient is number obtained by dividing numerical value of greatest diln (denominator of fraction expressing diln) of disinfectant capable of killing *S. typhi* in 10 min but not in 5 min by greatest diln of phenol showing same results.

*Example:*

| | Disinfectant (X): | | |
|---|---|---|---|
| Diln | 5 Min | 10 Min | 15 Min |
| 1–300 | 0 | 0 | 0 |
| 1–325 | + | 0 | 0 |
| 1–350 | + | 0 | 0 |
| 1–375 | + | + | 0 |
| 1–400 | + | + | + |
| | | Phenol: | |
| 1– 90 | + | 0 | 0 |
| 1–100 | + | + | + |

Phenol coefficient would be $\frac{350}{90} = 3.89$

Test is satisfactory only when phenol control gives one of following readings:

| Phenol | 5 Min | 10 Min | 15 Min |
|---|---|---|---|
| 1– 90 | + or 0 | + or 0 | 0 |
| 1–100 | + | + | + or 0 |

If none of dilns of disinfectant shows growth in 5 min and killing in 10 min, est. hypothetical diln only when any 3 consecutive dilns show following results: first, no growth in 5 min; second, growth in 5 and 10 min but not in 15 min; and third, growth in 5, 10, and 15 min.

*Example:*

| Diln | 5 Min | 10 Min | 15 Min |
|---|---|---|---|
| Disinfectant (X): | | | |
| 1–300 | 0 | 0 | 0 |
| 1–350 | + | + | 0 |
| 1–400 | + | + | + |
| Phenol: | | | |
| 1– 90 | 0 | 0 | 0 |
| 1–100 | + | + | 0 |

Phenol coefficient would be $\frac{325}{95} = 3.42$

To avoid giving impression of fictitious accuracy, calc. phenol coefficient to nearest 0.1. Thus, in examples cited above, phenol coefficients would be reported as 3.9 and 3.4, instead of 3.89 and 3.42.

*Note:* Although it is commonly accepted criterion that disinfectants be at diln equiv. in germicidal efficiency to phenol against *S. typhi* by calcg 20 × *S. typhi* coefficient to det. number of parts $H_2O$ in which 1 part disinfectant may be mixed, this should be regarded as presumptive and is subject to confirmation by use-dilution method.

Refs.: J. Roy. Sanit. Inst. **24,** 424(1903). Am. J. Public Health **3,** 575(1913). U.S. Dept. Agric. Circ. **198** (1931). JAOAC **32,** 408(1949); **38,** 465(1955). Soap Chem. Spec. **34,** No. 10, 79(1958); **47**, 176(1964); **53**, 860(1970); **56**, 308(1973).

## 955.12 Testing Disinfectants against *Staphylococcus aureus*

### Phenol Coefficient Method

### Final Action

Proceed as in **955.11**, except change phenol dilns and test organisms. Use 22–26 hr culture of *Staph. aureus* FDA 209, ATCC No. 6538, having at 20° at least resistance indicated by following:

| Phenol | 5 Min | 10 Min | 15 Min |
|---|---|---|---|
| 1–60 | + or 0 | + or 0 | 0 |
| 1–70 | + | + | + |

Refs.: J. Roy. Sanit. Inst. **24,** 424(1903). Am. J. Public Health **3,** 575(1913). U.S. Dept. Agric. Circ. **198** (1931). JAOAC **32,** 408(1949); **38,** 465(1955). Soap Chem. Spec. **34,** No. 10, 79(1958).

## 955.13 Testing Disinfectants against *Pseudomonas aeruginosa*

### Phenol Coefficient Method

### First Action

Proceed as in **955.11**. Use 22–26 hr culture of *Ps. aeruginosa* PRD 10 (ATCC 15442), having resistance to phenol at 20° at least as follows:

| Phenol | 5 Min | 10 Min | 15 Min |
|---|---|---|---|
| 1–80 | + or 0 | + or 0 | 0 |
| 1–90 | + | + | + |

## USE-DILUTION METHODS[1]

(Applicable to testing disinfectants miscible with $H_2O$ to confirm phenol coefficient results and to det. max. dilns effective for practical disinfection. These microbiological methods are technique-sensitive methods in which extreme adherence to the method with identified critical control points, good microbiological techniques, and quality controls is required for proficiency and validity of results. These methods have been validated using distd $H_2O$ only without soil challenge.)

## 955.14 Testing Disinfectants against *Salmonella choleraesuis*

### Use-Dilution Method

### First Action 1953
### Final Action 1959
### Repealed First Action 1988

#### *A. Reagents*

(**a**) *Culture media.—See* **955.11A**.

(**b**) *Test organism, Salmonella choleraesuis.*—(ATCC 10708). Obtain annually directly from ATCC. Maintain stock culture on nutrient agar slants by monthly transfers. Incubate new stock transfer 2 days at 37°; then store at 2–5°. From stock culture inoculate tube of nutrient broth and incubate at 37°. Make 3 consecutive 24 hr transfers; then inoculate tubes of nutrient broth (2 for each 10 carriers to be tested), using one loop of inoculum with each tube; incubate 48–54 hr at 37°.

(**c**) *Phenol.—See* **955.11B(f)**.

(**d**) *Sterile distilled water.*—Prep. stock supply of $H_2O$ in 1 L flasks, plug with cotton, sterilize 20 min at 121°, and use to prep. dilns of medicants.

(**e**) *Asparagine soln.*—Make stock supply of 0.1% asparagine ("Bacto") soln in $H_2O$ in erlenmeyer of convenient size, plug with cotton, and sterilize 20 min at 121°. Use to cover metal carriers for sterilization and storage.

(**f**) *Sodium hydroxide soln.*—Approx. 1*N* (4%). (For cleaning metal carriers before use.)

#### *B. Apparatus*

(**a**) *Glassware.*—As in **955.11B(a)**. Also: straight side Pyrex test tubes, 20 × 150 mm; 15 × 110 mm petri dishes; 100 mL, 300 mL, and 1 L erlenmeyers. Sterilize petri dishes in closed metal containers. Use 25 × 150 mm straight side tubes for disinfectant soln. (Smaller tubes can give high percentage of false positives when sides are touched.)

(**b**) *Water bath and racks.—See* **955.11B(b)** and (**c**).

(**c**) *Transfer loops and needles.*—(*1*) *See* **955.11B(d)**. (*2*) Make 3 mm right angle bend at end of 50–75 mm nichrome wire No. 18 B&S gage. Have other end in suitable holder (glass or Al rod).

(**d**) *Carriers.*—Polished stainless steel cylinders (penicillin cups), 8 ± 1 mm od, 6 ± 1 mm id, length 10 ± 1 mm, of type 304 stainless steel, SS 18-8. (Obtainable from S. & L. Metal Products Corp., 58-29 57 Drive, Maspeth, NY 11378.)

[1]The use-dilution methods for testing disinfectants, **955.14**, **955.15**, and **964.02**, are technique-sensitive and may produce questionable results unless conducted by experienced, trained analysts under strictly controlled conditions. Users of the methods are advised to consult the following reports of recent studies for current scientific data and interpretation: JAOAC **69,** 1003(1986); **70,** 635(1987); **70,** 903(1987); **71,** 9(1988); **71,** 288(1988); **71,** 868(1988); **71,** 1187(1988). Infect. Control **8,** 501(1987).

Discard cylinders that are visibly damaged (dull, chipped, dented, or gouged). Biologically screen remaining cylinders by performing use-dilution test with *Staphylococcus aureus* ATCC 6538 and 500 ppm alkyldimethylammonium chloride with alkyl chain distribution C14, 50%; C12, 40%; C16, 10% (e.g., BTC-835 Onyx Chemical Co., Jersey City, NJ 07302). Discard those cylinders giving pos. results in screening procedure. In subsequent testing of samples, cylinders in tubes showing growth must be rescreened and may not be reused unless screen tests result in no growth.

(**e**) *Petri dishes*.—Have available ca 6 sterile petri dishes matted with 2 layers of S&S No. 597 or Whatman No. 2, 9 cm filter paper.

(**f**) *Pipets*.—Use only disposable pipets. (Reusable pipets may have residues or chips.)

### C. Operating Technic

Soak ring carriers overnight in 1*N* NaOH, rinse with tap $H_2O$ until rinse $H_2O$ is neut. to phthln, then rinse twice with distd $H_2O$; place cleaned ring carriers in multiples of 10 in cotton-plugged erlenmeyers or 25 × 150 mm cotton plugged Pyrex test tubes, cover with asparagine soln, **955.14A(e)**, sterilize 20 min at 121°, cool, and hold at room temp. Vortex-mix nutrient broth test culture 3–4 s and let stand 10 min at room temp. before continuing. Transfer 20 sterile ring carriers, using flamed nichrome wire hook, into 20 mL 38–54 hr nutrient broth test culture in sterile 25 × 150 mm medication tube. One or 2 addnl carriers may be added at same inoculum rate to serve as reserves. Carriers that fall over in petri dishes cannot be used in test. After 15 min contact period remove cylinders, using flamed nichrome wire hook, shake carrier vigorously against side of tube to remove excess culture, and place on end in vertical position in sterile petri dish matted with filter paper, **955.14B(e)**, making sure that carriers do not touch to prevent improper drying. Cover and place in incubator at 37° and let dry 40 min. Hold broth culture for detn of its resistance to phenol by phenol coefficient method, **955.11C**.

From 5% stock phenol soln (1–20) make 1–90 and 1–100 dilns directly into medication tubes. Place tube for each diln in $H_2O$ bath and let come to 20° (10 min). Make stock soln of germicide to be tested in sterile g-s cylinder. From this soln make 10 mL dilns to be tested, depending upon phenol coefficient found and/or claimed against *S. typhi* at 20°, directly into each of ten 25 × 150 mm medication tubes; place the 10 tubes in $H_2O$ bath at 20° and let come to temp. Prep. diln of germicide to be tested by diln in sterile $H_2O$, **955.14A(d)**. Diln of sample should be made using ≥1.0 mL of sample. Use v/v dilns for liq. products and w/v dilns for solids. Round to 2 decimal places toward a stronger product. To ensure stable product, soln should be prepd ≤3 hr prior to use. Place tubes in 20° $H_2O$ bath ≥10 min. Det. diln to be tested by multiplying phenol coefficient number found and/or claimed by 20 to det. number of parts $H_2O$ in which 1 part germicide is to be incorporated. This detn is not required when disinfectant under test yields phenol coefficient that cannot be converted validly to presumptive use-diln, or when analyst dets that use-diln range can be found without resort to phenol coefficient test.

Add 0.5 mL of test culture suspension to 1–90 diln of phenol control; after 30 sec interval, add 0.5 mL to 1–100 diln of control, using sterile cotton-plugged pipets. After adding culture, agitate tubes gently but thoroly to distribute bacteria evenly, and replace in bath; 5 min after seeding first medication tube, transfer 1 loopful of mixt. of culture and dild phenol from medication tube to corresponding subculture tube. After 30 sec, transfer loopful from second medication tube; 5 min after making first set of transfers begin second set of transfers for 10 min period; and finally repeat for 15 min period. Use technic of loop sampling, flaming loop and mouths of tubes, and agitating medication and subculture tubes as in phenol coefficient method, **955.11C**. Incubate subcultures 48 hr at 37° and read results. Resistance in 48–54 hr culture of *S. choleraesuis* should fall within range specified for 24 hr culture of *S. typhi* in phenol coefficient method.

Without touching sides of tube with contaminated carrier or hook, either when placing carrier in tube or when withdrawing hook, add 1 contaminated dried cylinder carrier at 1 min intervals to each of the 10 tubes of use-diln of germicide to be tested. (*Note:* Proper execution of transfer step is one of the most critical, technique-sensitive areas of method. False positives will result if sides of tube are touched.) Thus, by time 10 tubes have been seeded, 9 min will have elapsed, plus 1 min interval before transfer of first carrier in series to individual tube of subculture broth. This interval is const for each tube with prescribed exposure period of 10 min. The 1 min interval between transfers allows adequate time for flaming and cooling nichrome wire hook and making transfer in manner so as to drain all excess medication from carrier by shaking carrier against side of tube. Shorter intervals may be used in adding and removing carriers if 2 alternately flamed and cooled hooks are used. Individual manipulation of carriers is required; use of semiautomated ring carrier is prohibited. (*Note:* Above step is one of the most critical, technique-sensitive areas of method. False positives can result from transfer of live organisms to sides of tube due to aerosol formation.) Flame lips of medication and subculture tubes in conventional manner. Immediately after placing carrier in medication tube, swirl tube 3 times before placing it back in bath. Thoroly shake subculture tubes, incubate 48 hr at 37°, and report results as + (growth) or − (no growth) values. Growth in tubes should be checked by gram stain to ensure that no contamination is present. Check ≥20% of pos. tubes. Confirm all pos. results by duplicate testing to assure against false pos. tests.

Where there is reason to suspect that lack of growth at conclusion of incubation period may be due to bacteriostatic action of medicant adsorbed on carrier that has not been neutzd by subculture medium used, transfer each ring to new tube of sterile medium and reincubate for addnl 48 hr at 37°. Where soln under test is such that material adsorbed on ring carriers and transferred into subculture medium makes it unsuitable for growth of test organism, as may be case with concd acids and alkalies, products carrying antibiotics, and wax emulsions, transfer each ring to new tube of sterile medium 30 min after initial transfer and incubate both primary and secondary subculture tubes 48 hr at 37°. Results showing no growth on all 10 carriers will confirm phenol coefficient number found. Results showing growth on any of the 10 carriers indicate phenol coefficient number to be unsafe guide to diln for use. In latter case, repeat test, using lower dilns (higher concns) of germicide under study. Max. diln of germicide which kills test organism on 10 carriers in 10 min interval represents presumed max. safe use-diln for practical disinfection.

Refs.: J. Bacteriol. **49,** 526(1945). Am. J. Vet. Res. **9,** 104(1948). JAOAC **36,** 466(1953); **70,** 318(1987); **71,** 117(1988); **72,** 116(1989).

## 955.15 Testing Disinfectants against *Staphylococcus aureus*

### Use-Dilution Method

**First Action 1953**
**Final Action 1959**
**Repealed First Action 1988**

Proceed as in **955.14C** except change phenol dilns and test organism to those specified in **955.12**. Use 48–54 hr culture of *Staph. aureus* FDA 209, ATCC No. 6538, having at least resistance specified for 24 hr culture at 20° in phenol coefficient method, **955.12**. Obtain organism annually, directly from ATCC. Prior to beginning use-dilution test, vortex-mix nutrient broth culture as in **955.14**. Results showing growth on any of 10 carriers indicate that diln is too high for use in disinfecting where pyogenic bacteria must be killed. In such cases repeat test, using lower dilns (higher concns). Max. diln of germicide which kills both this test organism and *S. choleraesuis* on 10 carriers in 10 min interval represents max. presumed safe use-diln for disinfecting in hospitals, clinics, and other places where pyogenic bacteria have special significance.

*Note:* While killing in 10 of 10 replicates specified provides reasonably reliable index in most cases, killing in 59 of 60 replicates is necessary for confidence level of 95%.

Refs.: J. Bacteriol. **49,** 526(1945). Am. J. Vet. Res. **9,** 104(1948). JAOAC **36,** 466(1953); **70,** 318(1987); **71,** 117(1988); **72,** 116(1989).

## 964.02 Testing Disinfectants against *Pseudomonas aeruginosa*

### Use-Dilution Method

### First Action 1964

Proceed as in **955.14C**. Use 48–54 hr nutrient broth culture *Ps. aeruginosa* PRD 10 (ATCC 15442). Carry stock culture on BBL CTA (cystine trypticase agar) in stab culture incubated 48 hr at 37° and stored at 5° with transfer every 30 days. Transfer nutrient broth test cultures daily for 30-day intervals with incubation at 37°. Make fresh transfer from stock culture every 30 days. Do not shake 48–54 hr test culture but decant liq. culture aseptically, leaving pellicle behind, to obtain 20 mL culture for inoculating 20 carriers in medication tube.

Proceed with vortex-mixing as in **955.14C** prior to use of culture. Alternatively, pellicle may be carefully suctioned off, and culture can be poured into clean, sterile tube before vortex-mixing. Any disruption of pellicle resulting in dropping, breaking up, or stringing of pellicle in culture before or during its removal renders that culture unusable in use-dilution test. This is extremely critical because any pellicle fragments remaining will result in uneven clumping and layering of organism on cylinder, allowing unfair exposure to disinfectant and causing false pos. results.

Refs.: J. Bacteriol. **49,** 526(1945). Am. J. Vet. Res. **9,** 104(1948); JAOAC **47,** 29, 176(1964); **70,** 318(1987); **72,** 116(1989).

## OTHER TESTS

## 955.16 Chlorine (Available) in Disinfectants

### Germicidal Equivalent Concentration

### Final Action

(Applicable to $H_2O$-miscible disinfectants for detg available Cl germicidal equiv. concns with products offered for use as sanitizing rinses for previously cleaned nonporous surfaces, especially where speed of action and capacity are essential considerations)

### *A. Reagents*

Use reagents specified in **955.11A** and **955.11B(e)** and **(f)**, and in addn:

**(a)** *Sterile distilled $H_2O$.—See* **955.14A(d)**.

**(b)** *Sterile phosphate buffer soln.*—pH 8.0. Add 97.5 mL soln contg 11.61 g anhyd. $K_2HPO_4$ in 1 L $H_2O$ to 2.5 mL soln contg 9.08 g anhyd. $KH_2PO_4$ in 1 L $H_2O$ and autoclave 20 min at 121° in cotton-plugged erlenmeyer.

**(c)** *NaOCl std stock soln.*—Approx. 5%. Store NaOCl stock soln in tightly closed bottle in refrigerator, and det. exact available Cl concn at frequent intervals by $As_2O_3$ titrn: Transfer 20 mL sample to 1 L vol. flask and dil. to vol. Pipet 50 mL aliquot of mixt. into 200 mL erlenmeyer. Add excess $As_2O_3$ soln and then decided excess $NaHCO_3$. Titr. excess $As_2O_3$ with std I soln, using starch soln (mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring const., and immediately discontinue heating; add ca 1 mL Hg, shake, and let soln stand over Hg), or use the I as its own indicator. Subtract vol. I soln, corrected to 0.1*N*, from vol. $As_2O_3$ soln used, and from this value and sp gr of soln, calc.% NaOCl.

$$1 \text{ mL } 0.1N \ As_2O_3 = 0.003722 \text{ g NaOCl}$$

**(d)** *Test organisms.*—Use *S. typhi* ATCC No. 6539 or *Staph. aureus* ATCC No. 6538 or both.

### *B. Apparatus*

*See* **955.11B**.

### *C. Operating Technic*

Det. resistance of test culture to phenol as in **955.11**, and use cultures with resistance specified. Prep., in sterile g-s cylinders, NaOCl solns contg 200, 100, and 50 ppm available Cl in sterile buffer soln, **955.16A(b)**. Transfer 10 mL of each soln to 25 × 150 mm medication tubes, place tubes in 20° $H_2O$ bath, and let come to temp.

Starting with tube contg 200 ppm available Cl, add 0.05 mL test culture prepd as in **955.11B(e)**, shake, and return to $H_2O$ bath. After 1 min, make transfer to tube of appropriate subculture medium, **955.11A(d)**, using flamed 4 mm loop. At 1.5 min, add another 0.05 mL culture to the 200 ppm Cl soln, shake, and return to bath. After addnl 1 min interval (2.5 min in test), make second subculture in same manner, and in 30 sec, or at 3 min time in test, add another 0.05 mL culture, shaking and returning to $H_2O$ bath. After another 1 min interval (4 min in test), make another transfer to tube of subculture medium.

Repeat operation to give total of 10 added increments. This requires total time of 14.5 min for each soln and addn of 0.5 mL total culture with subculture at std 1 min intervals after addn of culture aliquots. At conclusion of test shake all subculture tubes and incubate 48 hr at 37°.

Repeat operation with solns contg 100 and 50 ppm available Cl. Prep. soln of germicide to be tested at concn recommended or selected for study in sterile $H_2O$ in g-s graduate. Transfer 10 mL to 25 × 150 mm medication tubes, place in $H_2O$ bath, and let come to temp. Repeat operation with this soln.

To be considered equiv. in disinfecting activity to 200 ppm available Cl, unknown germicide must show absence of growth in as many consecutive tubes of subculture tube series as 200 ppm available Cl std. Det. activity equiv. to 100 and 50 ppm available Cl in same manner. *See* example, Table **955.16**.

In this example, 25 ppm soln of germicide X could be considered equiv. to 200 ppm soln of available Cl, and 20 ppm soln equiv. to 100 ppm of available Cl, but 10 ppm soln of germicide X would not be considered equiv. in germicidal activity to 50 ppm of available Cl.

Draw conclusions relative to germicidal equiv. concns only when resistance of test culture to NaOCl control is such that ≥1 neg. increment is obtained at 50 ppm concn and 1 pos. increment is obtained at 200 ppm level.

Refs.: Soap Sanit. Chem. **27,** No. 2, 133(1951). JAOAC **38,** 274(1955); **40,** 755(1957).

**Table 955.16 Example for Determination of Chlorine Germicidal Equivalent Concentration**

| Germicide | Concn, ppm Avail. Cl | Subculture Series 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 200 | − | − | − | − | − | + | + | + | + | + |
| NaOCl control | 100 | − | − | − | + | + | + | + | + | + | + |
| | 50 | − | − | + | + | + | + | + | + | + | + |
| | 25 | − | − | − | − | − | + | + | + | + | + |
| Unknown (X) | 20 | − | − | − | − | + | + | + | + | + | + |
| | 10 | − | + | + | + | + | + | + | + | + | + |

− = No growth + = growth

## 955.17 Fungicidal Activity of Disinfectants Using *Trichophyton mentagrophytes*

**Final Action**

(Applicable for use with $H_2O$-miscible type fungicides used to disinfect inanimate objects)

### *A. Test Organism*

Use as test fungus typical strain of *Trichophyton mentagrophytes* isolated from dermatophytosis of foot. Strain must sporulate freely on artificial media, presence of abundant conidia being manifested by powdery appearance on surface of 10-day culture, particularly at top of agar slant, and confirmed by microscopic examination. Conidia-bearing mycelium should peel easily from surface of glucose agar. Conidia of required resistance survive 10 min exposure at 20° to phenol diln of 1:70, but not to one of 1:60. Strain No. 640, ATCC No. 9533, is suitable.

### *B. Culture Medium*

Carry fungus on agar slants of following composition: Glucose 2%, Neopeptone (Difco) 1%, agar 2%, adjusted to pH 6.1–6.3. Use same culture medium to prep. cultures for obtaining conidial suspension, and use fluid medium of same nutrient composition (without agar) to test survival and viability of conidia after exposure to fungicide.

### *C. Care of Fungus Strain*

Store stock culture of fungus on glucose agar slants at 2–5°. At intervals ≤3 months, transfer to fresh agar slants, incubate 10 days at 25–30°, and store at 2–5° until next transfer period. Do not use culture that has been kept at or above room temp. >10 days as source of inoculum for culture. (Cultures may be kept at room temp. to preserve strain and to inoculate cultures if transferred at intervals ≤10 days.)

### *D. Preparation of Conidial Suspension*

Prep. petri dish cultures by planting inoculum at center of agar plate and incubating culture at 25–30° for ≥10, but ≤15 days. Remove mycelial mats from surface of 5 agar plate cultures, using sterile spatula or heavy flattened wire. Transfer to heat-sterilized glass tissue grinder, **966.04B(e)**, and macerate with 25 mL sterile physiological NaCl soln (0.85% NaCl), or to heat-sterilized erlenmeyer contg 25 mL sterile saline with glass beads, and shake thoroly. Filter suspension thru sterile absorbent cotton to remove hyphal elements. Est. density of conidial suspension by counting in hemacytometer and store at 2–10° as stock spore suspension (125–155 × $10^6$ conidia/mL) for ≤4 weeks for use in prepg test suspensions of conidia. Stdze test conidial suspensions as needed by dilg stock spore suspension with physiological NaCl soln so that it contains 5 × $10^6$ conidia/mL.

### *E. Operating Technic*

Prep. dilns of fungicide. (Tests are similar to those described in **955.11C**.) Place 5 mL of each fungicide soln and of phenol control solns in 25 × 150 mm test-culture tubes, arrange in order of ascending dilns, place tubes in 20° $H_2O$ bath, and let come to temp. With graduated pipet, place 0.5 mL spore suspension in first tube of fungicidal soln, shake, and immediately replace in $H_2O$ bath; 30 sec later add 0.5 mL conidial suspension to second tube. Repeat at 30 sec intervals for each fungicidal diln. If more convenient, run test at 20 sec intervals. After 5, 10, and 15 min exposure to fungicide, remove sample from each conidia-fungicide mixt. with 4 mm loop and place in 10 mL glucose broth, **955.17B**. To eliminate risk of faulty results due to possible fungistatic action, make subtransfers from the initial glucose broth subculture tubes to fresh tubes of glucose broth, using the 4 mm loop before incubation, or make initial subcultures in glucose broth contg either 0.05% Na thioglycolate, 1.5% isooctylphenoxy-polyethoxy-ethanol, or mixt. of 0.07% lecithin (Asolectin, Associated Concentrates, Inc., 32–34 61st St, Woodside, NY 11377) and 0.5% polysorbate 80 (Tween 80), whichever gives lowest result. Incubate inoculated tubes at 25–30°. Read final results after 10 days, altho indicative reading can be made in 4 days.

*Note:* Highest diln that kills spores within 10 min is commonly considered as highest diln that could be expected to disinfect inanimate surfaces contaminated with pathogenic fungi.

Refs.: Arch. Dermatol. Syphilol. **28,** 15(1933). J. Bacteriol. **42,** 225(1941); **47,** 102(1944). JAOAC **37,** 616(1954); **38,** 274(1955); **56,** 308(1973).

## 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants

**Final Action**

(Suitable for detg min. concn of chem. that can be permitted for use in sanitizing precleaned, nonporous food contact surfaces. Min. recommended starting concn is 2–4× this concn. Test also dets max. water hardness for claimed concns. As control, check accuracy of hard-water tolerance results with pure $C_{14}$ alkyl dimethyl benzyl $NH_4$ chloride at 700 and 900 ppm hardness, and pure $C_{16}$ alkyl dimethyl benzyl $NH_4$ chloride (Cetalkonium Chloride, at 400 and 550 ppm hardness, expressed as $CaCO_3$.)

### *A. Reagents*

(**a**) *Culture media.*—(*1*) *Nutrient agar A.*—Boil 3 g beef ext, 5 g peptone (Bacto, from Difco or equiv.; special grades must not be used), and 15 g salt-free agar in 1 L $H_2O$. Do not use premixed, dehydrated media. Tube, and autoclave 20 min at 121°. Use for daily transfer of test culture. (*2*) *Nutrient agar B.*—Prep. as above but use 30 g agar. Use for growing test cultures in French square bottles. (*3*) *Nutrient agar (AOAC).*—*See* **955.11A(c)**. Use for prepg stock culture slants.

(**b**) *Subculture media.*—(*1*) Use tryptone glucose ext agar (Difco), adding 25 mL stock neutralizer, (**c**)/L. (*2*) Tryptone glucose ext agar (Difco).

**Table 960.09A Percent Light Transmission at Various Wavelengths Corresponding to Bacterial Concentrations**

| % Light Transmission with Filters, nm | | | | | | | Av. Bacterial Count/mL |
|---|---|---|---|---|---|---|---|
| 370 | 420 | 490 | 530 | 550 | 580 | 650 | |
| 7.0 | 4.0 | 6.0 | 6.0 | 6.0 | 7.0 | 8.0 | $13.0 \times 10^9$ |
| 8.0 | 5.0 | 7.0 | 7.0 | 7.0 | 8.0 | 9.0 | 11.5 |
| 9.0 | 6.0 | 8.0 | 8.0 | 8.0 | 9.0 | 10.0 | 10.2 |
| 10.0 | 7.0 | 9.0 | 9.0 | 9.0 | 11.0 | 11.0 | 8.6 |
| 11.0 | 8.0 | 10.0 | 10.0 | 10.0 | 12.0 | 13.0 | 7.7 |
| 13.0 | 9.0 | 12.0 | 12.0 | 12.0 | 13.0 | 15.0 | 6.7 |

(**c**) *Neutralizer stock soln.*—Mix 40 g Asolectin (Associated Concentrates, 32–34 61st St, Woodside, NY 11377), 280 mL polysorbate 80, and 1.25 mL phosphate buffer, (**e**); dil. with $H_2O$ to 1 L and adjust to pH 7.2. Dispense in 100 mL portions and autoclave 20 min at 121°.

(**d**) *Neutralizer blanks.*—For use with ≤200 ppm quaternary $NH_4$ compd. Mix 100 mL neutralizer stock soln, (**c**), 25 mL 0.25*M* phosphate buffer stock soln, (**e**), and 1675 mL $H_2O$. Dispense 9 mL portions into 20 × 150 mm tubes. Autoclave 20 min at 121°.

(**e**) *Phosphate buffer stock soln.*—0.25*M*. Dissolve 34.0 g $KH_2PO_4$ in 500 mL $H_2O$, adjust to pH 7.2 with 1*N* NaOH, and dil. to 1 L.

(**f**) *Phosphate buffer dilution water.*—Add 1.25 mL 0.25*M* phosphate buffer stock soln, (**e**), to 1 L $H_2O$ and dispense in 99 mL portions. Autoclave 20 min at 121°.

(**g**) *Test organisms.*—Use *Escherichia coli* ATCC No. 11229 or *Staphylococcus aureus* ATCC 6538. Incubate 24 and 48 hr, resp. Maintain stock cultures on nutrient agar (AOAC), (**a**)(*3*), at refrigerator temp.

### B. Resistance to Phenol of Test Cultures

Det. resistance to phenol at least every 3 months by **955.11**. Resistance of *E. coli* should be equiv. to that specified for *S. typhi* in **955.11D** and that for *Staph. aureus* equiv. to that specified for this organism in **955.12**.

### C. Apparatus

(**a**) *Glassware.*—250 mL wide-mouth erlenmeyers; 100 mL graduate; Mohr, serological, and/or bacteriological (APHA specification) pipets; 20 × 150 mm test tubes. Sterilize at 180° in hot air oven ≥2 hr.

(**b**) *Petri dishes.*—Sterile.

(**c**) *French square bottles.*—175 mL, borosilicate (Thomas Scientific). Use of other containers will give variable results.

(**d**) *Water bath.*—Controlled at 25°.

### D. Preparation of Culture Suspension

From stock culture inoculate tube of nutrient agar *A*, **960.09A**(**a**)(*1*), and make ≥3 consecutive daily transfers (≤30), incubating transfers 20–24 hr at 35–37°. Do not use transfers >30 days. If only 1 daily transfer has been missed, no special procedures are required; if 2 daily transfers are missed, repeat with 3 daily transfers.

Prep. 175 mL French square culture bottles contg 20 mL nutrient agar *B*, **960.09A**(**a**)(*2*), autoclave 20 min at 121°, and let solidify with bottle in horizontal position. Inoculate culture bottles by washing growth from slant with 5 mL phosphate buffer diln $H_2O$, **960.09A**(**f**), into 99 mL phosphate buffer diln $H_2O$, and adding 2 mL of this suspension to each culture bottle, tilting back and forth to distribute suspension; then drain excess liq. Incubate 18–24 hr at 35–37°, agar side down. Remove culture from agar surface of 4 or more bottles, using 3 mL phosphate buffer diln $H_2O$ and glass beads in each bottle to suspend growth. Filter suspension thru Whatman No. 2 paper prewet with 1 mL sterile phosphate buffer, and collect in sterile tube. (To hasten filtration, rub paper gently with sterile policeman.) Stdze suspension to give av. of $10 \times 10^9$ organisms/mL by diln with sterile phosphate buffer diln $H_2O$, **960.09A**(**f**).

If Lumetron colorimeter is used, dil. suspension in sterile Lumetron tube to give % *T* according to Table **960.09A**.

If McFarland nephelometer and $BaSO_4$ stds are used, select 7 tubes of same id as that contg test culture suspension. Place 10 mL of each suspension of $BaSO_4$, prepd as indicated in Table **960.09B**, in each tube and seal tube. Stdze suspension to correspond to No. 4 std.

### E. Synthetic Hard Water

Prep. *Soln 1* by dissolving 31.74 g $MgCl_2$ (or equiv. of hydrates) and 73.99 g $CaCl_2$ in boiled distd $H_2O$ and dilg to 1 L. Prep. *Soln 2* by dissolving 56.03 g $NaHCO_3$ in boiled distd $H_2O$ and dilg to 1 L. *Soln 1* may be heat sterilized; *Soln 2* must be sterilized by filtration. Place required amt *Soln 1* in sterile 1 L flask and add ≥600 mL sterile distd $H_2O$; then add 4 mL *Soln 2* and dil. to 1 L with sterile distd $H_2O$. Each mL *Soln 1* will give a water equiv. to ca 100 ppm of hardness calcd as $CaCO_3$ by formula:

$$\text{Total hardness as ppm } CaCO_3 = 2.495 \times \text{ppm Ca} + 4.115 \times \text{ppm Mg}$$

pH of all test waters ≤2000 ppm hardness should be 7.6–8.0. Check prepd synthetic waters chemically for hardness at time of tests, using following method or other methods described in 14th ed. of *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes*.

### F. Hardness Method

(**a**) *EDTA std soln.*—Dissolve 4.0 g $Na_2H_2EDTA.2H_2O$ and 0.10 g $MgCl_2.6H_2O$ in 800 mL $H_2O$ and adjust by subsequent diln so that 1 mL of soln is equiv. to 1 mg $CaCO_3$ when titrd as in (**c**). Check EDTA soln after prepn or, if com. purchased, against $CaCO_3$ std at least every 2 months.

(**b**) *Calcium std soln.*—1 mL = 1 mg $CaCO_3$. Weigh 1.00 g $CaCO_3$, dried overnight or longer at 105°, into 500 mL erlenmeyer and add dil. HCl thru funnel until $CaCO_3$ is dissolved. Add 200 mL $H_2O$, boil to expel $CO_2$, and cool. Add few drops Me red indicator and adjust color to intermediate orange with dil. $NH_4OH$ or HCl as required. Transfer quant. to 1 L vol. flask and dil. to vol.

(**c**) *Determination.*—Dil. 5–25 mL sample (depending on hardness) to 50 mL with $H_2O$ in erlenmeyer or casserole. Add 1 mL *buffer soln* (67.5 g $NH_4Cl$ and 570 mL $NH_4OH$ dild to

**Table 960.09B Preparation of $BaSO_4$ Suspensions Corresponding to Bacterial Concentrations**

| Std No. | 2% $BaCl_2$ Soln, mL | 1% $H_2SO_4$ (v/v) Soln, mL | Av. Bacterial Count/mL |
|---|---|---|---|
| 1 | 4.0 | 96.0 | $5.0 \times 10^9$ |
| 2 | 5.0 | 95.0 | 7.5 |
| 3 | 6.0 | 94.0 | 8.5 |
| 4 | 7.0 | 93.0 | 10.0 |
| 5 | 8.0 | 92.0 | 12.0 |
| 6 | 10.0 | 90.0 | 13.5 |
| 7 | 12.0 | 88.0 | 15.0 |

1 L with $H_2O$), 1 mL *inhibitor* (5.0 g $Na_2S.9H_2O$ or 3.7 g $Na_2S.5H_2O$ dissolved in 100 mL $H_2O$), and 1 or 2 drops *indicator soln* (0.5 g Chrome Black T in 100 mL 60–80% alcohol). Titr. with EDTA std soln slowly, stirring continuously, until last reddish tinge disappears from soln, adding last few drops at 3–5 sec intervals.

Hardness as mg $CaCO_3$ /L
= (mL std soln × 1000)/mL sample

### G. Preparation of Samples

Use composition declared or detd as guide to sample wt required for vol. sterile $H_2O$ used to prep. 20,000 ppm soln. From this stock diln, transfer 1 mL into 99 mL of the water to be used in test to give concn of 200 ppm. In making transfer, fill 1 mL pipet and drain back into stock soln; then refill, to correct for adsorption on glass. After mixing, discard 1 mL to provide 99 mL of the test water in **960.09H**.

### H. Operating Technic

Measure 99 mL water to be used in test, contg bactericide at concn to be tested, into chem. clean, sterile, 250 mL widemouth erlenmeyer and place in const temp. bath until it reaches 25°, or ≥20 min. Prep. duplicate flasks for each germicide to be tested. Also prep. similar flask contg 99 mL sterile phosphate buffer diln $H_2O$, **960.09A(f)**, as "initial numbers" control.

Add 1 mL culture suspension to each test flask as follows: Whirl flask, stopping just before suspension is added, creating enough residual motion of liq. to prevent pooling of suspension at point of contact with test water. Add suspension midway between center and edge of surface with tip of pipet slightly immersed in test soln. Avoid touching pipet to neck or side of flask during addn. Transfer 1 mL portions of this exposed culture to neutralizer blanks exactly 30 and 60 sec after addn of suspension. Mix well immediately after transfer.

For "numbers control" transfer, add 1 mL culture suspension to 99 mL sterile phosphate diln $H_2O$ in same manner. In case of numbers control, plants need be made only immediately after adding and mixing thoroly ≤30 sec. (It is advantageous to use milk pipets to add culture and withdraw samples.)

Plate from neutralizer tube to agar, using subculture medium **960.09A(b)**(*1*) for quaternary $NH_4$ compds and **960.09A(b)**(*2*) with numbers control. Where 0.1 mL portions are planted, use 1 mL pipet graduated in 0.1 mL intervals. For dilns to give countable plates, use phosphate buffer diln $H_2O$, **960.09A(f)**. For numbers control, use following diln procedure: Transfer 1 mL exposed culture (1 mL culture suspension transferred to 99 mL phosphate buffer diln $H_2O$ in $H_2O$ bath) to 99 mL phosphate buffer diln $H_2O$, **960.09A(f)**, (*diln 1*). Shake thoroly and transfer 1 mL *diln 1* to 99 mL phosphate buffer diln $H_2O$, **960.09A(f)**, (*diln 2*). Shake thoroly and transfer 1 mL *diln 2* to 99 mL phosphate buffer diln $H_2O$ (*diln 3*). Shake thoroly and transfer four 1 mL and four 0.1 mL aliquots from *diln 3* to individual sterile petri dishes.

For test samples, use following diln procedure: Transfer 1 mL exposed culture into 9 mL neutralizer, **960.09A(d)**. Shake and transfer four 1 mL and four 0.1 mL aliquots to individual sterile petri dishes. For numbers control, use subculture medium **960.09A(b)**(*2*); for tests with quaternary $NH_4$ compds, use medium **960.09A(b)**(*1*). Cool agar to solidify, and then invert and incubate 48 hr at 35° before counting.

### I. Results

To be considered valid, results must meet std effectiveness: 99.999% reduction in count of number of organisms within 30 sec. Report results according to actual count and % reduction over numbers control. Counts on numbers control for germicide test mixt. should fall between 75 and 125 × $10^6$/mL for % reductions to be considered valid.

### J. Sterility Controls

(**a**) *Neutralizer*.—Plate 1 mL from previously unopened tube.
(**b**) *Water*.—Plate 1 mL from each type of water used.
(**c**) *Sterile distilled water*.—Plate 1 mL.

After counting plates, confirm that surviving organisms are *E. coli* by transfer to brilliant green bile broth fermentation tubes or lactose broth and EMB agar; confirm *Staph. aureus* by microscopic examination.

Refs.: Am. J. Public Health **38,** 1405(1948). J. Milk Food Technol. **19,** 183(1956). Fed. Regist. **21,** 7020(1956). JAOAC **41,** 541(1958); **56,** 308(1973).

## 961.02 Germicidal Spray Products as Disinfectants

**First Action 1961**
**Final Action 1964**

(Suitable for detg effectiveness of sprays and pressurized spray products as spot disinfectants for contaminated surfaces)

### A. Reagents

Use culture media and reagents specified in **955.11A**, **955.11B(e)** and **(f)**, and **955.14A** except that test organism *Salmonella typhi* is not used.

Use as test organisms *Trichophyton mentagrophytes* ATCC No. 9533, prepd as in **955.17D**, to which has been added 0.02 mL octyl-phenoxy-polyethoxy-ethanol (Triton ×100, Rohm & Haas Co.)/10 mL suspension to facilitate spreading, *Salmonella choleraesuis* ATCC No. 10708, **955.14A(b)**, *Staphylococcus aureus* ATCC No. 6538, maintained as in **955.14A(b)**, and *Pseudomonas aeruginosa* ATCC No. 15442, maintained as in **964.02**.

### B. Apparatus

Use app. specified in **955.11B** and **955.14B**, and in addn:

(**a**) *Capillary pipets*.—0.1 mL, graduated to deliver 0.01 mL. Sterilize in air oven 2 hr at 180°.

(**b**) *Microscope slides*.—Non-corrosive, 25 × 25 mm (1 × 1″), or 18 × 36 mm glass slide. Sterilize by placing individual slides in petri dish matted with 2 pieces 9 cm filter paper (Whatman No. 2, or equiv.) in air oven 2 hr at 180°.

(**c**) *Bacteriological culture tubes*.—Pyrex, 32 × 200 mm.

(**d**) *Metal forceps*.—Sharp points, straight, 115 mm long.

### C. Operating Technic

Thoroly shake 48 hr nutrient broth cultures of *S. choleraesuis* and *Staph. aureus* and let settle 10 min. With sterile capillary pipet or sterile 4.0 mm loop, transfer 0.01 mL culture onto 1 sq in. sterile test slide in petri dish and immediately spread uniformly over entire area. Cover dish immediately and repeat operation until 12 slides have been prepd for each organism. (Use 2 slides as control.) Dry all slides 30–40 min at 37°.

Spray 10 slides for specified time and distance. Hold each slide 10 min, drain off excess liq., and transfer slide to individual 32 × 200 mm tube contg 20 mL appropriate subculture medium, **955.11A(d)**, with flamed forceps. Shake culture thoroly. If broth appears cloudy after 30 min, make subculture to fresh individual tubes of subculture broth. Transfer 2 unsprayed slides, as viability controls, to individual subculture tubes in same manner.

Incubate all tubes used for primary and secondary transfers

48 hr at 37°. Read as + (growth) or − (no growth). Killing of test organisms in 10 of 10 trials is presumptive evidence of disinfecting action.

Det. resistance of *S. choleraesuis* as in **955.11C**; with *S. aureus* as in **955.12**; with *Ps. aeruginosa* as in **955.13**; and with *T. mentagrophytes* as in **955.17A**.

If there is reason to believe that lack of growth in subtransfer tubes is due to bacteriostasis, inoculate all incubated subculture tubes with loop needle inoculation of respective test culture and reincubate. Growth of these inocula eliminates bacteriostasis as cause of lack of growth. If there is question as to possibility of contamination as source of growth in subculture tubes, make gram stains and/or subculture for identification, according to respective test culture.

If fungicidal activity as well as germicidal activity is involved, use test suspension of *T. mentagrophytes* spores, **955.17D**, and prep. 12 slides, using 0.01 mL std spore suspension, spraying and subculturing exactly as above. Make subcultures in glucose broth, **955.17B**, incubating 7 days at 25–30°.

Refs.: JAOAC **44,** 422(1961); **50,** 763(1967). Soap Chem. Spec. **38**(2), 69(1962); **61,** 400(1978).

## 966.04 Sporicidal Activity of Disinfectants

**First Action 1966**
**Final Action 1967**

(Suitable for detg sporicidal activity of liq. and gaseous chems. Applicable to germicides for detg presence or absence of sporicidal activity against specified spore-forming bacteria in various situations and potential efficacy as sterilizing agent.)

### A. Reagents

(**a**) *Culture media.*—(*1*) *Soil extract nutrient broth.*—Ext 1 lb garden soil in 1 L $H_2O$, filter several times thru S&S No. 588 paper, and dil. to vol. (pH should be ≥5.2). Add 5 g beef ext. (Difco), 5 g NaCl, and 10 g peptone (Anatone, **955.11A(a)**). Boil 20 min, dil. to vol., adjust with 1*N* NaOH to pH 6.9, and filter thru paper. Dispense in 10 mL portions into 25 × 150 mm tubes, and autoclave 20 min at 121°. Use this broth to propagate test culture of *Bacilli.*

(*2*) *Nutrient agar.*—*See* **955.11A(c)**. Use slants of this medium to maintain stock culture of *Bacilli.*

(*3*) *Modified fluid thioglycolate medium USP.*—Prep. as in **955.11A(d)**(*2*), except add 20 mL 1*N* NaOH to each L before dispensing for sterilization. Use this medium to subculture spores exposed to 2.5*N* HCl. For spores exposed to unknown germicides, use fluid thioglycolate medium, **955.11A(d)**(*2*).

(*4*) *Soil extract-egg-meat medium.*—Add 1.5 g Bacto Egg-Meat Medium dehydrated (Difco No. 0042–17) to 25 × 150 mm tube; then add 15 mL garden soil ext, (*1*), and sterilize 20 min at 121°. Use this medium to propagate test cultures of *Clostridia* and maintain stock cultures of species of this genus.

(**b**) *Test organisms.*—Use *Bacillus subtilis,* ATCC No. 19659, or *Clostridium sporogenes,* ATCC No. 3584, for routine evaluation. Method is also applicable for use with strains of *B. anthracis, Cl. tetani,* or other spore forming species.

(**c**) *Dilute hydrochloric acid.*—2.5*N*. Use to det. resistance of dried spores. Stdze and adjust to 2.5*N* as in **936.15B**.

### B. Apparatus

(**a**) *Glassware.*—Bacteriological culture tubes, unflared, 25 × 150 mm; 100 mL g-s cylinders graduated in 1 mL divisions; 65 mm id funnels; supply of 15 × 110 mm petri dishes matted with 2 sheets 9 cm S&S No. 597 or Whatman No. 2 filter paper. Sterilize all glassware and matted petri dishes 2 hr in air oven at 180°.

(**b**) *Water bath.—See* **955.11B(b)**.

(**c**) *Racks.—See* **955.11B(c)**.

(**d**) *Transfer loop, hook, and forceps.—See* **955.14B(c)**.

(**e**) *Tissue grinder.*— Thomas Scientific, No. 3431-E20, Size B, or equiv.

(**f**) *Suture loop carrier.*—From spool of size 3 surgical silk suture (silk black braid A-59, USP, Ethicon, Inc., Rte 22, Sommerville, NJ 08876), prep. std loops by wrapping the silk around ordinary pencil 3 times, slipping coil so formed off end of pencil, and holding it firmly with thumb and index finger of left hand while passing another piece of suture through coil, knotting, and tying securely. Then shear off end of coil and knotted suture to within 2 mm. This should provide overall length of ca 65 mm of suture in 2-loop coil that can be conveniently handled in ordinary aseptic transfer procedure.

Ext loops in groups of 100–200 in Soxhlet extn app., using $CHCl_3$, for 24 h. Air-dry 12–18 h at room temp. in hood. Place 100 loops in 100 mL 0.5N HCl for 10 min or until all loops are completely submerged in soln. Decant, and rinse repeatedly with distd $H_2O$ for 15 min. Check rinse $H_2O$ for absence of HCl, using litmus paper. Air-dry on filter paper mats under ambient conditions or in incubator.

(**g**) *Cylinder carriers.*—"Penicylinders," porcelain, 8±1 mm od, 6±1 mm id, 10±1 mm long. (Available from Fisher Scientific Co., No. 7-907.) Sterilize 2 hr in 180° air oven. Wash used Penicylinders with Triton X-100 and rinse with $H_2O$ 4 times.

### C. Operating Technic

Grow all *Bacilli* in soil ext nutrient broth and all *Clostridia* in soil ext-meat-egg medium. Inoculate 3 tubes, using 1 loop stock culture, and incubate 72 hr at 37°. Place supply of suture loops and cylinder carriers in sep. petri dishes matted with filter paper, and sterilize 20 min at 121°. Use new loops for each test. Penicylinders must be free from chips or cracks. Filter *Cl. sporogenes* thru funnel contg 2 × 5 × 5 cm sq piece of moist cotton or glass wool into sterile 25 × 150 mm test tubes, using same funnel. In prepg *B. subtilis* culture, pour tube of 72 hr culture into tissue grinder and macerate to break up pellicle. Filter thru sterile funnel contg moist cotton or glass wool into sterile 25 × 150 mm tube, repeating operation for other 2 tubes. Place 10 sterile suture loops or Penicylinders into each of 3 tubes contg 10 mL filtrate from 72 hr culture of *Cl. sporogenes,* agitate, and let stand 10–15 min. Using this technic, contaminate 35 loops or cylinders. Place contaminated suture loops and/or cylinders into petri dish matted with 2 layers of filter paper. Drain. Proceed similarly for *B. subtilis*.

Place the 35 suture loops or cylinders contaminated with *Cl. sporogenes* or *B. subtilis* in vac. desiccator contg $CaCl_2$ and draw vac. of 69 cm (27") Hg for 20 min. Dry 24 hr under vac. (Spores dried and held under these conditions will retain resistance ≥7 days.)

Transfer 10 mL 2.5*N* HCl, **966.04A(c)**, into sterile 25 × 150 mm tube. Place tube in 20° const temp. $H_2O$ bath and let come to temp. Rapidly transfer 4 dried, contaminated loop or cylinder carriers to acid tube. Transfer remaining dried, contaminated suture loop or cylinder carriers to tube of thioglycolate subculture medium, **966.04A(a)**(*3*), as viability control. After 2, 5, 10, and 20 min, withdraw individual loops or cylinders from acid and transfer to individual tubes of subculture medium. Rotate each tube vigorously 20 sec and resubtransfer. Incubate 21 days at 37°. Test spores should resist HCl ≥2 min, and many may resist HCl for full 20 min.

When testing sporicidal or sterilizing activity of gas, place carriers in polyethylene bags or in petri dishes with lids ajar. Certain gases may require rehydration of spores before exposure to gas. Rehydrate spores on carriers by 1 hr immersion in $H_2O$, using ≤20 mL $H_2O$/6 carriers. Drain carriers 20 min on petri dishes matted with filter paper. After exposure to gas, remove carriers, using aseptic technic to subculture media as specified in next par.

For aq. sporicides and sterilizers, place 10 mL product at diln recommended for use or under investigation into each of six 25 × 150 mm tubes. Place tubes in 20° $H_2O$ bath and let come to temp. Using flamed forceps, place 5 suture loops or cylinders, contaminated with *Cl. sporogenes* or *B. subtilis* and dried 24 hr under vac., into each of the 6 tubes contg disinfectant, using 2-min intervals for seeding each tube. Five suture loops or cylinders can be placed into each tube within 5 sec. This seeding operation will take 10 min. After contact period specified for disinfectant has been achieved, remove suture loops or cylinders, using sterilized needle hook, from each tube of disinfectant to subculture medium or other subculture medium specified in **955.11A(d)** (select medium contg most suitable neutralizer), placing 1 suture loop or cylinder per tube. Five cylinders can be removed within each 2 min interval. Flame transfer needle hook after each carrier has been transferred to subculture medium. After completing transfer, resubtransfer each suture loop or cylinder to fresh tube of thioglycolate medium and incubate 21 days at 37°. If no growth is observed after 21 days, heat-shock tubes 20 min at 80° and reincubate 72 hr at 37°. Report results as + (growth) or − (no growth) values.

Killing in 59 of 60 replicates on 1 carrier at diln and time specified is considered evidence of sporicidal efficacy against 1 test spore and for confidence level of 95%. Tests with both *B. subtilis* and *Cl. sporogenes*, using 30 replicates with each of 2 carriers specified to provide min. of 120 carriers, are required to presumptively support unqualified sporicidal claim or for presumptive evidence of sterilizing activity at concn, time, and conditions specified. For sporicidal claims, no more than 2 failures can be tolerated in this 120 carrier trial. For sterilizing claims, no failures can be tolerated.

Refs.: JAOAC **36,** 480(1953); **39,** 480(1956); **40,** 759(1957); **49,** 721(1966); **50,** 194(1967); **56,** 308(1973); **61,** 371(1978); **68,** 279(1985).

## 965.12 Tuberculocidal Activity of Disinfectants

**First Action 1965**
**Final Action 1967**
**Repealed First Action 1988**

(Suitable for detg max. tuberculocidal diln of disinfectants used on inanimate surfaces. This method has not been validated for glutaraldehyde-based products)

### *I. Presumptive In Vitro Screening Test Using Mycobacterium smegmatis*

#### *A. Reagents*

(**a**) *Test organism.—Mycobacterium smegmatis* (PRD No. 1) (available from Microbiology Lab., U.S. Environmental Protection Agency, Benefits & Use Div., Bldg 306, BARC-East, Beltsville, MD 20705). Maintain on nutrient agar slants by monthly transfers. Incubate new stock transfer 2 days at 37°; then store at 2–5°. From stock culture inoculate tubes of Proskauer-Beck broth, (**b**)(*1*), incubate 48 hr in slanting position, carry 30 days, using 48 hr transfers, and use these 48 hr cultures to start test cultures. Inoculate 1 or 2 tubes of Proskauer-Beck broth. Incubate 6–7 days at 37°. Incubate tubes 48 hr in slanting position to provide max. surface aeration and then in upright position 4–5 days. Add 1.5 mL sterile 2.0% Bacto-Gelatin soln and homogenize culture with sterilized glass tissue grinder, **966.04B(e)**. Adjust to 20% *T* at 650 nm with sterile Proskauer-Beck broth for use in testing.

(**b**) *Culture media.—*(*1*) *Modified Proskauer-Beck broth.*—Dissolve 2.5 g $KH_2PO_4$, 5.0 g asparagine, 0.6 g $MgSO_4.7H_2O$, 2.5 g Mg citrate, 20.0 mL glycerol, 0.0046 g $FeCl_3$, and 0.001 g $ZnSO_4.7H_2O$ in 1 L $H_2O$. Adjust to pH 7.2–7.4 with 1*N* NaOH. Filter thru paper, place 10 mL portions in sep. 20 × 150 mm tubes, and sterilize 20 min at 121°. Use for propagating 48 hr test starter cultures and 6–7 day test cultures.

(*2*) *Subculture media.*—Use (*1*) with addn of suitable neutzg agents such as purified lecithin (Azolectin) or Na thioglycolate, where necessary.

(*3*) *Nutrient agar.*—Prep. as in **955.11A(c)**. Use to maintain stock culture.

(*4*) *Sterile distilled water.—See* **955.14A(d)**.

#### *B. Apparatus*

(**a**) *Glassware, water bath, transfer loops and needles, and petri dishes.—See* **955.14B(a)**, (**b**), (**c**), and (**e**).

(**b**) *Carriers.—See* **966.04B(g)**.

#### *C. Operating Technic*

Transfer 20 sterile Penicylinder carriers, using flamed nichrome wire hook, into 20 mL 6–7 day homogenized stdzd broth culture, **965.12A(a)**, in sterile 25 × 150 mm medicant tube. After 15 min contact, remove cylinders and place on end in vertical position in sterile petri dish matted with filter paper, **955.14B(e)**. Cover and place in incubator at 37° and let dry ≥20 min but ≤60 min. This will provide dried test carriers in groups of 20 in individual petri dishes. With each group of 20 carriers, add 1 dried cylinder at 30 sec intervals to each of 20 tubes contg 10 mL diln of germicide to be tested (at 20° in $H_2O$ bath). Flame lips of medicant and subculture tubes. Immediately after placing carrier in medicant tube, swirl tube 3 times before placing it back in $H_2O$ bath. (Thus, by time 20 tubes have been seeded, 9 min and 30 sec have elapsed, leaving 30 sec interval prior to subculturing series at 10 min exposure for each carrier. The 30 sec interval between transfers allows adequate time for flaming and cooling transfer hook and making transfer in manner so as to drain all excess medicant from carrier.) Transfer carrier to 10 mL subculture media, **965.12A(b)**(*2*). Shake all subculture tubes thoroly and incubate 12 days at 37°. Report results as + (growth) or − (no growth). Where there is reason to suspect that results may be affected by bacteriostatic action of medicant carried over in subculture tubes, use suitable neutralizer in subculture media.

Make ≥30 carrier exposures at each of 3 relatively widely spaced dilns of germicide under test between no response and total response diln levels. Calc. % of carriers on which organism is killed at each diln. Using log % probit paper (3 cycle logarithmic normal No. Y3 213 HG, Codex Book Co., Inc., 74 Broadway, Norwood, MA 02062), locate % kill points on diln lines employed (log scale). Draw best fitting straight line thru these 3 points and extend to intercept 99% kill line. Read diln line (log scale) at point of intercept. This is presumed 95% confidence end point for product. (Do not use presumptive test organism for checking validity of this presumptive end point.)

### *II. Confirmative In Vitro Test for Determining Tuberculocidal Activity—First Action 1965*

#### *D. Reagents*

(**a**) *Culture media.—*(*1*) *Modified Proskauer-Beck medium.*—Prep. as in **966.12A(b)**(*1*), and in addn, place 20 mL portions in 25 × 150 mm tubes. Use 10 mL portions for daily transfers of test cultures and 20 mL portions for subculturing porcelain cylinders.

(2) *Middlebrook 7H9 Broth Difco A*.—Dissolve 4.7 g in 900 mL $H_2O$ contg 2 mL glycerol and 15.0 g agar. Heat to bp to dissolve completely. Distribute in 180 mL portions and autoclave 15 min at 121°. To each 180 mL sterile medium at 45°, add 20 mL Middlebrook ADC Enrichment (Difco) under aseptic conditions and distribute in 10 mL portions in sterile 20 × 150 mm tubes. Slant. Use to maintain test culture.

(3) *Middlebrook 7H9 Broth Difco B*.—Dissolve 4.7 g in 900 mL $H_2O$ contg 2 mL glycerol and 1.0 g agar. Heat to bp to dissolve completely. Distribute in 18 mL portions in 25 × 150 mm tubes, and autoclave 15 min at 121°. To each 18 mL sterile medium at 45° add 2 mL Middlebrook ADC Enrichment under aseptic conditions. Use to subculture for survival.

(4) *Kirchners Medium Difco*.—Dissolve 13.1 g in 1 L $H_2O$ contg 20 mL glycerol and heat to bp to dissolve completely. Distribute in 18 mL portions in 25 × 150 mm tubes and autoclave 15 min at 121°. If commercial medium is not available, add 5 g asparagine (Difco), 2.5 g Na citrate, 0.6 g $MgSO_4$, 2.5 g monopotassium phosphate, 1.5 g dipotassium phosphate, 1 g Bacto agar in 1 L $H_2O$, and add 20 mL glycerol. To each 18 mL sterile medium at 50–55° add 2 mL Middlebrook ADC Enrichment under aseptic conditions. Use to subculture for survival.

(5) *TB Broth Base Difco (without Polysorbate 80)*.—Dissolve 11.6 g in 1 L $H_2O$ contg 50 mL glycerol and 1.0 g agar. Heat to bp to dissolve completely. Distribute in 18 mL portions in 25 × 150 mm tubes, and autoclave 15 min at 121°. To each 18 mL sterile medium at 50° add 2 mL Dubos Medium Serum (Difco) under aseptic conditions. Use to subculture for survival.

(**b**) *Test organism*.—*Mycobacterium bovis* (BCG) (Bionetics Research, Inc., 115 S Sangamon St, Chicago, IL 60607). Maintain stock cultures on culture medium (**a**)(*2*) by monthly or 6 weeks transfer. Incubate new stock transfer 15–20 days at 37° until sufficient growth is indicated; then store at 2–5°. From stock culture, inoculate tube of culture medium (**a**)(*1*) and incubate 21–25 days at 37°. Allow to remain quiescent until 21–25th day. Make daily transfers from 21 day cultures. Transfer culture to heat-sterilized glass tissue grinder, add 1.0 mL 0.1% Tween 80 in saline soln (Difco), grind, and dil. with culture medium (**a**)(*1*) to give 20% *T* at 650 nm. Use to inoculate porcelain cylinders used in test. Tests will be satisfactory only when organism is killed on all 10 carriers by aq. phenol (1+50) and shows survival after exposure to aq. phenol (1+75) control. Prep. dilns from 5% std phenol soln, **955.11B(f)**.

(**c**) *Sterile distilled water*.—*See* **955.14A(d)**.

(**d**) *Normal horse serum*.—Difco Laboratories.

### E. Apparatus

(**a**) *Glassware, water bath, transfer loops and needles, and petri dishes*.—*See* **955.14B(a)**, (**b**), (**c**), and (**e**).

(**b**) *Carriers*.—*See* **966.04B(g)**.

### F. Operating Technic

Soak ring carriers overnight in 1*N* NaOH; rinse with tap $H_2O$ and then with distd $H_2O$ until distd $H_2O$ is neut. to phthln; then rinse twice with distd $H_2O$. Place clean ring carriers in multiple of 10 or 20 in capped erlenmeyer or 20 × 150 mm tubes. Autoclave 20 min at 121°, cool, and hold at room temp. Transfer 10 sterile ring carriers, using flamed wire hook, into enough (ca 15–20 mL) 21–25 day stdzd test culture, **965.12D(b)**, in 25 × 150 mm medication tube. After 15 min contact period, remove cylinders, using flamed wire hook, and place on end in vertical position in sterile petri dish matted with filter paper, **955.14B(e)**. Cover, place in incubator at 37°, and let dry 30 min.

Let 10 tubes contg 10 mL use-diln germicide sample to be tested come to 20° (or desired temp., if germicide use is recommended for temp. other than room temp.) in $H_2O$ bath and add 1 contaminated cylinder carrier at either 30 sec or 1 min intervals to each tube. (Thus, by time 10 tubes have been seeded, 9 min will have elapsed, plus 1 min interval before transfer of first carrier in series to individual tube of 10 mL neutralizer appropriate for germicide tested, or 10 mL neutralizer blank, **960.09A(d)**, if 1 min intervals are used. This interval is constant for each tube with prescribed exposure period of 10 min. Interval between transfers allows adequate time for flaming and cooling wire hook and making transfer in manner so as to drain all excess medication from carrier.) Transfer carrier to 10 mL neutralizer appropriate for germicide tested, after exactly 10 min contact. Shake tube contg carrier in neutralizer thoroly and place carrier in tube contg 20 mL broth, **965.12D(a)**(*1*). From same tube, take 2 mL portions serum and place in any 2 of the subculture media, **965.12D(a)**(*3*), (*4*), (*5*). Repeat this with each of the 10 carriers. Incubate 1 tube of each subculture medium with 2 mL sterile serum as control. Where there is reason to suspect that germicide to be tested may possess bacteriostatic action, use suitable neutralizer in lieu of serum. Shake each subculture tube thoroly, incubate 60 days at 37°, and report results as + (growth) or − (no growth). If no growth or only occasional growth is observed in subculture, incubate addnl 30 days before making final reading. Max. diln of germicide which kills test organism on the 10 carriers, and no growth in each of the 2 mL aliquots for 2 extra media, represents max. safe use-diln for practical tuberculocidal disinfection.

Refs.: JAOAC **48,** 635(1965); **50,** 767(1967); **53,** 860(1970); **70,** 318(1987).

## 972.04 Bacteriostatic Activity of Laundry Additive Disinfectants

**First Action 1972**

(Applicable to antimicrobial products, recommended for use during laundering operations, which are intended to provide residual bacteriostatic treatment to laundered fabric. Method includes treatment of fabric with product and subsequent bacteriostatic testing of treated fabric.)

### A. Reagents

(**a**) *Culture media*.—(*1*) *Nutrient broth*.—*See* **955.11A(a)**.

(*2*) *Nutrient agar A*.—*See* **955.11A(c)**. Use for monthly transfer of stock cultures.

(*3*) *Nutrient agar B*.—Boil 3 g beef ext, 5 g peptone (Anatone), 8 g NaCl, and 10 g agar (Difco) in 1 L $H_2O$. Transfer 100 mL portions to erlenmeyers, and autoclave 20 min at 121°. Use for agar plate tests to evaluate bacteriostatic activity of treated fabric. *See also* (**c**).

(**b**) *Test organisms*.—Use *Staphylococcus aureus* ATCC No. 6538 and *Klebsiella pneumoniae*, aberrant ATCC No. 4352 (formerly *Escherichia coli*), and maintain as in **955.11B(e)**.

(**c**) *2,3,5-Triphenyl tetrazolium chloride*.—Use as optional biological indicator. With *S. aureus*, use 0.15% soln; with *K. pneumoniae*, aberrant, use 0.25% soln. Autoclave each 20 min at 121°. Apply as in **972.04D**.

(**d**) *Alkaline nonionic wetting agent*.—Prep. aq. soln contg 0.5% alkyl phenol polyglycol ether wetting agent and 0.5% $Na_2CO_3$. Use to scour test fabric.

### B. Apparatus

(a) *Test fabric.*—80 × 80 threads/sq in. plain weave cotton print cloth, completely desized, bleached, and without bluing or optical brighteners. Scour before use by boiling ca 300 g 1 hr in 3 L $H_2O$ contg 1.5 g nonionic wetting agent and 1.5 g $Na_2CO_3$. Then rinse fabric, first in boiling $H_2O$ and then in cold $H_2O$, until all visual traces of wetting agent are removed. Air-dry and cut into long strip 5 cm (2") wide and weighing exactly 15 g.

(b) *Stainless steel spindle.*—Fabricate from single continuous piece of stainless steel wire $^1/_{16}$" diam. and bent to contain 3 horizontal extensions 5 cm (2") long connected by 2 vertical sections ca 5 cm (2") long. Shape so that vertical sections form 150° angle, and sharpen free ends of 2 outer horizontal extensions to point (*see* Fig. **972.04**). Use as carrier for test fabric. Primary objective of spindle is to prevent wadding or lodging of test fabric during agitation in exposures to test chem. solns.

(c) *Exposure chamber.*—Clean, dry 1 pt Mason jar with rubber washer or gasket and metal screw cap.

(d) *Agitator.*—Device to rotate Mason jar thru 360° vertical orbit of 10–20 cm (4–8") diam. at 45–60 rpm for 5 min. Launderometer or Tumble Jar described in AATCC70 B-1967, 43, B154, B155, or ASTM D583-63 is adequate.

(e) *Water bath.*—Thermostatically controlled at 25°.

(f) *Petri dishes.*—Sterile, 100 × 15 mm.

(g) *Glassware.—See* **955.11B(a)**.

(h) *Transfer loops and needles.—See* **955.11B(d)**.

### C. Preparation of Fabric

(a) *Fabric mounting.*—Pierce one end of prescoured, 15 g test fabric strip and secure onto an outer horizontal extension of test spindle; then wind strip around 3 horizontal extensions with enough tension to obtain 12 (but not 13) entire laps. Secure final end of test fabric strip to previous laps with stainless steel safety pin.

(b) *Fabric treatment with product.*—Dil. product as directed to 75 mL (most frequently, use directions are based on dry wt of laundry fabric equiv. to 15 g test fabric), add to Mason jar (exposure chamber), and maintain in $H_2O$ bath at 25°. Add addnl materials to Mason jar as required by use directions for product. These are:

(*1*) *Product recommended as final rinse additive in industrial laundering operation.*—Add no addnl materials; 5:1 (v/w) treatment product soln to dry fabric ratio is representative of industrial laundering operations.

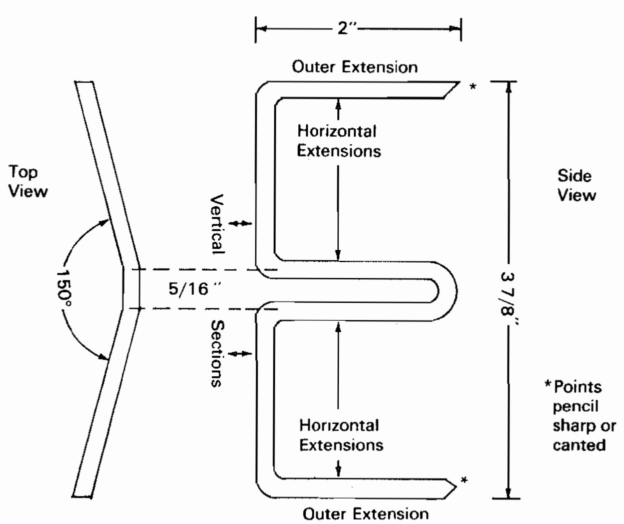


**FIG. 972.04—Stainless steel spindle for winding test fabric**

(*2*) *Product recommended as final rinse additive in home or coin-operated laundering operations.*—Add 150 mL $H_2O$ to Mason jar. Resultant 10:1 (v/w) treatment product soln to dry fabric ratio is representative of home and coin-operated laundering operations.

(*3*) *Product recommended as final rinse additive in both industrial and home laundering operations.*—Prep. 2 jars contg product soln according to (*1*) and (*2*) so that 2 test fabric strips may be treated at different treatment product soln to dry fabric ratios (5:1 and 10:1 (v/w)).

(*4*) *Product recommended as final rinse additive and described as compatible with adjunct chemicals which may be used in this cycle (sours, bleaches, optical brighteners, softeners, etc).*—Prep. so that required vol. of product treatment soln contains adjunct chemicals according to description and amts specified on product label or advertising literature.

### D. Operating Technic

Place test spindle with test fabric in Mason jar contg product soln. Secure rubber gasket and Mason jar cap, remove from $H_2O$ bath, place jar in agitator, and rotate 5 min. Addnl manipulation with test spindle is required if use directions do not specify addn of product in final rinse phase of laundry cycle. In this instance, to det. durability of antimicrobial agent in fabric, execute 3 rinse operations as follows: Immediately after end of initial 5 min agitation, drain treatment soln from Mason jar and replace with 100 mL $H_2O$. Secure Mason jar contg test spindle, return to agitator, and rotate 2 min. Repeat operation twice more.

Following all required fabric treatment operations, remove test spindle from Mason jar and unwind test fabric strip from spindle. Let test fabric strip air dry with long axis of strip in horizontal position.

When test strip is dry, remove 1 sq in. bacteriological test samples. Five test samples are required for single bacteriostatic test against 1 test organism. In each such instance, at least 2 test samples must be removed from middle 20% of length of test strip.

Perform bacteriostatic agar plate tests as follows: Prep. 5 replicate plates in each test for each organism. Sep. inoculate flasks contg 100 mL sterile, liq. (≤40°) nutrient agar B with 1 mL 24 hr nutrient broth culture of *S. aureus* and *K. pneumoniae,* aberrant. Immediately thereafter, if desired, add 1 mL appropriate soln of 2,3,5-triphenyl tetrazolium chloride to inoculated nutrient agar B. Vigorously swirl contents of erlenmeyers to ensure complete mixing. Add 10 mL portions of inoculated agar to 100 mm sterile petri dishes, distribute evenly, and let cool and harden. As soon as plates harden, implant single 1 sq in. treated fabric test sample on center of 1 test agar plate surface. Using blunt forceps, press each fabric test sample onto agar surface to ensure complete and uniform contact. Incubate test plates 48 hr at 37°. If desired, test plates may be refrigerated 18–20 hr before incubation. Following incubation, examine test plates to det. presence or absence of zones of inhibition along each side of test fabric sample.

### E. Interpretation

Use clear zone of inhibition adjacent to each side of test fabric sample as index of bacteriostatic activity. Size of zone is not considered important, but zone is required to extend along entire edge to be acceptable. Score zone of inhibition along single side of sq test fabric samples as 1, so that for 5-replicate plate test, a score of 20 shows that bacteriostasis occurs along all 4 sides of each sample. Total score of 18/20 sides demonstrating bacteriostasis is required for effective demonstration of residual bacteriostatic activity of laundry fabric treated with antimicrobial laundry additive product during laundering operation. Unless qualified residual bacteriostatic claim is made, residual treatment must be bacteriostatic against both *S. aureus* and *K. pneumoniae*, aberrant.

Ref.: JAOAC **52,** 836(1969).

## 965.13 Disinfectants (Water) for Swimming Pools

**First Action 1965**
**Final Action 1970**

(Suitable for presumptive evidence of acceptability of products for disinfecting swimming pool water)

### *A. Test Culture Media*

(**a**) *Nutrient Agar A.—See* **960.09A(a)**(*1*).
(**b**) *Nutrient Agar B* (Trypticase Soy Agar, BBL).*—See* **965.13B(b)**.
(**c**) *Nutrient Agar C.*—Prep. as in **955.11A(c)**.

### *B. Subculture Media*

(**a**) *Tryptone glucose extract agar* (*Difco*).—Dissolve 24 g in 1 L freshly distd $H_2O$ and heat to bp to dissolve completely. Autoclave 15 min at 121°. Use for plate counts of *E. coli* survivors.
(**b**) *Trypticase soy agar* (*BBL*).—Suspend 40 g powder in 1 L $H_2O$. Let stand 5 min and mix thoroly. Heat gently with occasional agitation and boil ca 1 min or until soln is complete. Autoclave 15 min at 121°. Let cool and reautoclave 15 min at 121°. Use for plate counts of *S. faecalis* survivors.
(**c**) *Fluid thioglycolate medium* (*Difco*).*—See* **955.11A(d)**(*2*).
(**d**) *Lactose broth* (*Difco*).—Dissolve 19 g in 1 L $H_2O$. Dispense 10 mL portions into tubes with fermentation vials. Autoclave 15 min at 121°. Use for detg presence of *E. coli* survivors.
(**e**) *Eosin methylene blue agar* (*Difco*).—Suspend 36 g in 1 L $H_2O$ and heat to bp to dissolve completely. Autoclave 15 min at 121°. Use for confirming *E. coli* survivors.
(**f**) *S-F agar* (*Difco*).—Dissolve 36 g in 1 L $H_2O$. Add 15 g agar and heat to bp to dissolve completely. Autoclave 15 min at 121°. Use for confirming *S. faecalis* survivors.

### *C. Neutralizer Stock Solns*

(**a**) *Sodium thiosulfate soln.*—Dissolve 1 g $Na_2S_2O_3$ in 1 L $H_2O$. Dispense in 100 mL portions and autoclave 20 min at 15 lb.
(**b**) *Azolectin soln.—See* **960.09A(c)**.
(**c**) *Other preparations.*—Prepns found to be suitable and necessary, depending upon nature of germicidal prepns to be tested.

### *D. Neutralizer Blanks*

(**a**) *With 0.6 ppm residual chlorine or less.*—Dil. 10 mL neutralizer stock soln, **965.13C(a)**, with 90 mL sterile $H_2O$. Dispense aseptically in 9 mL portions into sterile 25 × 150 mm tubes.
(**b**) *With quaternary ammonium compounds and phenolic derivatives.*—Mix 10 mL neutralizer stock soln, **965.13C(b)**, 2.5 mL 0.25*M* phosphate buffer stock soln, **965.13E(a)**, and 167.5 mL $H_2O$. Dispense in 9 mL portions into 20 × 150 mm tubes. Autoclave 20 min at 121°.
(**c**) *Other preparations.*—Use dilns of **965.13C(c)** as suitable.

### *E. Reagents*

(**a**) *Phosphate buffer stock soln.*—0.25*M. See* **960.09A(e)**.
(**b**) *Phosphate buffer dilution water.—See* **960.09A(f)**.
(**c**) *Sodium thiosulfate std solns.*—(*1*) *0.1N.* Dissolve exactly 24.820 g $Na_2S_2O_3.5H_2O$ in $H_2O$ and dil. to 1 L. Stdze as in **942.27B**.
(*2*) *0.001N.*—Dil. 10 mL soln (*1*) to 1 L with $H_2O$.
(**d**) *Starch indicator soln.*—Mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring constantly, and immediately discontinue heating. Add a few drops of $CHCl_3$ as preservative.
(**e**) *Sterile phosphate buffer stock solns.*—(*1*) Dissolve 11.61 g anhyd. $K_2HPO_4$ in 1 L $H_2O$ and autoclave 20 min at 121°. (*2*) Dissolve 9.08 g anhyd. $KH_2PO_4$ in 1 L $H_2O$ and autoclave 20 min at 121°.
(**f**) *NaOCl stock soln.*—Approx. 5%. Store NaOCl stock soln in tightly closed bottle in refrigerator and det. exact available Cl at frequent intervals by $As_2O_3$ titrn, **955.16A(c)**.
(**g**) *Test organism.*—Use *Escherichia coli* ATCC 11229 and *Streptococcus faecalis* ATCC No. 6569 (American Type Cultures Collection, 12301 Parklawn Dr, Rockville, MD 20852). Maintain, by monthly transfer, stock cultures of *E. coli* on *Nutrient Agar C*, **955.11A(c)**, and *S. faecalis* on *Nutrient Agar B*, **965.13B(b)**; store at 4–5°.

### *F. Apparatus*

(**a**) *Glassware.*—500 mL wide-mouth erlenmeyers; 100 mL graduates; Mohr pipets; milk pipets; 20 × 150 mm tubes; Board of Health tubes (6mm × 50mm); 200, 500, and 1000 mL vol. flasks. Wash in strong, fresh chromic acid cleaning soln, and fill and drain with $H_2O$ ≥3 times. Heat ≥2 hr at 180° in hot air oven.
(**b**) *Petri dishes.*—Sterile.
(**c**) *Water bath.*—Controlled at 20 or 25°.

### *G. Preparation of Culture Suspension*

From stock culture, inoculate tube Nutrient Agar A for *E. coli* and Nutrient Agar B for *S. faecalis;* make ≥3 consecutive daily transfers (≤30), incubating transfer 20–24 hr at 35–37°. Do not transfer >30 days. If only 1 daily transfer has been missed, no special procedures are required; if 2 daily transfers are missed, repeat with 3 daily transfers. Remove culture from agar surface, using 5 mL phosphate buffer diln $H_2O$, **965.13E(b)**. Transfer culture suspension to sterile centrf. tube and centrf. 1–2 min at speed necessary to settle agar particles. Transfer supernate to another sterile centrf. tube and centrf. to obtain complete sepn of cells. Discard supernate and resuspend cells in 5 mL buffer diln $H_2O$. With *S. faecalis*, centrf., discard supernate, and resuspend cells in 5 mL buffer diln $H_2O$ 2 addnl times. Finally, stdze suspension to give av. of $2.0 \times 10^8$ organisms/mL by diln with sterile phosphate diln $H_2O$.

If Lumetron is used, dil. suspension in sterile Lumetron tube to give % *T* according to Table **965.13**. Make serial diln plate count of each culture suspension before use, using phosphate buffer diln $H_2O$, **965.13E(b)**, and subculture medium, **965.13B(a)**, with *E. coli*, and (**b**) with *S. faecalis*. Incubate diln plates in inverted position 48 hr at 35–37°. Use Quebec Colony Counting Chamber and report results in terms of number of bacteria/mL suspension. Count of $2.0 \times 10^8$ is desired so that 1 mL test culture suspension + 199 mL test soln will provide soln contg $1 \times 10^6$ organisms/mL. Permitted variation

**Table 965.13 Percent Light Transmission at Various Wavelengths Corresponding to Bacterial Concentrations**

| | % Light Transmission with Filter, nm | | | | | | Av. Bacterial Count/mL |
|---|---|---|---|---|---|---|---|
| Bacteria | 370 | 420 | 490 | 530 | 580 | 650 | |
| *E. coli* | 90 | 88 | 89 | 88 | 91 | 92 | $2.0 \times 10^8$ |
| *S. faecalis* | 86 | 82 | 85 | 85 | 87 | 89 | $2.0 \times 10^8$ |

in test culture suspension is +500,000 and −100,000/mL of 200 mL test soln. Use actual count for calcg zero time count in later tests.

### *H. Determining Chlorine Demand of Freshly Distilled Test Water*

Place 200 mL $H_2O$ in each of five 500 mL erlenmeyers. To flasks 1–5, resp., add 0.025, 0.05, 0.075, 0.1, and 0.15 mL of 200 ppm available Cl prepd from NaOCl soln, **965.13E(f)**. Shake each flask, and let stand several min. Add crystal KI and 1 mL HOAc, and swirl. Add 1 mL starch soln, **965.13E(d)**. Flask showing perceptible blue indicates Cl demand has been satisfied.

### *I. Operating Technic*

Place ca 600 mL freshly sterilized distd $H_2O$ in 1 L vol. flask. Add ca 1.5–3.0 mL $K_2HPO_4$ buffer, **965.13E(e)**(*1*), and 0.5 mL $KH_2PO_4$, **965.13E(e)**(*2*), and dil. to 900 mL. Add enough NaOCl from suitable diluent of std stock soln, **965.13E(f)**, to satisfy Cl demand of 1 L test $H_2O$, **965.13H**, and to provide ca 0.6 ppm residual available Cl. Dil. to vol. (Example: If Cl demand of $H_2O$ is 0.1 ppm, add 3.5 mL of 200 ppm soln of available Cl made from std stock NaOCl soln, **965.13E(f)**, and dil. to vol. This should provide soln with ca 0.6 ppm residual available Cl at pH 7.5±0.1.) Transfer 199 mL of this test soln to each of three 500 mL erlenmeyers and place in $H_2O$ bath at either 20 or 25°. Let come to temp.

To first flask, add 1 mL boiled distd $H_2O$ and det. residual available Cl as follows: Add small crystal KI and 1 mL HOAc; then add 1 mL starch soln, **965.13E(d)**. Blue soln indicates presence of Cl. Titr. with 0.001*N* $Na_2S_2O_3$, **965.13E(c)**(*2*), until color disappears; mL 0.001*N* $Na_2S_2O_3 \times 0.1773$ = ppm residual available Cl. This represents available Cl at 0 time in test. Result should be ≥0.58 but ≤0.62.

To each of remaining flasks add 1 mL test culture suspension, **965.13G**, as follows: Swirl flask, stopping just before suspension is added, to create enough centrifugal motion to prevent pooling of suspension at point of contact with test $H_2O$. Add suspension midway between center and edge of liq. surface, immersing tip of pipet slightly below surface of $H_2O$. Avoid touching pipet to neck or side of test flask during operation.

From one of these 2 flasks transfer 1 mL aliquots to neutralizer blanks, **965.13D(a)**, after intervals of 0.5, 1, 2, 3, 4, 5, and 10 min. Shake neutralizer blank thoroly immediately after adding sample. Prep. serial diln plate counts from neutralizer blanks, using phosphate buffer diln $H_2O$, **965.13E(b)**, and subculture medium, **965.13B(a)** for *E. coli,* and **(b)** for *S. faecalis*.

After prepg diln plate counts, inoculate 5 lactose broth tubes, **965.13B(d)**, with 1.0 mL aliquots from each neutralizer blank tube for each time interval when *E. coli* is used as the test organism, and 5 thioglycolate broth tubes, **965.13B(c)**, with 1.0 mL aliquots from each neutralizer blank tube for each time interval when *S. faecalis* is test organism.

Incubate all diln plates in inverted position and subculture tubes 48 hr at 37°. Use Quebec Colony Counting Chamber in reading diln plates and report results in terms of number of surviving bacteria/mL test $H_2O$. Absence of colony growth on diln plates and absence of growth in all 5 lactose or thioglycolate tubes, as case may be, is necessary to show complete kill of test organism.

Immediately after transferring 10 min interval sample from second flask to neutralizer blank tube, remove third flask from $H_2O$ bath and det. residual available Cl exactly as specified for first flask. Results should represent residual available Cl present at 10 min exposure interval. To be acceptable, concn of available Cl in this flask should be >0.4 ppm. Results in Cl control test described above should show complete kill of *E. coli* and *S. faecalis* within 0.5 min.

With unknown sample, prep. 2 flasks contg 199 mL each of soln at concn recommended or to be studied, using Cl demand-free, unbuffered, freshly distd $H_2O$ previously prepd in 1 L vol. flask where Cl demand, as detd above, has been satisfied by addn of NaOCl soln. Place flasks in $H_2O$ bath at 20 or 25°; let come to temp. Inoculate 1 flask with 1 mL std test culture suspension of *E. coli* and other with 1 mL std test culture suspension of *S. faecalis*. Subculture at exactly same time intervals and in same manner used with NaOCl control except vary composition of neutralizer blank depending upon nature of chem. or mixt. of chems under investigation. For example, mixt. of Cl-contg chem. and quaternary $NH_4$ compd would require special neutralizer blank prepd by using both neutralizer stock solns, **965.13C(a)** and **(b)**.

Where no concn of chem. under study has been recommended and objective of study is to det. concn of unknown necessary to provide result equiv. to that obtained with Cl control std, use series of three or four 500 mL flasks contg 199 mL of various concns of chem. and 1 mL stdzd culture suspension with each test organism. Report results as log (number of survivors) at each time interval both for Cl controls and various concns of unknown under test.

Lowest concn of unknown germicide or germicidal mixt. providing results equiv. to those obtained with NaOCl as Cl std is considered lowest concn which could be expected to provide acceptable disinfecting activity in swimming pool water.

Refs.: JAOAC **47,** 540(1964); **48,** 640(1965).

# 7. Pesticide Formulations


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(Chemical names for pesticides mentioned in this chapter are given at end of chapter.)

(Pesticide ref. stds may be available from the following: Alltech Associates, 2051 Waukegan Rd, Deerfield, IL 60015; Analabs, Inc.; Applied Science; Chem Service Inc., PO Box 194, West Chester, PA 19380; RFR Corp., 1 Main St, Hope, RI 02831; and Supelco.)

## GENERAL METHODS

### 935.06 Sampling of Pesticide Formulations
**Procedure**

(*Caution:* See safety notes on pesticides.)

Examine shipping cases closely for code numbers, different labels, and other pertinent information. Give special attention to products subject to deterioration.

*Caution:* Use care in sampling and transporting toxic materials to avoid personal injury and contamination of transportation facilities in case of breakage. Avoid mutual contamination with other products during transportation.

Mark each sample container according to laboratory requirements.

(**a**) *Small package retail units.*—Take one unopened unit (1 lb if dry, 1 pt if liq.), except take min. of 2 units of small baits in cake form. Size of sample is governed by composition of material and anal. methods.

(**b**) *Large package dry products (25 lb or more).*— Sample unopened containers, using trier long enough to reach bottom of container by inserting into container at one edge or corner and probing diagonally toward opposite edge or corner. Take cores by code or batch number. Analyze cores from same code or batch number as composite or individually. Clean trier thoroly after sampling each batch.

Store samples in air-tight glass, metal, plastic, or cardboard containers.

(**c**) *Large package liquid products (5 gallons or more).*—Use glass, plastic tubing, or stainless steel trier with plunger, or rubber tubing for certain materials. Store samples in glass or containers of other noncorrosive material with screw top caps lined with Teflon or other inert material. Plastic containers may be used only for carefully selected products.

### 984.03 Fertilizer-Pesticide Formulation Mixtures
**Sampling Methods**
**First Action 1984**

*See* **929.01** and **969.01**.

### 920.11 Pesticide Formulations
**Preparation of Sample**
**Final Action**

Thoroly mix all samples before analysis. Det. $H_2O$-sol. As on samples as received, without further pulverization or drying. In case of lye, NaCN, or KCN, weigh large amts in weighing bottles and analyze aliquots of their aq. solns.

### 920.12 Moisture in Pesticide Formulations
**Final Action**

(Applicable to Paris green, powd Pb arsenate, Ca arsenate, Mg arsenate, Zn arsenite, powd Bordeaux mixt., and Bordeaux mixt. with arsenicals)

Dry 2 g to const wt at 105–110° and report loss in wt as moisture.

### 922.03 Arsenic (Total) in Pesticide Formulations
**Hydrazine Sulfate Distillation Method**
**Final Action**

(Nitrates do not interfere. Applicable to detn of total As in Paris green, Pb arsenate, Ca arsenate, Zn arsenite, Mg arsenate, and Bordeaux mixt. with arsenicals)

#### *A. Reagents*

(**a**) *Arsenious oxide std soln.*—0.1 or 0.05*N*. *See* **939.12**.

(**b**) *Iodine std soln.*—0.1 or 0.05*N*. *See* **939.13**.

(**c**) *Bromate std soln.*—0.1 or 0.05*N*. Dissolve ca 2.8 or 1.4 g $KBrO_3$ in boiled $H_2O$ and dil. to 1 L. Stdze as follows: Pipet 25 mL aliquots $As_2O_3$ soln, (**a**), into 500 mL erlenmeyers. Add 15 mL HCl, dil. to 100 mL, heat to 90°, and titr. with the $KBrO_3$ soln, using 10 drops Me orange, (**g**). Do not add indicator until near end of titrn, and agitate soln continuously to avoid local excess of $KBrO_3$ soln. Add $KBrO_3$ soln very slowly near end point; at end point soln changes from red to colorless.

(**d**) *Hydrazine sulfate-sodium bromide soln.*—Dissolve 20 g $N_2H_4.H_2SO_4$ and 20 g NaBr in 1 L HCl (1+4).

(**e**) *Sodium hydroxide soln.*—Dissolve 400 g NaOH in $H_2O$ and dil. to 1 L.

(**f**) *Starch indicator.*—Mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring constantly, and immediately discontinue heating. Add ca 1 mL Hg, shake, and let soln stand over the Hg.

(**g**) *Methyl orange indicator.*—0.05%. Dissolve 0.5 g Me orange in $H_2O$ and dil. to 1 L.

### *B. Apparatus*

*See* Fig. **922.03**. Set 500 mL distn flask on metal gauze that fits over circular hole in heavy sheet of asbestos board, which in turn extends out far enough to protect sides of flask from direct flame of burner. First receiving flask holds 500 mL and contains 40 mL $H_2O$; second holds 500 mL and contains 100 mL $H_2O$. Vol. in first flask should be ≤40 mL, otherwise compd of As may sep. that is difficult to dissolve without danger of loss of $AsCl_3$. Keep both flasks cool by placing in pan of circulating $H_2O$, or contg $H_2O$ and ice.

### *C. Determination*

(*Caution*: See safety notes on pesticides and arsenic trioxide.)

Weigh sample contg ≤0.4 g As and transfer to distg flask. Add 50 mL $N_2H_4.H_2SO_4$-NaBr soln, close flask with stopper that carries funnel tube, and connect side tube with condenser. Boil 2–3 min, add 100 mL HCl from dropping funnel, and distil until vol. in distg flask is reduced to ca 40 mL; add 50 mL more HCl and continue distn until vol. is again reduced to ca 40 mL. Wash down condenser, transfer contents of receiving flasks to 1 L vol. flask, dil. to vol., mix thoroly, and proceed as in (**a**) or (**b**):

(**a**) Pipet 200 mL aliquot into erlenmeyer and nearly neutze with NaOH soln, using few drops phthln, and keeping soln well cooled. If neut. point is passed, add HCl until again slightly acid. Neutze with $NaHCO_3$, add 4–5 g excess, and add std I soln from buret, shaking flask continuously until yellow color disappears slowly from soln. Add 5 mL starch indicator and keep adding std I soln dropwise to permanent blue.

(**b**) Pipet 200 mL aliquot into erlenmeyer and titr. with $KBrO_3$ soln, (**c**), beginning ". . . heat to 90° . . ."

Calc. % As. Report as $As_2O_3$ or $As_2O_5$, according to whether As is present in trivalent or pentavalent form. If condition of arsenic is unknown, report as As.

Refs.: Ind. Eng. Chem. **14,** 207(1922). JAOAC **5,** 33, 402(1922); **6,** 313(1923); **48,** 564(1965).

CAS-7440-38-2 (arsenic)

## 924.04 Arsenic (Total) in Pesticide Formulations
### Iodometric Method
### Final Action

(Applicable in presence of sulfides, sulfites, thiosulfates, and large amts of S or org. matter)

### *A. Reagent*

*Sodium thiosulfate soln.*—Dissolve 13 g crystd $Na_2S_2O_3.5H_2O$ in $H_2O$ and dil. to 1 L.

*See* **922.03A** for other reagents and solns and **922.03B** for app.

### *B. Determination*

(*Caution:* See safety notes on pesticides and arsenic trioxides.)

Weigh sample contg ≤0.4 g As and transfer to distg flask. Add 50 mL $N_2H_4.H_2SO_4$-NaBr soln, **922.03A(d)**, and distil as in **922.03C**. Dil. distillate to vol. in 1 L vol. flask, mix thoroly, and transfer 200 mL aliquot to 400 mL Pyrex beaker or porcelain casserole. Add 10 mL $HNO_3$ and 5 mL $H_2SO_4$, evap. to sirupy consistency on steam bath, and then heat on hot plate to white fumes of $H_2SO_4$. Cool, and wash into 500 mL erlenmeyer. If vol. $H_2SO_4$ is appreciably lessened by fuming, add enough $H_2SO_4$ to make total vol. ca 5 mL. Dil. to 100–150 mL, add 1.5 g KI, and boil until vol. is reduced to ca 40 mL. Cool under running $H_2O$, dil. to 100–150 mL, and add $Na_2S_2O_3$ soln, **924.04A**, dropwise until I color just disappears. Nearly neutze $H_2SO_4$ with NaOH soln, **922.03A(e)**, finish neutzn with $NaHCO_3$, add 4–5 g excess, and titr. with std I soln as in

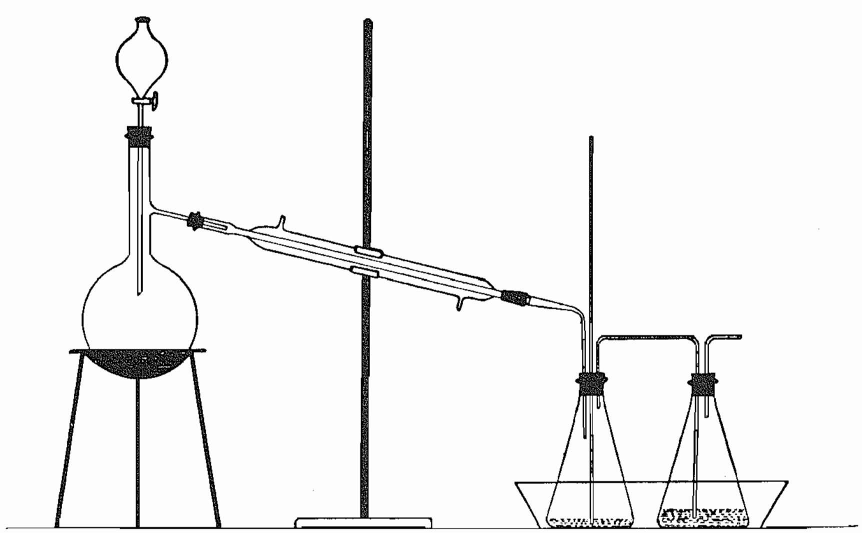

**FIG. 922.03—Apparatus for distilling arsenious chloride**

**922.03C(a)**. From mL std soln used, calc. % As in sample. Report as $As_2O_3$, $As_2O_5$, or As as in **922.03C**.

Ref.: JAOAC **7,** 313(1924).

CAS-7440-38-2 (arsenic)

## 963.06 Arsenic (Total) in Pesticide Formulations

### Ion Exchange Method

**First Action 1963**
**Final Action 1968**

(Applicable to inorg. arsenates and arsenites)

### A. Apparatus

*Ion exchange column.*—Use Allihn filter tube 10 × 2.7 cm od with coarse filter disk. Attach piece of rubber tubing to bottom of filter tube and regulate flow with Hoffman clamp. To tube add aq. slurry of Dowex 50W-X8, 50–100 mesh, using resin bed vol. of 12 mL, and place 500 mL separator above tube.

Regenerate resin bed before each run by first back-washing column few min with $H_2O$; then elute with 350 mL 2*N* HCl followed by 200 mL $H_2O$ at 20 mL/min.

### B. Preparation of Sample

(*Caution:* See safety notes on pesticides.)

Weigh 200 mg sample (100 mg if As content is >30%) into 150 mL beaker, add 7 mL $HNO_3$, and bring to bp. Add 3 mL 2*N* $KBrO_3$ and evap. to dryness, avoiding spattering. Backwash and regenerate resin during this evapn. Dissolve cooled residue in 2 mL 6*N* HCl without heat and add 8 mL $H_2O$. Filter into separator, and wash filter with three 10 mL portions $H_2O$. (If residue dissolves completely in 2 mL 6*N* HCl, omit filtration, and dil. directly to 40 mL.) Pass soln thru resin column at 20 mL/min and collect eluate in 250 mL erlenmeyer. Wash separator and column with 20 and 40 mL portions $H_2O$ into same erlenmeyer.

### C. Determination

Add 50 mL HCl to eluate to make 4*N*. Add 1 g $NaHCO_3$, 0.2 g at time, swirling constantly. Add 1 g KI, stopper, and swirl until all KI dissolves. After 5 min, titr., without starch indicator, with 0.05*N* $Na_2S_2O_3$, **942.27**, to disappearance of I. (Recognition of end point is facilitated by titrg on porcelain stand. In presence of starch, reaction between I and $Na_2S_2O_3$ is retarded, so appreciable amt of $Na_2S_2O_3$ reacts with acid. End point becomes indistinct if >30 mL $Na_2S_2O_3$ is used in titrn.) 1 mL 0.05*N* $Na_2S_2O_3$ = 1.873 mg As.

Refs.: Anal. Chem. **22,** 1066(1950). JAOAC **46,** 672(1963).

CAS-7440-38-2 (arsenic)

## 925.02 Arsenic (Water-Soluble) in Pesticide Formulations

### Titrimetric Method

**Final Action**

(Applicable to detn of $H_2O$-sol. arsenic in Pb arsenate, Ca arsenate, Zn arsenite, Mg arsenate, and Bordeaux mixt. with arsenicals)

(*Caution:* See safety notes on pesticides.)

To 2 g original sample if powder, or 4 g if paste, in 1 L Florence flask, add 1 L recently boiled $H_2O$ that has been cooled to 32°. Stopper flask and place in constant temp. $H_2O$ bath at 32°. Digest 24 hr, shaking hourly 8 hr during this period. Filter thru dry filter. If filtrate is not clear, refilter thru buchner contg paper and enough Filter-Cel coating to give clear soln. Discard first 50 mL.

Transfer 250–500 mL *clear* filtrate to erlenmeyer, add 3 mL $H_2SO_4$, and evap. to ca 100 mL on hot plate. Add 1 g KI, and continue boiling until vol. is ca 40 mL. Cool, dil. to ca 200 mL, and add $Na_2S_2O_3$ soln, **924.04A**, dropwise, until I color is exactly removed. (Avoid use of starch indicator at this point.) Neutze with $NaHCO_3$, add 4–5 g excess, titr. with std I soln, shaking flask continuously, until yellow disappears slowly, add 5 mL starch indicator, **922.03A(f)**, and continue titrn to permanent blue. Correct for amt std I soln necessary to produce same color, using same reagents and vol. From mL std I soln used, calc. % $H_2O$-sol. As in sample.

CAS-7440-38-2 (arsenic)

## 922.04 Lead in Pesticide Formulations

### Gravimetric Method

**Final Action**

(Applicable to such prepns as Bordeaux-Pb arsenate, Bordeaux-Zn arsenite, Bordeaux-Paris green, and Bordeaux-Ca arsenate)

(*Caution:* See safety notes on nitric acid, fuming acids, pesticides, hydrogen sulfide, and arsenic trioxide.)

Weigh 1 g powd sample and transfer to beaker. Add 5 mL *HBr* (ca 1.38 sp gr) and 15 mL HCl, and evap. to dryness to remove As. Repeat treatment; add 20 mL HCl, and again evap. to dryness. Add 25 mL 2*N* HCl to residue, heat to bp, filter immediately to remove $SiO_2$, and wash with boiling $H_2O$ to vol. of 125 mL. See that all $PbCl_2$ is in soln before filtering; if it will not dissolve completely in 25 mL 2*N* acid, add 25 mL more and dil. filtrate to 250 mL. Pass in $H_2S$ until pptn is complete. Filter, and wash ppt thoroly with 0.5*N* HCl satd with $H_2S$. Save filtrate and washings for Zn detn.

Transfer paper with sulfides of Pb and Cu to 400 mL Pyrex beaker and completely oxidize all org. matter by heating on steam bath with 4 mL $H_2SO_4$ and ca 20 mL *fuming* $HNO_3$ in covered beaker. Evap. on steam bath, and then completely remove $HNO_3$ by heating on hot plate to copious white fumes of $H_2SO_4$. Cool, add 2–3 mL $H_2O$, and again heat to fuming. Cool, add 50 mL $H_2O$ and 100 mL alcohol, and let stand several hr (preferably overnight). Filter thru gooch, previously washed with $H_2O$, then with *acidified alcohol* (100 parts $H_2O$, 200 parts alcohol, and 3 parts $H_2SO_4$), and finally with alcohol, and dried at 200°. Wash ppt of $PbSO_4$ in crucible ca 10 times with acidified alcohol, and then with alcohol, to remove $H_2SO_4$. Retain filtrate and washings for Cu detn, if desired.

Dry at 200° to const wt, keeping crucible covered to prevent loss from spattering. From wt $PbSO_4$, calc. % Pb in sample, using factor 0.6832.

Ref.: JAOAC **5,** 398(1922).

CAS-7439-92-1 (lead)

## 922.05 Copper in Pesticide Formulations

**Final Action**

(Applicable to such prepns as Bordeaux-Pb arsenate, Bordeaux-Zn arsenite, Bordeaux-Paris green, and Bordeaux-Ca arsenate)

### *A. Electrolytic Method*

Evap. filtrate and washings from $PbSO_4$ pptn, **922.04**, to fuming; add few mL *fuming $HNO_3$* to destroy org. matter, and continue evapn to ca 3 mL. Take up with ca 150 mL $H_2O$, add 5 mL $HNO_3$, and filter if necessary. Wash into 250 mL beaker, adjust vol. to 200 mL, and electrolyze, using rotating anode and weighed gauze cathode with current of 2–3 amp. After all Cu has apparently deposited (ca 30 min), add 15–20 mL $H_2O$ to electrolyte and continue electrolysis few min. If no further deposition occurs on newly exposed surface of electrode, wash with $H_2O$ without breaking current either by siphoning or quickly replacing beaker with electrolyte successively with 2 beakers of $H_2O$. Interrupt current, rinse cathode with alcohol, dry few moments in oven, and weigh. Calc. % Cu in sample.

### *B. Volumetric Thiosulfate Method*

Proceed as in **922.05A** to point at which filtrate and washings from $PbSO_4$ pptn are treated with fuming $HNO_3$ and evapd to vol. of ca 3 mL. Take up in ca 50 mL $H_2O$, add $NH_4OH$ in excess, and boil to expel excess $NH_3$, as shown by color change in liq. and partial pptn. Add 3–4 mL HOAc (4+1), boil 1–2 min, cool, add 10 mL 30% KI soln, and titr. with std $Na_2S_2O_3$ soln until brown color becomes faint. Add starch indicator, **922.03A(f)**, and continue titrn cautiously until blue color due to free I entirely disappears. From mL std $Na_2S_2O_3$ soln used, calc. % Cu in sample.

*Thiosulfate std soln.*—Prep. soln contg 39 g $Na_2S_2O_3.5H_2O$/L. Accurately weigh 0.2–0.4 g pure electrolytic Cu and transfer to 250 mL erlenmeyer roughly marked at 20 mL intervals. Dissolve Cu in 5 mL $HNO_3$ (1 + 1), dil. to 20 or 30 mL, boil to expel red fumes, add slight excess satd Br-$H_2O$, and boil until Br is completely removed. Cool, and add 10 mL NaOAc soln (574 g trihydrate/L). Prep. 42 g/100 mL KI soln made very slightly alk. to avoid formation and oxidn of HI. Add 10 mL of the KI soln and titr. with $Na_2S_2O_3$ soln to light yellow. Add enough starch indicator, **922.03A(f)**, to produce marked blue. As end point nears, add 2 g KSCN and stir until completely dissolved. Continue titrn until ppt is perfectly white. 1 mL $Na_2S_2O_3$ soln = ca 10 mg Cu.

It is essential for $Na_2S_2O_3$ titrn that concn of KI in soln be carefully regulated. If soln contains <320 mg Cu, at completion of titrn 4.2–5 g KI should have been added for each 100 mL total soln. If greater amts of Cu are present, add KL soln slowly from buret with const agitation in amts proportionately greater.

Ref.: JAOAC **5**, 398(1922).

CAS-7440-50-8 (copper)

## 918.01 Zinc in Pesticide Formulations
### Gravimetric Method
### Final Action

(Applicable to such prepns as Bordeaux-Pb arsenate, Zn arsenite, Bordeaux-Zn arsenite, Bordeaux-Paris green, and Bordeaux-Ca arsenate)

### *A. Reagent*

*Mercury-thiocyanate soln.*—(*Caution:* See safety notes on mercury salts.) Dissolve 27 g $HgCl_2$ and 30 g $NH_4SCN$ in $H_2O$ and dil. to 1 L.

### *B. Determination*

Conc. filtrate and washings from sulfide pptn, **922.04**, by gentle boiling to ca 50 mL; then evap. on steam bath to dryness. Dissolve residue in 100 mL $H_2O$ contg 5 mL HCl, and add 35–40 mL Hg-thiocyanate soln with vigorous stirring. Let stand ≥1 hr with occasional stirring. Filter thru weighed gooch, wash with $H_2O$ contg 20 mL Hg-thiocyanate soln/L, and dry to const wt at 105°. Calc. to % Zn, using factor 0.1312.

*Note:* Some Fe is usually present and during Zn detn should be in ferrous condition. In pptg sulfides pass $H_2S$ into soln long enough to reduce Fe as well as to ppt Cu and Pb. $ZnHg(SCN)_4$ ppt normally is white, and occluded $Fe(SCN)_3$ should not give more than faint pink color.

Refs.: J. Am. Chem. Soc. **40,** 1036(1918). JAOAC **5,** 398(1922).

CAS-7440-66-6 (zinc)

## 929.04 Fluorine (Total) in Pesticide Formulations
### Lead Chlorofluoride Method
### Final Action

### *A. Reagents*

(**a**) *Fusion mixture.*—Mix 30 g anhyd. $Na_2CO_3$ with 40 g anhyd. $K_2CO_3$.

(**b**) *Lead chlorofluoride wash soln.*—Dissolve 10 g $Pb(NO_3)_2$ in 200 mL $H_2O$, dissolve 1 g NaF in 100 mL $H_2O$ and add 2 mL HCl, and mix these 2 solns. Let ppt settle and decant. Wash ppt 4 or 5 times with 200 mL $H_2O$ by decanting; then add ca 1 L cold $H_2O$ to ppt and let stand ≥1 hr, with occasional stirring. Filter and use clear filtrate. (Prep. more wash soln as needed by adding more $H_2O$ to ppt of PbClF and stirring.)

(**c**) *Silver nitrate std soln.*—0.1 or 0.2*N*. Stdze as in **941.18E**.

(**d**) *Potassium or ammonium thiocyanate std soln.*—0.1*N*. Stdze against std $AgNO_3$ soln under same conditions as in detn.

(**e**) *Ferric indicator.*—To cold satd Cl-free $FeNH_4(SO_4)_2.12H_2O$ soln add enough colorless $HNO_3$ to bleach brown color.

(**f**) *Bromophenol blue indicator.*—Grind 0.1 g powder with 1.5 mL 0.1*N* NaOH and dil. to 25 mL.

### *B. Determination*

(**a**) *Samples difficult to decompose such as cryolite, and others that contain aluminum or appreciable amounts of siliceous material.*—Mix 0.5 g sample (or less if necessary to contain 0.01–0.10 g F) with 5 g fusion mixt. and 0.2–0.3 g *powd. $SiO_2$* in Pt dish, cover with 1 g fusion mixt., and heat to fusion over Bunsen burner. (Use of blast lamp is unnecessary since it is preferable not to heat much beyond melting temp. If much Al is present, uniform, clear, liq. melt cannot be obtained; particles of white solid will sep. in melt. Cooled melt should be colorless, or at least should not have more than gray color.)

Leach cooled melt with hot $H_2O$ and when disintegration is complete, filter into 400 mL beaker. Return insol. residue to Pt dish with jet of $H_2O$, add 1 g $Na_2CO_3$, dil. to 30–50 mL, and boil few min, disintegrating any lumps with flat-end rod. Filter thru same paper, wash thoroly with hot $H_2O$, and adjust vol. of filtrate and washings to ca 200 mL. Add 1 g *ZnO* dissolved in 20 mL $HNO_3$ (1+9), boil 2 min, stirring constantly, filter, and wash thoroly with hot $H_2O$. During this washing return gelatinous mass to beaker 3 times and thoroly disintegrate in wash soln because proper washing of this ppt on filter is difficult. (Mass can easily be returned to beaker by rotating funnel above beaker while cutting ppt loose from paper with jet of wash soln.)

Add 2 drops bromophenol blue to filtrate, and with cover glass almost entirely over beaker, add $HNO_3$ (1+4) until color just changes to yellow. Make soln slightly alk. with 10% NaOH soln, and with cover glass on beaker, boil gently to expel $CO_2$. Remove from burner; add the $HNO_3$ until color just changes to yellow and then 10% NaOH until color just changes to blue; then add 3 mL *10% NaCl soln*. (Vol. of soln at this point should be ca 250 mL.)

Add 2 mL HCl (1+1) and 5 g *$Pb(NO_3)_2$* and heat on steam bath. As soon as $Pb(NO_3)_2$ is in soln, add 5 g NaOAc, stir vigorously, and digest on steam bath 30 min with occasional stirring. Let stand overnight, filter, and wash ppt, beaker, and paper once with cold $H_2O$, then 4 or 5 times with PbClF wash soln, and then once more with cold $H_2O$.

Transfer ppt and paper to beaker in which pptn was made, stir paper to pulp, add 100 mL $HNO_3$ (5+95), and heat on steam bath until ppt dissolves. (5 min is ample to dissolve ppt. If sample contains appreciable amt of sulfates, ppt will contain $PbSO_4$, which will not dissolve. In such case heat 5–10 min with stirring and consider PbClF to be dissolved.) Add slight excess 0.1*N* or 0.2*N* $AgNO_3$, digest on steam bath 30 min, and cool to room temp., protecting from light; filter, wash with cold $H_2O$, and det. $AgNO_3$ in filtrate by titrn with std thiocyanate soln, using 10 mL ferric indicator. Subtract amt of $AgNO_3$ found in filtrate from that originally added. Difference is amt required to combine with Cl in the PbClF; from this difference calc. % F in sample. 1 mL 0.1*N* $AgNO_3$ = 0.00190 g F.

**(b)** *Water-soluble fluorides in presence of organic matter.*—In presence of ≤50% org. matter such as flour, pyrethrum, tobacco powder, and derris or cubé powders, which readily decompose without addn of powd $SiO_2$ and contain little or no sulfates, Al, or siliceous compds, mix 0.5 g sample (or less if necessary to contain 0.01–0.1 g F) with 5 g fusion mixt., cover with 1 g fusion mixt., and heat to fusion over Bunsen burner. Leach cooled melt with hot $H_2O$, and when disintegration is complete, filter into 600 mL beaker. Wash thoroly with hot $H_2O$ and proceed as in **(a)**, third par.

In presence of >50% org. matter or org. matter that is impractical to free without preliminary ashing, such as apple peel and pulp, transfer enough sample to Pt crucible to be representative of mixt. and to contain 0.01–0.1 g F. Add 15 mL $H_2O$ and enough *F-free CaO* (0.3–0.4 g) to make mixt. distinctly alk. to phthln, mix with glass rod, and evap. to dryness on steam bath and in oven at 105°. Ignite at low heat, preferably in furnace (≤600°), until org. matter is thoroly charred. Pulverize, with glass rod, any lumps present in charred ash, mix with 5 g of the fusion mixt., and proceed as in **(a)**, first par., beginning ". . . cover with 1 g fusion mixt., . . ."

**(c)** *Water-soluble samples in absence of organic matter and appreciable quantities of sulfates or aluminum salts.*—In absence of org. matter or other interfering substances, fusion may be omitted and detn made on aliquot of aq. soln contg 0.01–0.1 g F, as in **(a)**, third par.

In presence of Al, as in samples contg $Na_2SiF_6$ and $KAl(SO_4)_2 12H_2O$, transfer sample to 400 mL beaker, dissolve in 150 mL hot $H_2O$, add 6 g fusion mixt., and boil. Add 1 g *ZnO* dissolved in 20 mL $HNO_3$ (1+9), boil 2 min with const stirring, filter into 500 mL vol. flask, and wash thoroly with hot $H_2O$. Cool to room temp. and dil. to vol. Transfer 200 mL aliquot contg 0.01–0.10 g F to 600 mL beaker and proceed as in **(a)**, third par.

**(d)** *Sodium and magnesium fluosilicates, or samples containing more than 5% sulfates in absence of aluminum and boron, with or without moderate amounts of organic matter.*—With large amts of $Na_2SiF_6$ and some other more volatile fluosilicates, *e.g.*, $MgSiF_6$, where there is possibility of some F being evolved as $SiF_4$ before fusion is effected, or in samples contg appreciable amts of sulfates, distil F as in **933.03B**, and det. F in distillate as follows: Add several drops bromophenol blue, make alk. with NaOH, and adjust vol. to ca 250 mL by gently boiling down vol. from 400 to 250 mL. Proceed as in **(a)**, third par., beginning "Remove from burner; . . ."

*Notes:* These methods give accurate results for 0.01–0.10 g F. Below 0.01 g, results tend to be slightly low, and above 0.10 g, slightly high. Convenient sample to fuse is one contg 0.07–0.08 g F; too large sample may result in incomplete fusion. Large amts of B compds and alkali salts retard or prevent complete pptn of PbClF. B has greater effect when amt of F is large than when it is small. In methods described B has little effect, and it may be disregarded in analysis of insecticides if amt of F to be pptd is ≤0.03 g. With some prepns contg $Na_2B_4O_7$ or $H_3BO_3$, where it is difficult to obtain representative mixt. when extremely small sample (0.1 g) is used for analysis, take larger sample and ppt PbClF from aliquot of fusion soln. Amt of alkali carbonates specified in fusion and in washing of insol. residue is not large enough to cause low results. If sample contains S, remove it with $CS_2$ and det. F on air-dried residue, allowing in calcns for % S removed. (*Caution:* See safety notes on flammable solvents, toxic solvents, and carbon disulfide.)

Refs.: J. Res. Natl. Bur. Standards **3,** 581(1929). JAOAC **25,** 670(1942); **27,** 74(1944); **28,** 72(1945).

CAS-7782-41-4 (fluorine)

## 921.04 Fluorine (Total) in Pesticide Formulations

### Modified Travers Method

### Final Action

(Applicable in absence of B, Al, and large amts of pyrethrum powder)

(*Caution:* See safety notes on asbestos.)

#### *A. Reagents*

**(a)** *Alcoholic potassium chloride soln.*—Dissolve 60 g KCl in 400 mL $H_2O$, add 400 mL alcohol, and test with phthln; if soln is not neut., adjust to exact neutrality with NaOH or HCl soln.

**(b)** *Sodium hydroxide std soln.*—0.2*N*. Prep. and stdze as in **936.16**.

#### *B. Determination*

Treat 0.5 g sample in small beaker with 20–25 mL $H_2O$. Add 0.3 g finely divided *pptd $SiO_2$* and few drops Me orange. Add HCl dropwise until soln assumes apparently permanent pink; then add 2 mL excess, cover beaker with watch glass, and boil 1 min. Cool to room temp., add 4 g KCl, and stir until KCl dissolves. Add 25 mL alcohol and let stand 1 hr, stirring frequently. Filter thru gooch contg disk of filter paper covered with medium pad of asbestos. Wash ppt with alc. KCl soln until one washing does not destroy color made by 1 drop 0.2*N* NaOH and phthln (usually 3–4 washings). Transfer crucible and contents to 400 mL beaker, add 100 mL recently boiled $H_2O$ and 1–2 mL phthln, heat, and titr. with std NaOH soln. Finish titrn with the F soln actively boiling. Calc. % F. 1 mL 0.2*N* NaOH = 0.0057 g F.

Refs.: Compt. rend. **173,** 714, 836(1921). JAOAC **14,** 253(1931).

CAS-7782-41-4 (fluorine)

## 933.03 Fluorine (Total) in Pesticide Formulations

### Distillation Method

### Final Action

(Applicable to $H_2O$-sol. or $H_2O$-insol. insecticides in absence of gelatinous $SiO_2$, B, and Al)

#### *A. Reagents*

(**a**) *Sodium alizarin sulfonate indicator.*—Dissolve 0.1 g Na alizarin sulfonate in 200 mL $H_2O$.

(**b**) *Thorium nitrate soln.*—Approx. 0.05*N*. Stdze in terms of g F/mL by titrg F obtained by distn from std NaF as in **933.03B**. In stdzg for use with **933.03B(b)**, add 5 mL satd $KMnO_4$ soln in addn to other reagents in distn flask.

#### *B. Determination*

(**a**) *In absence of organic matter.*—Weigh sample contg ca 0.09 g F, and with aid of little $H_2O$ transfer to 250 mL Claisen distn flask (B) contg 12 glass beads. Adjust to ca 30 mL and close flask with 2-hole rubber stopper, thru which pass thermometer (D) and 4 mm glass tube, both of which extend into soln. (The 4 mm glass tube extends ca 5 cm above rubber stopper and by means of rubber tube, *E*, connects still with 1 L Florence flask (A) contg $H_2O$ for steam generation. Flask is equipped with steam discharge, *H*, and pressure tube, *G*. *See* Fig. **933.03**.)

Bring $H_2O$ in steam generating flask to boil with pinchcock, *F*, in release tube open. Connect distg flask to condenser, *C*, and add 25 mL $H_2SO_4$ thru top of 4 mm tube, using pipet or special funnel. With pinchcock, *F*, open, connect rubber tubing to 4 mm tube. Light burner under Claisen flask. Regulate flow of steam by adjusting burner flames and pinchcock, *F*, so that vol. of soln is held const and temp. in flask, *B*, is kept at 145–150°. Continue distn until 400 mL distillate collects. Dil. to 500 mL in vol. flask, transfer 50 mL aliquot to tall-form 150 mL beaker, and add 5 drops indicator, **933.03A(a)**. Adjust acidity with 1% NaOH soln and HCl (1+249) until pink just disappears. Add 2 mL of the HCl, and titr. with 0.05*N* $Th(NO_3)_4$ to permanent pink, using buret graduated in 0.05 mL.

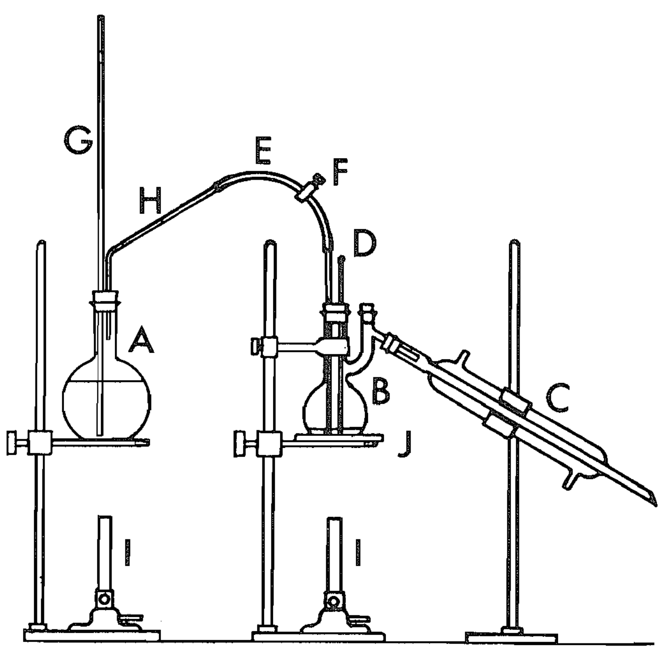


FIG. 933.03—Apparatus for determining fluorine

(**b**) *In presence of organic matter.*—(*Caution:* See safety notes on permanganates.) In presence of moderate amts of org. matter, transfer sample contg ca 0.09 g F and contg ≤0.2 g org. matter, with aid of little $H_2O$, to 250 mL Claisen distn flask contg 12 glass beads. Add 5 mL satd $KMnO_4$ soln, adjust to ca 30 mL, and proceed as in (**a**), beginning ". . . close flask with 2-hole rubber stopper, . . ."

In presence of large amts of org. matter, transfer sample to medium-size Pt dish, add 15 mL $H_2O$ and enough *F-free CaO* to make mixt. distinctly alk. to phthln, mix with glass rod, and evap. to dryness on steam bath and in oven at 105°. Ignite at low heat, preferably in furnace (≤600°), until org. matter is thoroly charred. Pulverize any lumps present in charred ash with glass rod, transfer to 250 mL Claisen distn flask by brushing, and finally wash out dish with 30 mL $H_2SO_4$ (1+9). Except to add 22 mL instead of 25 mL $H_2SO_4$, proceed as in (**a**), par. 2.

*Note:* If coating of pptd $SiO_2$ forms on inside of distn flask, remove by treatment with hot concd alkali soln, as it is capable of retaining F during distn of some samples and giving it up, at least in part, in later distns.

Refs.: J. Am. Chem. Soc. **55**, 1741(1933). Ind. Eng. Chem. Anal. Ed. **5**, 7(1933); **9**, 551(1937); **11**, 21(1939). JAOAC **21**, 459(1938); **53**, 378(1970).

CAS-7782-41-4 (fluorine)

## 945.05 Fluorine Present as Sodium Fluosilicate in Pesticide Formulations

### Final Action

(B, CaO, and alum absent)

#### *A. Reagents*

*Alcoholic potassium chloride and sodium carbonate soln.*—Dissolve 1.0 g $Na_2CO_3$ in 100 mL alc. KCl reagent, **921.04A(a)**. For other reagent *see* **921.04A**.

#### *B. Determination*

Weigh 1 g sample into Pt dish, and add rapidly, with continuous stirring, 50 mL of the alc. KCl-$Na_2CO_3$ reagent. Do not let soln become acid, and if necessary, use more reagent to insure alky.

Continue stirring until all sol. portions of sample dissolve. Proceed as in **921.04B**, beginning: "Filter thru gooch . . ." Calc. % $Na_2SiF_6$ (1 mL 0.2*N* NaOH = 0.009403 g $Na_2SiF_6$).

CAS-16893-85-9 (sodium fluosilicate)

## 972.05 Organochlorine Pesticide Contamination of Pesticide Formulations

### Thin Layer Chromatographic Method

### First Action 1972
### Final Action 1977

### *AOAC-CIPAC Method*

(Applicable to detection of contamination by 0.01% chlorinated hydrocarbons such as aldrin, DDT, dieldrin, and endrin, and 0.05–0.10% of chlordane, strobane, and toxaphene)

#### *A. Apparatus*

(**a**) *Thin layer chromatographic apparatus.*—*See* **970.52F**.

(**b**) *Ultraviolet apparatus.*—Sterilamp G-15T8, or equiv.

### B. Reagents

(**a**) *Adsorbent.*—Aluminum oxide G, Type E (Brinkmann Instruments, Inc.).

(**b**) *Mobile solvents.*—(*1*) *n*-Hexane, (*2*) *n*-hexane-acetone (98+2), and (*3*) *n*-hexane-alcohol (98+2).

(**c**) *Pesticide std solns.*—1 μg/μL EtOAc, acetone, or any convenient solv.

### C. Preparation of Sample

Ext 8 g sample with 20 mL acetone in 250 mL erlenmeyer by shaking intermittently 5 min. Let solids settle. If soln is turbid, filter or centrf. to obtain clear supernate for spotting. For samples contg large amts S, use 8 g sample and 20 mL pet ether.

### D. Preparation of Plates

Dissolve 0.1–0.15 g $AgNO_3$ in 1–2 mL $H_2O$ in 100 mL beaker, add 58 mL MeOH, and mix. Weigh 40 g adsorbent, (**a**), in 250 mL flask, add $AgNO_3$-MeOH soln, and shake vigorously 20 sec. Apply slurry as 0.25 mm thick layer to five 20 × 20 cm (8 × 8″) plates positioned on plastic mounting board. After plates appear dry, store in desiccator over desiccant. When plate is dry, scrape 1 cm strip from side edges to ensure even solv. front. Use plate immediately after removal from desiccator.

### E. Detection

Pour *n*-hexane into glass chromatgc tank to depth of 10–20 mm. Place 2 paper blotters (ca 7.5 × 22 cm) on each side of tank or large blotter covering back of tank and let equilibrate ≥2 hr before use.

Spot 10 μL sample ext on plate with 100 μL syringe. Do *not* disturb adsorbent layer. Also spot std solns of pesticides declared as part of formulation. Spots should be ≤6 mm diam. and placed <30 mm from bottom of plate. Place plate in chromatgc tank, and let plate develop ≥10 cm. Remove plate and expose to shortwave UV, **972.05A(b)**. (*Caution:* See safety notes on hazardous radiations.) Chlorinated org. pesticides should be visible as dark spots against white or light gray background. Expose plates ≥1 hr. Longer exposure will not harm plates.

To confirm identification of pesticide, repeat TLC step with different mobile solv., **972.05B(b)**(*2*) or (*3*).

Ref.: JAOAC **55,** 851(1972).

## 960.10 Herbicide Formulations (Ester Forms of Hormone-Type)

**Volatility Determination**

**First Action 1960**
**Final Action 1961**

### A. Material

(**a**) *Paper bags.*—No. 20 to open with flat bottom. Close with paper clips.

(**b**) *Filter paper.*—7 cm diam.

(**c**) *Bacteriological loop.*—0.01 mL. Wash with acetone after each application or heat to cherry red in flame.

(**d**) *Test plants.*—Actively growing tomato seedlings 65–75 mm high in 3–4″ pots.

(**e**) *Formulation to be tested.*—Use 0.01 mL aliquot of 4 lb/gal. formulation or equiv. vol. of other concns.

(**f**) *High and low volatile ester stds.*—Use Bu ester of 2,4-D as high volatile ester and tetrahydrofurfural ester of 2,4-D as low volatile ester with same wt of acid/gal. as formulations to be tested.

### B. Operating Technic

Open bags with flat bottom and place plant toward one side on bottom of bag. Apply 0.01 mL of formulation to middle of filter paper by means of bacteriological loop, and for controls, apply 0.01 mL solv. only. Place treated paper in bottom of bag. Do not touch treated part of paper against plant, sides of bag, or pot. Close bag by folding top, secure with clips, and let stand 24 hr at 85–110°F (29–43°C).

Use 3 plants per treatment and 3 for controls. Repeat test on another day.

Remove plants from bag, let stand 24 hr, and read curvature (stem bending, epinasty) response. (Fold and discard used bags to prevent contamination.) Rate plants according to scale as follows:

(1) Normal growth of untreated check–no apparent response.
(2) Epinasty 1–20° compared to normal–no curling.
(3) Epinasty 21–40° compared to normal–slight curling.
(4) Epinasty 41–60° compared to normal–moderate curling.
(5) Epinasty 61–80° compared to normal–moderate curling.
(6) Epinasty 81 to >90° compared to normal–severe formative effects.

Mean response of 1 to 2.4 for all tests indicates low volatility. Mean response of 2.5 to 6 indicates volatile formulation.

To detect small differences between low volatile esters, or differences between 2,4-D and 2,4,5-T types, hold plants 7 days after treatment to allow time for modified leaves or stem lesions to develop. Absence of such responses indicates that formulation was a low volatile 2,4,5-T ester.

Ref.: JAOAC **43,** 367(1960).

# INORGANIC AND ORGANOMETALLIC PESTICIDES AND ADJUVANTS

## 920.13 Paris Green Pesticide Formulations

**Final Action**

### A. Moisture

*See* **920.12**.

### B. Total Arsenic

*See* **922.03**.

### C. Water-Soluble Arsenious Oxide★ —Surplus 1965

*See* **4.031**, 10th ed.

## 920.14★ Paris Green Pesticide Formulations

**Total Arsenious Oxide**

**Final Action**
**Surplus 1965**

(Following methods det. only As present in trivalent form ($As_2O_3$) and Sb present in trivalent form ($Sb_2O_3$) in absence of ferrous and cuprous salts.)

### A. Method I

*See* **4.028–4.029**, 10th ed.

### B. Method II

*See* **4.030**, 10th ed.

## 920.15★ Paris Green Pesticide Formulations

### Total Copper

**Final Action**
**Surplus 1965**

***A. Electrolytic Method***

*See* **4.032**, 10th ed.

***B. Volumetric Thiosulfate Method***

*See* **4.033**, 10th ed.

## 920.16 Lead Arsenate Pesticide Formulations

### Moisture

**Final Action**

(*Caution:* See safety notes on pesticides and arsenic trioxide.)

(**a**) *Powder.*—Dry 2 g to const wt at 105–110°. Report loss in wt as $H_2O$.

(**b**) *Paste.*—Proceed as in (**a**), using 50 g. Grind dry sample to fine powder, mix well, transfer small portion to sample bottle, and again dry 1–2 hr at 105–110°. Use this anhyd. material to det. total Pb and total As.

## 920.17 Lead Arsenate Pesticide Formulations

### Total Arsenic

**Final Action**

***A. Method I***

*See* **922.03C**.

***B. Method II***

(Not applicable in presence of Sb)

Dissolve 1 g powd sample with $HNO_3$ (1+4) in porcelain casserole or evapg dish, add 5 mL $H_2SO_4$, and heat on hot plate to copious evolution of white fumes. Cool, add little $H_2O$, and again evap. until white fumes appear, to assure removal of last trace of $HNO_3$. Wash into 200 mL vol. flask with $H_2O$, cool, dil. to vol., and filter thru dry filter. Transfer 100 mL filtrate to erlenmeyer and proceed as in **925.02**, beginning ". . . add 1 g KI, . . ." From mL std I soln used, calc. % total As as $As_2O_5$.

Ref.: USDA Bur. Chem. Bull. **105,** p. 167.

CAS-7440-38-2 (arsenic)

## 920.18 Lead Arsenate Pesticide Formulations

### Total Arsenious Oxide

**Final Action**

Weigh 2 g powd sample and transfer to 200 mL vol. flask, add 100 mL $H_2SO_4$ (1+6), and boil 30 min. Cool, dil. to vol., shake thoroly, and filter thru dry filter. Nearly neutze 100 mL filtrate with NaOH soln, **922.03A(e)**, using few drops phthln. If neut. point is passed, make acid again with the dil. $H_2SO_4$. Continue as in **925.02**, beginning "Neutze with $NaHCO_3$, . . ." From mL std I soln used, calc. % $As_2O_3$.

Ref.: JAOAC **3,** 332(1920).

## 920.19 Lead Arsenate Pesticide Formulations

### Total Arsenic Oxide

**Final Action**

***A. Reagents***

(**a**) *Potassium iodide soln.*—Dissolve 20 g KI in $H_2O$ and dil. to 100 mL.

(**b**) *Thiosulfate std soln.*—0.05*N*. Prep. daily by dilg 0.1*N* soln, **942.27**. 1 mL 0.05*N* $Na_2S_2O_3$ = 2.873 mg $As_2O_5$.

***B. Determination***

Weigh 0.5 g powd sample and transfer to erlenmeyer. Add 25–30 mL HCl and evap. to dryness on steam bath. Add 50 mL HCl and if necessary to effect soln, heat on steam bath, keeping flask covered with watch glass to prevent evapn of acid. Cool to 20–25°, add 10 mL of the KI soln and 50 mL (or more if necessary to produce clear soln) *25% $NH_4Cl$ soln,* and immediately titr. liberated I with std $Na_2S_2O_3$ soln. When color becomes faint yellow, dil. with ca 150 mL $H_2O$ and continue titrn carefully, dropwise, until colorless, using starch indicator, **922.03A(f)**, near end point. From mL $Na_2S_2O_3$ soln used, calc. % $As_2O_5$.

Ref.: JAOAC **3,** 333(1920).

## 920.20 Lead Arsenate Pesticide Formulations

### Water-Soluble Arsenic

**Final Action**

Proceed as in **925.02**, and calc. results as $As_2O_5$.

## 920.21 Lead Arsenate Pesticide Formulations

### Total Lead

**Final Action**

In 600 mL beaker on hot plate heat 0.5 g powd sample and ca 25 mL $HNO_3$ (1+4). Filter to remove any insol. residue. Dil. to ≥400 mL, heat nearly to bp, and add $NH_4OH$ to slight pptn, then $HNO_3$ (1+9) to redissolve ppt, adding 1–2 mL excess. Into this soln, kept almost boiling, pipet 50 mL hot *10% $K_2CrO_4$ soln,* stirring constantly. Decant while hot thru weighed gooch, previously heated to 140–150°, and wash ppt several times by decanting and then on filter with boiling $H_2O$ until washings are colorless. Dry $PbCrO_4$ at 140–150° to const wt. From wt $PbCrO_4$, calc. % Pb, using factor 0.6411. ($PbCrO_4$ ppt may contain small amt $PbHAsO_4$, which will cause slightly high results, but this error rarely is >0.1–0.2%.)

Refs.: USDA Bur. Chem. Bull. **137,** p. 40; **152,** p. 68.

CAS-7439-92-1 (lead)

## 921.05 Calcium Arsenate Pesticide Formulations

**Final Action**

(*Caution:* See safety notes on pesticides.)

***A. Moisture***

*See* **920.12**.

***B. Total Arsenic***

*See* **922.03**.

### C. Total Arsenious Oxide

(**a**) *Not applicable in presence of nitrates*.—Weigh 1 g sample, transfer to 500 mL erlenmeyer, and dissolve in 100 mL HCl (1+3). Heat to 90° and titr. with std $KBrO_3$ soln, **922.03A(c)**, using 10 drops Me orange, **922.03A(g)**. From mL std $KBrO_3$ soln used, calc. % $As_2O_3$.

(**b**) *Applicable in presence of small amounts of nitrates*.—Proceed as in (**a**) except to titr. at room temp.

Refs.: JAOAC **5,** 33(1921); **6,** 392(1922).

### D. Water-Soluble Arsenic

Proceed as in **925.02**, and calc. results as $As_2O_5$. (In testing Ca arsenate by this method, low value for $H_2O$-sol. As is not assurance against plant injury when using this product.)

## 921.06 Calcium Arsenate Pesticides Formulations
**Total Calcium**
**Final Action**

### A. Reagents

(**a**) *Ammonium oxalate soln*.—Dissolve 40 g $(NH_4)_2C_2O_4 \cdot H_2O$ in 1 L $H_2O$.

(**b**) *Potassium permanganate std soln*.—0.1*N*. Prep. and stdze as in **940.35**.

### B. Method I

Dissolve 2 g sample in 80 mL HOAc (1+3), transfer to 200 mL vol. flask, dil. to vol., and filter thru dry filter. Transfer 50 mL aliquot to beaker, dil. to ca 200 mL, heat to bp, and ppt Ca with $(NH_4)_2C_2O_4$ soln. Let beaker stand 3 hr on steam bath, filter, and wash ppt with hot $H_2O$. Dissolve ppt in 200 mL $H_2O$ contg 25 mL $H_2SO_4$ (1+4), heat to ca 70°, and titr. with std $KMnO_4$ soln. From mL $KMnO_4$ soln used, calc. % Ca.

### C. Method II

(Not applicable in presence of Pb. *Caution:* See safety notes on arsenic trioxide.)

Weigh 2 g sample, transfer to beaker, add 5 mL *HBr* (ca 1.38 sp gr) and 15 mL HCl, and evap. to dryness under hood to remove As. Repeat treatment, add 20 mL HCl, and again evap. to dryness. Take up with $H_2O$ and little HCl, filter into 200 mL vol. flask, wash, and dil. to vol. Transfer 50 mL aliquot to beaker, add 10 mL HCl and few drops $HNO_3$, boil, and make slightly alk. with $NH_4OH$. Let stand few min and filter. Dissolve ppt in HCl (1+4), reppt, filter thru same paper, and wash with hot $H_2O$. To combined filtrates and washings add 20 mL HOAc (1+3) and adjust to ca 200 mL. Heat to bp, ppt with $(NH_4)_2C_2O_4$ soln, and let stand 3 hr on steam bath. Filter, and wash with hot $H_2O$. Ignite at 950°, and weigh as CaO; or dissolve and titr. as in **921.06B**. From wt CaO or mL $KMnO_4$ soln used, calc. % Ca.

Refs.: JAOAC **5,** 33(1921); **6,** 392(1922).

CAS-7440-70-2 (calcium)

## 920.22 Zinc Arsenite Pesticide Formulations
**Final Action**

### A. Moisture

*See* **920.12**.

### B. Total Arsenic

Proceed as in **922.03** and calc. as $As_2O_3$.

### C. Water-Soluble Arsenic

Proceed as in **925.02**, and calc. results as $As_2O_3$.

### D. Total Zinc★ —Surplus 1965

*See* **4.054**, 10th ed.

## 920.23★ Zinc Arsenite Pesticide Formulations
**Total Arsenious Oxide**
**Final Action**
**Surplus 1965**

### A. Method I

*See* **4.051**, 10th ed.

### B. Method II

*See* **4.052**, 10th ed.

## 920.24★ Copper in Copper Carbonate Pesticide Formulations
**Final Action**
**Surplus 1965**

### A. Electrolytic Method

*See* **4.055**, 10th ed.

### B. Volumetric Thiosulfate Method

*See* **4.056**, 10th ed.

## 964.03 Copper in Copper Naphthenate Pesticide Formulations
**First Action 1964**
**Final Action 1982**

(*Caution:* See safety notes on pesticides.)

### A. Titrimetric Method

Accurately weigh sample contg ca 0.2 g Cu into dry g-s flask. Add 5 mL pet ether to concd products. Add 100 mL $H_2O$, 1.5 g $NH_4HF_2$, and 5–10 g KI. Stopper and shake vigorously until reaction is complete (usually ca 2 min). Wash stopper and sides of flask with $H_2O$ and titr. with std 0.1*N* $Na_2S_2O_3$ (stdzd against Cu) to light brown. Add starch indicator, **922.03A(f)**, titr. almost to end point, add 2 g KSCN, shake to dissolve, and complete titrn to starch end point.

### B. Electrolytic Method

Accurately weigh sample contg ca 0.2 g Cu into 200 mL separator. Add 50 mL pet ether and 25 mL $HNO_3$ (1+4), and shake 2 min. Drain aq. phase into 250 mL beaker and save. Wash pet ether with 15 and 10 mL $HNO_3$ (1+4), and combine acid exts. Neutze with $NH_4OH$, acidify with 6 mL $H_2SO_4$ and 4 mL $HNO_3$, and proceed as in **922.05A**, beginning ". . . adjust vol. to 200 mL, . . ." using ca 0.5 amp during first 10 min and 1.5–2.0 amp for ca 20 min.

Ref.: JAOAC **47,** 253(1964).

CAS-7440-50-8 (copper)

## 981.01 Copper (Water-Soluble) in Water-Insoluble Copper Fungicides

**First Action 1981**

***CIPAC-AOAC Method***

(*Caution:* See safety notes on atomic absorption spectrophotometer and chloroform.)

### *A. Principle*

Water-soluble Cu is isolated by dispersing sample in $H_2O$, shaking, centrifg, and filtering. Cu in filtrate is detd either by spectrophtric bathocuproine method or by atomic absorption.

### *B. Apparatus*

(**a**) *Filter paper.*—7 cm. (Whatman No. 1 chromatgc or filter paper, S&S No. 2043b, and Albet No. 305 have been satisfactory; the following papers have not been suitable: Whatman Nos 32, 40, 42, and Albet No. 240. If other papers are used, check adsorption of Cu before use.)

(**b**) *Filter.*—13 mm Millipore, HA.WP.013-porosity 0.45 μm, Sartorius, or equiv.

(**c**) *Atomic absorption spectrophotometer.*—With oxidizing air–$C_2H_2$ flame.

### *C. Reagents*

(**a**) *Deionized water.*—For prepg all aq. solns and rinsing glassware and app.

(**b**) *Sodium acetate-acetic acid.*—0.05*M*, buffered at pH 6.5. Dissolve 3.4 g $NaOAc.3H_2O$ in $H_2O$, add 1.8 mL 0.2*M* HOAc, adjust to pH 6.5, and dil. to 500 mL with $H_2O$.

(**c**) *Cupric sulfate.*—$CuSO_4.5H_2O$, contg 25.4% Cu. Check Cu content, e.g., by electrolysis.

(**d**) *Copper std stock soln.*—25 μg Cu/mL. Dissolve 98.3 mg $CuSO_4.5H_2O$ in $H_2O$ and dil. to 1 L with $H_2O$.

(**e**) *Ascorbic acid soln.*—Dissolve 10 g ascorbic acid in $H_2O$ and dil. to 100 mL with $H_2O$.

(**f**) *Sodium acetate soln.*—Dissolve 40 g $NaOAc.3H_2O$ in $H_2O$ and dil. to 100 mL with $H_2O$.

(**g**) *Chloroform.*—Redistd.

(**h**) *Bathocuproine soln.*—Dissolve 0.050 g in 500 mL redistd $CHCl_3$ and store in dark glass bottles.

### *D. Isolation of Water-Soluble Copper*

Accurately weigh ca 0.350 g sample (*w*) and transfer to 250 mL conical flask. Add 100 mL $H_2O$, stopper tightly, and shake 1 min. Let stand 1 h in 20° $H_2O$ bath, shaking suspension every 15 min. Centrf. ca 50 mL suspension 20 min at 3000 rpm, taking care to avoid increase in temp. Filter ca 40 mL supernate thru 13 mm filter.

#### *Bathocuproine Method*

### *E. Preparation of Standard Curve*

Pipet 25.0 mL Cu std stock soln, (**d**), into 500 mL vol. flask and dil. to vol. with NaOAc–HOAc buffer soln (1.25 μg Cu/mL). Transfer 0, 4, 6, 8, 10, 12, 14, and 16 mL aliquots of intermediate soln to 100 mL separators and dil. each to 20 mL with NaOAc-HOAc buffer soln. Add 1 mL ascorbic acid soln to each separator and shake 1 min. Add 2.5 mL NaOAc soln and shake 1 min. Develop color by pipetting in 10.0 mL bathocuproine soln. Ext. complex by vigorously shaking 1 min (vigorous extn is necessary to ext all complex). Let layers sep., filter $CHCl_3$ ext thru 7 cm paper into clean, dry tube, and stopper tube. Exactly 15 min after extn, measure *A* at 465 nm, using $CHCl_3$ as ref. Plot *A* vs μg Cu. (Curve is linear ≤20 μg Cu, but does not necessarily pass thru origin.)

### *F. Determination*

Pipet 10.0 mL sample filtrate into 100 mL vol. flask, dil. to vol. with NaOAc–HOAc buffer soln, and mix.

Transfer 20.0 mL aliquot to 100 mL separator and continue as in *Preparation of Standard Curve,* starting with "Add 1 mL ascorbic acid soln . . ."

If *A* of sample is higher than *A* of highest point of calibration curve, dil. sample soln with buffer soln so *A* falls on calibration curve. Read μg Cu (*Q*) corresponding to *A* found.

$$\% \text{ Soluble Cu (w/w)} = Q/200w$$

If soln has been dild, correct formula accordingly. Carry out blank detn on reagents, using 20 mL NaOAc-HOAc buffer soln. *A* must be of same order as that for *A* of point 0 on calibration curve.

#### *Atomic Absorption Spectrophotometric Method*

### *G. Preparation of Standard Curve*

Transfer 0, 4.0, 8.0, 12.0, 16.0, and 20.0 mL aliquots of Cu std stock soln (25 μg Cu/mL) to 100 mL vol. flasks. Add 2 mL $HNO_3$ to each, dil. to vol. with $H_2O$, and mix. (These solns contain 0, 1, 2, 3, 4, and 5 μg Cu/mL.)

Det. *A* at 324.7 nm of solns in oxidizing air–$C_2H_2$ flame, and plot calibration curve.

### *H. Determination*

Pipet 25.0 mL sample filtrate into 50 mL vol. flask, add 1 mL $HNO_3$, dil. to vol. with $H_2O$, and mix. Det. *A* at 324.7 nm as in *Preparation of Standard Curve.* Obtain μg Cu/mL from std curve,

$$\% \text{ Soluble Cu (w/w)} = (\mu g \text{ Cu/mL})/50w$$

If *A* of sample is higher than highest point of calibration curve, dil. sample with 2% $HNO_3$ and correct calcn for diln factor.

Ref.: JAOAC **64,** 75(1981); corr. 233.

CAS-7440-50-8 (copper)

## 979.02 Fentin in Pesticide Formulations

**Potentiometric Titration Method**

**First Action 1979**
**Final Action 1982**

***CIPAC-AOAC Method***

### *A. Principle*

Org. Sn compds are extd with acetone, diphenyltin compds are quant. converted to insol. oxide with alk. alumina and filtered, and acetone soln is titrd potentiometrically.

### *B. Apparatus*

(**a**) *Filtration apparatus.*—Glass bell with neck and removable plate to permit glass buchner with fine porosity fritted disk and long stem to drain into beaker under vac. (Fig. **979.02**).

(**b**) *Potentiometric titration apparatus.*—pH meter with glass and satd calomel electrodes is satisfactory.

### *C. Reagents*

(**a**) *Alkaline alumina.*—Mix 150 g neutral $Al_2O_3$ (Woelm 4649, or equiv.) with 150 mL alcohol contg 15 g KOH in 1 L r-b flask. Reflux 30 min, cool, and filter with suction thru buchner. Dry powder in vac. 1 hr at 100° and 3-3.5 hr at 130°. Pour warm powder into bottle and stopper tightly. Com. alk. $Al_2O_3$ is not satisfactory.

(**b**) *Cellulose powder.*—Whatman CF 11, or equiv.

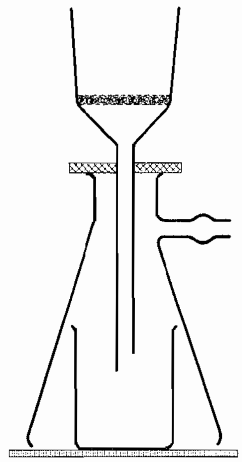

**FIG. 979.02—Filtration apparatus**

### *D. Determination*

Accurately weigh into 100 mL glass beaker sample contg ca 0.30 g triphenyltin compd. Add 2 g alk. $Al_2O_3$ and 25 mL acetone and stir with mag. stirrer 10 min. Prep. and process blank of 2 g alk. $Al_2O_3$ and 25 mL acetone in same manner. Place 1 g cellulose powder and 1 g alk. $Al_2O_3$ in funnel and mix thoroly. Assemble filtration app. contg 250 mL beaker and filter suspension thru funnel. Wash beaker and funnel with four 20 mL portions acetone. Titr. filtrate potentiometrically with stdzd 0.1*N* HCl.

$$\% \text{ Triphenyltin compd} = (S - B) \times N \times (M/W) \times 10$$

where $S$ = mL HCl used for sample, $B$ = mL HCl used for blank, $N$ = normality of HCl, $M$ = mol. wt of compd (367.0 for triphenyltin hydroxide and 409.0 for the acetate), and $W$ = g sample.

Ref.: JAOAC **61,** 1504(1978).

## 984.04 Fentin in Fentin-Maneb Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1984**

***CIPAC-AOAC Method***

### *A. Principle*

Fentin is converted with *n*-butylmagnesium chloride to butyltriphenyltin, which is extd with toluene and detd by GC with docosane as internal std.

### *B. Reagents and Apparatus*

(**a**) *Dilute acetic acid.*—Weigh 0.750 g HOAc in 250 mL vol. flask and dil. to vol. with toluene.

(**b**) *n-Butylmagnesium chloride.*—2.5M in tetrahydrofuran (Alfa Products, Div. of Morton Thiokol, Inc., 152 Andover St, Danvers, MA 01923), or equiv.

(**c**) *Butyltriphenyltin.*—Prep. as follows: Purify triphenyltin chloride by repeated crystn from isopropanol until diphenyltin dichloride and/or tetraphenyltin are absent by GC or TLC. (For TLC, use silica gel F254 (Merck) plates and develop in $CH_2Cl_2$-acetone-HOAc-$H_2O$ (80 + 5 + 2.5 + 0.5) or $CH_2Cl_2$-*n*-hexane-HOAc (30 + 20 + 2.5). Expose developed plates to UV radiation (254 nm) or I vapors and spray with 0.1% pyrocatechol violet soln in alcohol.) Dry salt at 50° under vac. to remove isopropanol. Flush 250 mL 3-necked flask equipped with stirrer, reflux condenser, and dropping funnel with N. Dissolve 20 g triphenyltin chloride in 80 mL toluene, add 40 mL *n*-butylmagnesium chloride, and let react 15 min under N. Carefully destroy excess Grignard reagent (cool in $H_2O$ bath) by adding 50 mL 5% $H_2SO_4$ and transfer mixt. to sep. funnel. Discard lower (aq.) layer. Wash org. layer with 20 mL 5% $H_2SO_4$ and then with $H_2O$ until acid-free. Dry org. layer with anhyd. $Na_2SO_4$. Filter soln into 500 mL r-b flask and evap. solv. in rotary vac. evaporator at 50°. Dissolve residue in 400 mL hot isopropanol, cool, and filter butyltriphenyltin in buchner funnel; wash with 25 mL cold isopropanol and dry at 50° under vac. Check purity by GC and mp (corrected mp 60.0–60.5°).

(**d**) *Internal std soln.*—Accurately weigh 2.25 g pure docosane into 250 mL vol. flask, and dissolve and dil. to vol. with toluene.

(**e**) *Calibration soln.*—Accurately weigh ca 0.20 g pure butyltriphenyltin into g-s flask, pipet in 10.0 mL internal std soln, and add 40 mL toluene.

(**f**) *Gas chromatograph.*—With on-column injection, flame ionization detector, and 1.5 m × 3 mm (id) glass column packed with 5% SE-30, or equiv. on 100–200 mesh Chromosorb W (HP) or Gas-Chrom Q. Operating conditions: temps (°): column 220, injection port 250, detector 260; flow rates (mL/min): N carrier gas 30, air 400, H 40. Alternatively, thermal conductivity detector may be used with He carrier gas, bridge current at 150 mamp, and detector at 300°.

### *C. Preparation of Sample*

(**a**) *Fentin acetate-maneb mixtures.*—Accurately weigh sample contg 205 ± 5 mg fentin acetate into 100 mL erlenmeyer. Pipet in 10.0 mL internal std soln and 15 mL toluene and stir 5 min on mag. stirrer. Add 8 mL *n*-butylmagnesium chloride and let react 15 min under N. Destroy excess Grignard reagent by careful and dropwise addn of 1.5 mL $H_2O$ by syringe or microburet. Dry dispersion with 2 g anhyd. $Na_2SO_4$. Add 25 mL toluene (omit if thermal conductivity detector is used) and filter.

(**b**) *Fentin hydroxide-maneb mixtures.*—Accurately weigh sample contg 185 ± 5 mg fentin hydroxide into 100 mL erlenmeyer. Pipet in 10.0 mL internal std soln and 10 mL dil. HOAc. Add 5 mL toluene and stir 5 min on mag stirrer. Continue as described in (**a**).

### *D. Determination*

Inject 1 μL portions of calibration soln until peak area ratio (area butyltriphenyltin peak/area docosane peak) varies <1% for successive injections. Inject, in duplicate, 1 μL portions of sample solution followed by 1 μL portions of calibration soln. (For thermal conductivity detection, inject 10–20 μL.) Measure peak areas by triangulation (peak ht by retention time) or by electronic integration. Retention times: internal std 7 min; butyltriphenyltin 14 min.

### *E. Calculations*

$$\text{Fentin acetate, } \% = R/R' \times W'/W \times 100.5$$
$$\text{Fentin hydroxide, } \% = R/R' \times W'/W \times 90.2$$

where $R$ and $R'$ = av. peak area ratios for sample and calibration solns, resp.; $W$ and $W'$ = mg sample and butyltriphenyltin, resp.; 100.5 = 100 × mol. wt fentin acetate/mol. wt butyltriphenyltin = 100 × 409/407; 90.2 = 100 × mol. wt fentin hydroxide/mol. wt butyltriphenyltin = 100 × 367/407.

Refs.: JAOAC **61,** 1507(1978); **67,** 479(1984).

CAS-900-95-8 (fentin acetate)
CAS-76-87-9 (fentin hydroxide)

## 920.25 Moisture in Bordeaux Mixture Pesticide Formulations

**Final Action**

(*Caution:* See safety notes on pesticides.)

(**a**) *Powder.—See* **920.16(a)**.

(**b**) *Paste.*—Heat ca 100 g in oven at 90–100° until dry enough to powder readily and note loss in wt. Powder this partially dried sample and det. remaining $H_2O$ in 2 g as in (**a**). Det. $CO_2$ as in **920.26B**, both in original paste and in partially dried sample. Calc. total $H_2O$ by following formula:

$$M = a + \frac{(100 - a)(b + c)}{100} - d$$

where $M$ = % total $H_2O$ in original paste; $a$ = % loss in wt of original paste during first drying; $b$ = % loss in wt of partially dried paste during second drying; $c$ = % $CO_2$ remaining in partially dried paste after first drying; and $d$ = % total $CO_2$ in original paste.

## 920.26 Carbon Dioxide in Bordeaux Mixture Pesticide Formulations

**Final Action**

### A. Apparatus

Use 200 mL erlenmeyer with 2-hole stopper; in one hole fit dropping funnel with stem extending almost to bottom of flask, and thru other hole pass outlet of condenser that is inclined upward at 30° angle from horizontal. Connect upper end of condenser with $CaCl_2$ tube, which in turn connects with double U-tube filled in middle with pumice fragments, previously satd with *20% $CuSO_4.5H_2O$ soln* and subsequently dehydrated, and with $CaCl_2$ at either end. Connect 2 weighed U-tubes to absorb $CO_2$, first filled with porous soda-lime, and second, 1/3 with soda-lime and 2/3 with $CaCl_2$, placing the $CaCl_2$ at exit end of train. Attach Geissler bulb, partly filled with $H_2SO_4$, to last U-tube to show rate of gas flow, and connect aspirator with Geissler bulb to draw air thru app. Connect absorption tower filled with soda-lime to mouth of dropping funnel to remove $CO_2$ from air entering app.

### B. Determination

Weigh 2 g powder or 10 g paste into the erlenmeyer and add ca 20 mL $H_2O$. Attach flask to app., omitting the 2 weighed U-tubes, and draw $CO_2$-free air thru app. until it displaces original air. Attach weighed U-tubes as in **920.26A**, close stopcock of dropping funnel, pour into it 50 mL HCl (1+4), reconnect with soda-lime tower, and let acid flow into erlenmeyer, slowly if there is much $CO_2$, rapidly if there is little. When effervescence diminishes, place low Bunsen flame under flask and start flow of $H_2O$ thru condenser, letting slow current of air flow thru app. at same time. Maintain steady but quiet boil and slow air current thru app. Boil few min after $H_2O$ begins to condense, remove flame, and continue air flow at ca 2 bubbles/sec until app. is cool. Disconnect weighed absorption tubes, cool in balance case, and weigh. Increase in wt = $CO_2$.

Refs.: Fresenius, "Quantitative Chemical Analysis," Trans. 6th German Ed., 1906, amplified and revised, Vol. **2,** 1180. U.S. Geol. Survey Bull. **700,** p. 218.

CAS-124-38-9 (carbon dioxide)

## 920.27 Copper in Bordeaux Mixture Pesticide Formulations

**Final Action**

### A. Electrolytic Method

(Also applicable to $CuCO_3$ and $CuSO_4$)

Dissolve powd sample contg 0.2–0.25 g Cu in 45 mL $HNO_3$ (1+4). Filter if necessary, dil. to 200 mL, and electrolyze as in **922.05A**.

### B. Volumetric Thiosulfate Method

Dissolve 2 g powd sample in ca 25 mL $HNO_3$ (1+4), dil. to 50 mL, add $NH_4OH$ in excess, and heat. Without removing ppt that has formed, boil off excess $NH_3$, add 3–4 mL HOAc, cool, add 10 mL 30% KI soln, and titr. as in **922.05B**, beginning ". . . titr. with std $Na_2S_2O_3$ soln, . . ."

CAS-7440-50-8 (copper)

## 920.28 Bordeaux Mixture with Paris Green

**Final Action**

(*Caution:* See safety notes on pesticides.)

### A. Moisture

*See* **920.25**.

### B. Carbon Dioxide

*See* **920.26B**.

### C. Total Arsenic

Proceed as in **922.03**, using 2 g sample, and calc. results as $As_2O_3$.

### D. Total Arsenious Oxide★ —Surplus 1965

*See* **4.067**, 10th ed.

### E. Water-Soluble Arsenious Oxide★ —Surplus 1965

*See* **4.068**, 10th ed.

### *Copper*

### F. Electrolytic Method I

*See* **922.05A**.

### G. Electrolytic Method II—(Short Method)★ —Surplus 1965

*See* **4.070**, 10th ed.

### H. Volumetric Thiosulfate Method

*See* **922.05B**.

## 920.29 Bordeaux Mixture with Lead Arsenate

**Final Action**

(*Caution:* See safety notes on pesticides.)

### A. Moisture

*See* **920.25**.

### B. Carbon Dioxide

*See* **920.26B**.

***C. Total Arsenic***

Proceed as in **922.03**, using 2 g sample, and calc. results as $As_2O_5$.

***D. Water-Soluble Arsenic***

Proceed as in **925.02** and calc. results as $As_2O_5$.

***E. Copper—Electrolytic Method***

*See* **922.05A**.

***F. Copper—Volumetric Thiosulfate Method***

*See* **922.05B**.

***G. Lead***

*See* **922.04**.

***H. Lead and Copper—Electrolytic Method★ —Surplus 1965***

*See* **4.079–4.080**, 10th ed.

## 925.03 Bordeaux Mixture with Calcium Arsenate

**Final Action**

(*Caution:* See safety notes on pesticides.)

***A. Moisture***

*See* **920.25**.

***B. Carbon Dioxide***

*See* **920.26B**.

***C. Total Arsenic***

Proceed as in **922.03**, using 2 g sample, and calc. results as $As_2O_5$.

***D. Water-Soluble Arsenic***

Proceed as in **925.02** and calc. results as $As_2O_5$.

***Copper***

***E. Electrolytic Method I***

*See* **922.05A**.

***F. Electrolytic Method II★ —Surplus 1965***

*See* **4.070**, 10th ed.

***G. Volumetric Thiosulfate Method***

*See* **922.05B**.

## 930.12★ Calcium Cyanide Pesticide Formulations

**Final Action**
**Surplus 1965**

***A. Cyanide***

*See* **4.093–4.094**, 10th ed.

***Chloride***

***B. Method I***

*See* **4.095**, 10th ed.

***C. Method II***

See **4.096**, 10th ed.

## 952.01 Potassium Cyanate Pesticide Formulations

**Final Action**

(*Caution:* See safety notes on pesticides.)

***A. Reagent***

*Wash soln.*—Satd aq. soln of hydrazodicarbamide, $NH_2CONHNHCONH_2$. Prep. by mixing KOCN and semicarbazide.HCl, $NH_2CONHNH_2$.HCl, in $H_2O$, filter, and wash ppt with $H_2O$. Transfer ppt to flask, add small amt $H_2O$, shake vigorously, and filter. (Solubility of ppt in $H_2O$ is ca 1 part in 6600.)

***B. Determination***

Weigh sample contg 0.2–0.5 g KOCN into 100 mL beaker, add 20 mL wash soln and 1 g semicarbazide.HCl, and let stand 24 hr. Filter hydrazodicarbamide on gooch or fine fritted glass crucible, wash with 10 mL wash soln, and dry at 100° to const wt. KOCN = wt residue × 0.6868.

Ref.: JAOAC **35**, 377(1952).

CAS-590-28-3 (potassium cyanate)

## 920.30★ Sodium and Potassium Cyanide Pesticide Formulations

**Final Action**
**Surplus 1965**

***A. Cyanide***

*See* **4.088–4.089**, 10th ed.

***Chloride***

***B. Method I***

*See* **4.090–4.091**, 10th ed.

***C. Method II***

*See* **4.092**, 10th ed.

## 920.31 Sulfur (Soluble) in Lime Sulfur Solutions and Dry Lime Sulfur

**Gravimetric Method**
**Final Action**

(Use low S reagents.)

***A. Preparation of Sample***

(**a**) *Solns.*—Accurately weigh ca 10 g soln, transfer to 250 mL vol. flask, and immediately dil. to vol. with recently boiled and cooled $H_2O$. Mix thoroly and either take necessary aliquots in individual pipets in min. time for detns or transfer to small bottles, filling them completely and avoiding contact of soln with air as much as possible. Stopper bottles, seal with paraffin, and store in dark, cool place.

(**b**) *Dry lime-sulfur.*—Thoroly stir 5 g sample with ca 50 mL $H_2O$ in 250 mL beaker. Let settle and decant thru paper into 250 mL vol. flask. Repeat extn with $H_2O$ until filtrate is colorless and ca 200 mL is obtained. Transfer residue to paper, wash with hot $H_2O$, cool to room temp., and dil. to vol. Dry residue 1.5 hr at 105°, and reserve for free S and sulfite S detns in residue, if desired. (Ext S from dry residue with $CS_2$ (*Caution:* See safety notes on flammable solvents, toxic solvents, and carbon disulfide.), evap. on steam bath or in air current, dry 15 min at 105°, weigh, and calc. % S.)

Prep. soln in min. time and keep beaker and funnel covered as much as possible.

### B. Determination

With clean, dry pipet transfer 10 mL prepd soln, **920.31A(a)** or **(b)**, to 250 mL beaker. Partially cover with cover glass and add 2–3 g *$Na_2O_2$* in small portions, with stirring, from tip of spatula. Continue adding $Na_2O_2$ until all S appears to be oxidized to sulfate (yellow color disappears). Add slight excess $Na_2O_2$, completely cover beaker with cover glass, and heat on steam bath, stirring occasionally, 15–20 min.

Wash off cover glass and sides of beaker, acidify with HCl (1+4), and filter if necessary. Dil. to 150–200 mL, heat to bp, and add *10% $BaCl_2$ soln* (11 mL/1 g $BaSO_4$), with const stirring, at such rate that ca 4 min is required to add necessary amt. Let stand until clear and cool, filter thru quant. paper, wash until Cl-free, ignite carefully, and heat to const wt over Bunsen burner. Calc. % S from wt $BaSO_4$, using factor 0.1374.

Ref.: JAOAC **3,** 353(1920).

CAS-7704-34-9 (sulfur)

## 920.32 Sulfur (Thiosulfate) in Lime Sulfur Solutions and Dry Lime Sulfur

**Titrimetric Method**

**Final Action**

### A. Reagent

*Ammoniacal zinc chloride soln.*—Dissolve 50 g $ZnCl_2$ in ca 500 mL $H_2O$, add 125 mL $NH_4OH$ and 50 g $NH_4Cl$, and dil. to 1 L.

### B. Determination

To 50 mL $H_2O$ in 200 mL vol. flask add 50 mL prepd soln, **920.31A(a)** or **(b)**. Add slight excess of the ammoniacal $ZnCl_2$ soln and dil. to vol. Complete detn as rapidly as possible. Shake thoroly and filter thru dry filter. To 100 mL filtrate add few drops Me orange, **922.03A(g)**, or Me red (1 g Me red in 200 mL alcohol), and exactly neutze with 0.1*N* HCl. Titr. neut. soln with 0.05*N* I, **922.03A(b)**, using few drops starch indicator, **922.03A(f)**. From mL I soln used, calc. % thiosulfate S present. (Factor of I soln in terms of $As_2O_3 \times 1.296$ = equiv. in thiosulfate S.)

Ref.: JAOAC **3,** 353(1920).

## 920.33 Sulfur (Sulfide) in Lime Sulfur Solutions and Dry Lime Sulfur

**Final Action**

### A. Zinc Chloride Method

To 10–15 mL $H_2O$ in small beaker add 10 mL aliquot prepd soln, **920.31A(a)** or **(b)**. Calc. amt ammoniacal $ZnCl_2$ soln, **920.32A**, necessary to ppt all S in aliquot and add slight excess. Stir thoroly, filter, wash ppt twice with cold $H_2O$, and transfer paper and ppt to beaker in which pptn was made. Cover with $H_2O$, disintegrate paper with glass rod, and add ca 3 g *$Na_2O_2$*, keeping beaker well covered with watch glass. Warm on steam bath with frequent shaking until all S is oxidized to sulfate, adding more $Na_2O_2$ if necessary. Acidify slightly with HCl (1+4), filter to remove shreds of paper, wash thoroly with hot $H_2O$, and det. S in filtrate as in **920.31B**.

Ref.: JAOAC **3,** 353(1920).

### B. Indirect Method

Difference between sol. S and sum of thiosulfate S and sulfate S = sulfide S.

## 920.34 Lime Sulfur Solutions and Dry Lime Sulfur

**Final Action**

### A. Sulfate Sulfur

Slightly acidify soln from **920.32B** with HCl (1+4) and heat to bp. Add slowly, with const stirring, slight excess *10% $BaCl_2$ soln*, boil 30 min, let stand overnight, and filter. Calc. S from wt $BaSO_4$, and report as % sulfate S.

### B. Total Calcium

To 25 mL prepd soln, **920.31A(a)** or **(b)**, add 10 mL HCl, evap. to dryness on steam bath, add $H_2O$ and few mL HCl (1+4), warm until all $CaCl_2$ dissolves, and filter to remove S and any $SiO_2$ present. Dil. filtrate to 200–250 mL, heat to bp, add few mL $NH_4OH$ in excess, and then add excess *satd $(NH_4)_2C_2O_4$ soln*. Continue boiling until pptd $CaC_2O_4$ assumes well defined granular form, let stand 1 hr, filter, and wash few times with hot $H_2O$. Ignite at 950° in Pt crucible to const wt (CaO) and calc. % Ca. CaO × 0.7147 = Ca.

CAS-7440-70-2 (calcium)

## 935.07 Sodium Hypochlorite Solution Pesticide Formulations

**Final Action**

**Sodium Hypochlorite**

**Arsenious Oxide Titration Method**

### A. Reagents

**(a)** *Arsenious oxide std soln.*—0.1*N*. Prep. as in **939.12**.

**(b)** *Iodine std soln.*—Prep. as in **939.13A**. Stdze against **(a)**.

### B. Determination

Transfer 20 mL sample to 1 L vol. flask and dil. to vol. Pipet 50 mL aliquot of mixt. into 200 mL erlenmeyer. Add excess $As_2O_3$ soln and then decided excess $NaHCO_3$. Titr. excess $As_2O_3$ with std I soln, using starch soln, **922.03A(f)**, or the I as its own indicator. Subtract vol. I soln, corrected to 0.1*N*, from vol. $As_2O_3$ soln used, and from this value and sp gr of soln, calc. % NaOCl.

$$1 \text{ mL } 0.1N\ As_2O_3 = 0.003722 \text{ g NaOCl}$$

Refs.: JAOAC **18,** 63(1935).

CAS-7681-52-9 (sodium hypochlorite)

## 935.08 Sodium Hypochlorite Solution Pesticide Formulations

**Final Action**

### A. Chlorine (Available)

Calc. % available Cl from titrn, **935.07B**. 1 mL 0.1*N* $As_2O_3$ = 0.003545 g available Cl.

### B. Chlorine (Chloride)

Pipet 50 mL aliquot prepd soln, **935.07B**, into 200 mL erlenmeyer and add slight excess $As_2O_3$ soln, **935.07A(a)**, calcd from NaOCl titrn; add slight excess $HNO_3$, neutze with *$CaCO_3$*, and titr. with 0.1*N* $AgNO_3$, **941.18**, using $K_2CrO_4$ soln,

**941.18B(b)**, or the $Ag_3AsO_4$ formed in soln, as indicator. Det. blank on reagents and correct for any Cl found. From this corrected titrn and sp gr of sample, calc. % Cl. From this value subtract $^1/_2$ the % available Cl. Difference = % chloride Cl.

CAS-7782-50-5 (chlorine)

### *C. Sodium Hydroxide*

Stdze pH meter equipped with calomel and glass electrodes, using std pH 6.9 buffer soln, **964.24(d)**.

Place 50 mL 10% $BaCl_2.2H_2O$ soln and 30 mL 3% $H_2O_2$ soln in 250 mL beaker. Neutze to pH 7.5 with ca 0.1*N* NaOH, using pH meter. Add 10 mL sample from pipet, stir vigorously 1 min, and titr. to pH 7.5 with stdzd 0.1*N* HCl, using pH meter.

% NaOH = (mL HCl × normality × 4.0)/(mL sample × sp gr)

Ref.: JAOAC **18,** 63(1935); **43,** 346(1960).

CAS-1310-73-2 (sodium hydroxide)

### *D. Carbon Dioxide⋆ —Surplus 1965*

*See* **4.158–4.159**, 10th ed.

## 935.09 Chlorine (Available) in Calcium Hypochlorite and Bleaching Powder

**Arsenious Oxide Titration Method**

**Final Action**

Weigh 5–10 g thoroly mixed sample into porcelain mortar, add 30–40 mL $H_2O$, and triturate to smooth cream (high-test $Ca(OCl)_2$ will dissolve readily and not form a cream). Add more $H_2O$, stir well with pestle, and let insol. residue settle few moments. Pour mixt. off into 1 L vol. flask, add more $H_2O$, and thoroly triturate sample and pour off as before. Repeat operation until all material is transferred to flask. Rinse mortar and pestle, catch wash $H_2O$ in flask, dil. to vol., and mix. Without letting material settle, pipet 25–50 mL aliquot into 200 mL erlenmeyer. Add excess std $As_2O_3$ soln, **935.07A(a)**, and then decided excess of $NaHCO_3$. Titr. excess $As_2O_3$ with std I soln, **935.07A(b)**, using starch soln, **922.03A(f)**, or I as its own indicator. Subtract vol. I soln, corrected to 0.1*N*, from vol. $As_2O_3$ soln used, and calc. % available Cl. 1 mL 0.1*N* $As_2O_3$ = 0.003545 g available Cl.

Refs.: JAOAC **18,** 65(1935).

CAS-7778-54-3 (calcium hypochlorite)

## 935.10 Chloramine T Pesticide Formulations

**Final Action**

### *A. Active Chlorine*

Transfer 0.5 g sample to 300–500 mL erlenmeyer, dissolve in 50 mL $H_2O$, and add excess std $As_2O_3$ soln, **935.07A(a)**, and 5 mL $H_2SO_4$ (1+4). Add decided excess $NaHCO_3$ and titr. excess $As_2O_3$ with std I soln, **935.07A(b)**, using starch soln, **922.03A(f)**, or I as its own indicator. From this titrn, calc. active Cl in sample. 1 mL 0.1*N* $As_2O_3$ = 0.001773 g active Cl. (To convert active Cl to available Cl, multiply active Cl by 2.)

### *B. Total Chlorine*

Dissolve 0.5 g sample in 50 mL $H_2O$ in erlenmeyer and add slight excess std $As_2O_3$ soln as calcd from active Cl titrn, **935.10A**. Add 5 mL $HNO_3$ (1+4), neutze with *$CaCO_3$*, and titr. with std $AgNO_3$, **941.18**, using $K_2CrO_4$, **941.18B(b)**, as indicator. Det. blank on reagents and correct for any Cl found. From corrected titrn, calc. % total Cl in sample. 1 mL 0.1*N* $AgNO_3$ = 0.003545 g Cl. If total Cl exceeds active Cl, NaCl is indicated.

### *C. Sodium⋆ —Surplus 1974*

*See* **6.183**, 11th ed.

Refs.: JAOAC **18,** 63(1935).

## 927.01 Unsulfonated Residue of Mineral Oils (1)

**Final Action**

### *A. Reagent*

(*Caution:* See safety notes on sulfuric acid and fuming acids.)

*Fuming 38N sulfuric acid.*—In tared g-s bottle (2.5 L acid bottle is convenient) mix fuming $H_2SO_4$ (free from N oxides) (*x*) with $H_2SO_4$ (*y*) to obtain mixed acid (*z*), contg slightly >82.38% total $SO_3$. Depending on strength of fuming acid available, use following proportions of 2 acids: 100 parts *x* (15–20% free $SO_3$) to 50 parts *y*; 100 parts *x* (20–30% free $SO_3$) to 75 parts *y*; or 100 parts *x* (50% free $SO_3$) to 140 parts *y*. Mix thoroly (considerable heat is generated), let cool, and again weigh to det. amt mixed acid obtained. Det. exact strength of mixed acid (2) and also of reserve supply of acid (*y*) as follows:

Pour ca 50 mL into small beaker and fill ca 10 mL weighing bulb or pipet by slight suction, wiping off outside of bulb with moist, then with dry, cloth. Weigh on analytical balance and let acid flow slowly down sides of neck of 1 L vol. flask into ca 200 mL cold $H_2O$. (These sizes of bulb and flask give final soln ca 0.5*N*.) When bulb has drained, wash all traces of acid into flask, taking precautions against loss of $SO_3$ fumes. Dil. to vol. and titr. from buret with std alkali, using same indicator as used in stdzg. Calc. $SO_3$ content of both acids, and add calcd amt of reserve acid (*y*) to amt of mixed acid (*z*) on hand to bring *z* to 82.38% total $SO_3$ (equiv. to 100.92% $H_2SO_4$). After adding required amt of *y*, again analyze mixed acid to make certain it is of proper concn (±0.15% $H_2SO_4$). Keep acid in small bottles or in special dispenser bottle (*2*) to prevent absorption of $H_2O$ from air.

### *B. Determination*

Pipet 5 mL sample into 6″ Babcock cream bottle, **938.01B(b)**, either 9 g 50% or 18 g 30% type. To reduce viscosity of heavy oils, warm pipet after initial drainage by passing it several times thru flame; then drain thoroly. If greater accuracy is desired, weigh measured sample and calc. exact vol. from wt and sp gr. Slowly add 20 mL 38*N* $H_2SO_4$, gently shaking or rotating bottle and taking care that temp. does not rise above 60°. Cool in ice-$H_2O$ if necessary. When mixt. no longer develops heat on shaking, agitate thoroly, place bottle in $H_2O$ bath, and heat 10 min at 60–65°, keeping contents of bottle thoroly mixed by shaking vigorously 20 sec at 2 min intervals. Remove bottle from bath and add $H_2SO_4$ until oil is in graduated neck. Centrf. 5 min (or longer if necessary to obtain const vol. of oil) at 1200–1500 rpm. Read vol. of unsulfonated residue from graduations on neck of bottle and, to convert to mL, multiply reading from 9 g 50% bottle by 0.1 and reading from 18 g 30% bottle by 0.2. From result obtained, calc. % by vol. of unsulfonated residue.

Refs.: (1) JAOAC **10,** 30, 124(1927); **11,** 35(1928).
(2) USDA Bull. **898,** p. 48.

## 926.01 Soaps
**Final Action**

### A. Moisture

Weigh ca 20 g sample into 300–500 mL flask; add 50 mL toluene (tech. grade is satisfactory); and, to prevent foaming, add ca 10 g lump rosin (do not use powd). Distil into Dean and Stark type distg tube receiver and continue distn until no more $H_2O$ collects in receiver. Cool contents of tube to room temp., read vol. $H_2O$ under toluene in tube, and calc. % $H_2O$.

Ref.: JAOAC **9,** 27(1926).

### B. Sodium and Potassium★ —Surplus 1974

*See* **6.094**, 11th ed.

## 926.02 Mineral Oil-Soap Emulsions
**Final Action**

### A. Water

Weigh ca 25 g sample and proceed as in **926.01A**, except use less rosin.

Ref.: JAOAC **9,** 28(1926).

### B. Total Oil

Weigh ca 10 g sample into Babcock cream bottle, **920.111B(a)**. Dil. with ca 10 mL hot $H_2O$ and add 5–10 mL $H_2SO_4$ (1+1). Heat in hot $H_2O$ bath ca 5 min to hasten sepn of oil, add enough satd NaCl soln to bring oil layer within graduated neck of bottle, centrf. 5 min at 1200 rpm, and let cool. Read vol. of oil layer, det. density, and from these values calc. wt and %. From this % value deduct % fatty acids (and phenols if present), detd sep., to obtain % oil.

Ref.: USDA Bur. Chem. Bull. **105,** p. 165.

### C. Soap

(Error will result if apparent mol. wt of fatty acids varies appreciably from that of oleic acid.)

Weigh 20 g sample into separator, add 60 mL pet ether, and ext mixt. once with 20 mL and 4 times with 10 mL 50% alcohol. Break emulsion if necessary by letting 1 or 2 mL 20% NaOH soln run down wall of separator. Then gently swirl separator and let stand few min. Drain alc. layers and wash successively thru pet ether contained in 2 other separators. Combine alc. exts in beaker and evap. on steam bath to remove alcohol. Dissolve residue in ca 100 mL $H_2O$ made alk. with NaOH. Transfer to separator, acidify with HCl or $H_2SO_4$, ext 3 times with Et ether, and wash ether exts twice with $H_2O$. Combine ether exts, evap. in weighed beaker on steam bath, and weigh as fatty acids. From wt fatty acids, calc. % soap in sample as Na or K oleate.

Ref.: JAOAC **9,** 28(1926).

### D. Unsulfonated Residues

Using 5 mL of the recovered oil, **926.02B**, proceed as in **927.01B**.

### E. Ash

Evap. 10 g sample, or more if necessary, in Pt dish. Ignite, and leach charred mass with $H_2O$. Ignite residue, add leachings, evap. to dryness, ignite, and weigh. From this wt, calc. % ash. Test ash for Cu, Ca, $CaF_2$, etc.

Ref.: JAOAC **9,** 29(1926).

## 930.13★ Mercury in Organic Mercurial Seed Disinfectants
**Volatilization Method**
**Final Action**
**Surplus 1965**

*See* **4.150–4.151**, 10th ed.

## 930.14★ Mercury in Organic Mercurial Seed Disinfectants
**Precipitation Method**
**Final Action**
**Surplus 1974**

*See* **6.173**, 11th ed.

## 971.04 Mercury in Organic Mercurial Seed Disinfectants
**Titrimetric Method**
**First Action 1971**
**Final Action 1974**

### A. Principle

Sample is digested under $H_2O$-cooled condenser with fuming $H_2SO_4$-fuming $HNO_3$. Hg is detd by titrn with std SCN soln with ferric alum as indicator. Small amts of chloride are oxidized to Cl and expelled thru condenser. Not applicable in presence of large amts of Cl-contg materials.

### B. Reagents

(**a**) *Ferric indicator.*—Dissolve 8 g $FeNH_4(SO_4)_2.12H_2O$ in 80 mL $H_2O$. Add enough $HNO_3$ to destroy brown Fe color and dil. to 100 mL with $H_2O$.

(**b**) *Ferrous sulfate soln.*—Dissolve 1 g $FeSO_4.7H_2O$ in $H_2O$, add 1 mL $H_2SO_4$, and dil. to 100 mL with $H_2O$. Prep. fresh for each detn.

### C. Preparation of Sample

(**a**) *Solns.*—Mix thoroly and weigh, by difference, amt sample (max. 10 g) contg preferably 0.07 g Hg into 500 mL ₮ erlenmeyer.

(**b**) *Dusts.*—Mix thoroly and, using glass weighing dish, weigh amt sample as in (**a**). Transfer thru powder funnel into 500 mL ₮ erlenmeyer.

### D. Determination

(*Caution:* Conduct detn in well ventilated hood. Method is dangerous in presence of material which reacts violently with $H_2SO_4$ and/or $HNO_3$. See safety notes on wet oxidation, nitric acid, sulfuric acid, fuming acids, and mercury salts.)

Connect straight-tube, $H_2O$-cooled condenser to erlenmeyer contg sample. Place flask in cold $H_2O$ bath. Carefully add 10 mL $H_2SO_4$ thru top of condenser and mix by swirling. Add in small portions, swirling after each addn, 30–40 mL *fuming $H_2SO_4$* (20% free $SO_3$) thru top of condenser, followed by 10 mL red *fuming $HNO_3$* (98% $HNO_3$). Remove from bath and dry outside of flask. Heat with small flame to reflux at ca 30 drops/min with red fumes persisting in flask and condenser. Heat 30 min; if small amt chloride is present, heat 2 hr with occasional addn of fuming $HNO_3$ as required. Cool, and add 100 mL cold $H_2O$ slowly thru top of condenser while cooling flask in cold $H_2O$ bath. Add 2 or 3 glass beads or boiling chips and boil until N oxides have been expelled to top of condenser (ca 2 min). Wash condenser with 50 mL cold $H_2O$, disconnect flask, and add *satd $KMnO_4$ soln* until color remains purple. (If large amts insol. material are present, filter hot soln thru medium tight asbestos mat in gooch before addn of $KMnO_4$. Wash flask and filter 5 times with hot $H_2O$, and then add $KMnO_4$.) Cool flask, and destroy $KMnO_4$ with fresh 1% $FeSO_4$ soln. Add 10 mL ferric indicator and titr. with 0.1*N* $NH_4SCN$ or KSCN, **942.26**, to appearance of first permanent faint orange. 1 mL 0.1*N* $NH_4SCN$ or KSCN = 0.01003 g Hg.

Ref.: JAOAC **54**, 685(1971).

CAS-7439-97-6 (mercury)

## 973.11 Mercury in Organic Mercurial Seed Disinfectants

**Gravimetric Method**

**First Action 1973**
**Final Action 1975**

***AOAC-CIPAC Method***

(Applicable in presence of large amts Cl-contg materials; not applicable to chloro- or nitrophenols nor to materials not decomposed by digestion mixt.)

### *A. Reagents*

(**a**) *Dilute sulfuric acid.*—Add 30 mL $H_2SO_4$ to $H_2O$ in 100 mL vol. flask, cool, and dil. to vol. with $H_2O$.

(**b**) *Sodium sulfite soln.*—10%. Dissolve 10 g $Na_2SO_3$ in $H_2O$ in 100 mL vol. flask and dil. to vol. with $H_2O$.

(**c**) *Ammonium citrate soln.*—Should have sp gr of 1.09 at 20° and pH of 7.0 as detd potentiometrically.

Dissolve 370 g cryst. citric acid in 1.5 L $H_2O$ and nearly neutze by adding 345 mL $NH_4OH$ (28–29% $NH_3$). If concn of $NH_3$ is 28%, add correspondingly larger vol. and dissolve citric acid in correspondingly smaller vol. $H_2O$. Cool, and check pH. Adjust with $NH_4OH$ (1 + 7) or citric acid soln to pH 7. Dil. soln, if necessary, to sp gr of 1.09 at 20°. (Vol. will be ca 2 L.) Keep in tightly stoppered bottles and check pH from time to time. If pH has changed from 7.0, readjust.

(**d**) *Precipitating reagent.*—Add 20 mL 1,2-propanediamine (Eastman Kodak Co., P3170) to 100 mL 1*M* $CuSO_4$ soln. Store in g-s container.

(**e**) *Wash soln.*—Add 1 g KI and 2 mL pptg reagent to 1 L $H_2O$.

### *B. Preparation of Sample*

(**a**) *Solns.*—Mix thoroly and weigh, by difference, sample (max. 5 g) contg 0.02–0.08 g Hg into 125 mL ₮ erlenmeyer.

(**b**) *Dusts.*—Mix thoroly and, using glass weighing dish, weigh sample as in (**a**). Transfer thru powder funnel into 125 mL ₮ erlenmeyer.

### *C. Determination*

(*Caution:* Conduct detn in well ventilated hood.)

Add to sample in following order: 5 mL *ethylene glycol,* swirling to thoroly suspend solids, 4 g KI, 10 mL dil. $H_2SO_4$, 0.4 g I, and 2 glass beads. After thoro mixing, connect straight-tube, $H_2O$-cooled condenser and, with low flame, heat to slight boil so that liq. condenses in lower portion of condenser. Swirl occasionally, avoiding excessive heat and crystn of large amt I in condenser. Reflux 1 hr and, while cooling flask in $H_2O$ bath, immediately wash warm condenser with heavy stream of ca 25 mL $H_2O$. (If dye or I persists in condenser, loosen by reheating flask contents, without $H_2O$ in condenser, until liq. refluxes slightly beyond adhering material. Wash condenser again with ca 25 mL $H_2O$, and cool flask.) Disconnect condenser and wash connections directly into flask. Add ca 2 mL 10% $Na_2SO_3$ dropwise, with swirling, until I color slightly lightens. (Excess I must be present.) Neutze soln with $NH_4OH$, using pH test paper, until very slightly alk. (pH 7.0–7.3). Cool, and filter with vac. thru retentive paper (S&S Blue Ribbon, or equiv.) in buchner into 400 mL beaker. Wash flask and paper thoroly, keeping total filtrate <150 mL. Add 50 mL $NH_4$ citrate soln, bring mixt. just to bp, and stir in 5 mL pptg reagent. Cool and let stand ≥2 hr (preferably overnight); filter thru medium porosity glass crucible, previously dried at 105° and weighed. Transfer ppt with wash soln, and wash with same soln several times. Rinse I from ppt with ca 25 mL alcohol in 5 mL portions (some samples may require up to 50 mL) until filtrate is colorless. (Let alcohol stand few min with occasional swirling after each addn before applying suction. Ppt should be suspended in liq. each time.) Wash ppt with three 5 mL portions $CHCl_3$, suspending ppt each time as above until dye and pesticides are completely removed. Finally wash with 5 mL alcohol, dry 30 min at 105°, cool, and weigh.

$$\text{Wt Hg} = \text{wt ppt} \times 0.218$$

Ref.: JAOAC **56**, 572(1973); **58**, 309(1975).

CAS-7439-97-6 (mercury)

## 948.03★ Alpha-Naphthylthiourea in Rodenticide Formulations

**First Action**
**Surplus 1965**

*See* **4.132**, 10th ed.

## 939.01 Thallous Sulfate in Rodenticide Formulations

**Final Action**

(*Caution:* See safety notes on asbestos, wet oxidation, nitric acid, fuming acids, and pesticides.)

Weigh sample contg 0.1–0.15 g $Tl_2SO_4$ (usually 10 g), transfer to 800 mL Kjeldahl flask, and add 25 mL $H_2SO_4$ followed by 5–10 mL $HNO_3$. After first violent reaction ceases, heat until white fumes of $H_2SO_4$ appear. Add few drops *fuming $HNO_3$* and continue heating and adding $HNO_3$ until org. matter is destroyed, as shown by colorless or light yellow soln. Cool, add 10–15 mL $H_2O$, again cool, and wash contents of flask into 400 mL beaker, continuing washing until vol. is 60–70 mL. Boil several min to remove all $HNO_3$, cool, and filter into 400 mL beaker. Wash with hot $H_2O$ until vol. in beaker is 175 mL, neutze with $NH_4OH$, and then slightly acidify with $H_2SO_4$ (1+4). Add 1 g *$NaHSO_3$* to ensure reduction of thallic to thallous state. Heat to bp, add 50 mL *10% KI soln,* stir, and let stand overnight. Filter thru tight gooch contg 2 disks S&S 589 white ribbon paper covered by medium pad of asbestos. Wash 4 or 5 times with 10 mL portions *1% KI soln,* and finally with absolute alcohol. Dry to const wt at 105° (1–1.5 hr), and weigh as TlI.

$$\% \ Tl_2SO_4 = (\text{g TlI} \times 0.7619 \times 100)/\text{g sample}$$

Refs.: JAOAC **22,** 411(1939); **25,** 79(1942); **28,** 72(1945).

CAS-7446-18-6 (thallous sulfate)

## 966.05 Fumigant Mixtures

### Gas Chromatographic Method

**First Action 1966**
**Final Action 1985**

(Applicable to org. components of $CS_2$, $CCl_4$, $(CH_2)_2Cl_2$, and $(CH_2)_2Br_2$ mixts. *Precautions:* Handle with care in hood or well-ventilated area. Mixts are volatile, poisonous, and sometimes flammable and may be fatal if inhaled or swallowed. They cause skin and eye irritation. In case of contact, immediately remove contaminated clothing and flush affected area with copious amts of $H_2O$. Do not reuse clothing until free of contamination. Do not use containers or equipment of Al, Mg, or their alloys.)

#### A. Principle

Components are detd by GC. Peak area of each component is measured and compared to stds of same fumigant mixt. Precision of method is ±0.6% for each component.

#### B. Sampling

Obtain representative 1 L sample from container. Sample bulk containers by means of weighted bottle, lowered toward bottom and raised at such rate that it is $^3/_4$ full when withdrawn. Sample drums or small containers with thief or thru tap or valve located so that sample comes from well below surface. Prevent contamination of product or sample.

Place sample in clean, dry, and solv. vapor-tight glass bottle of such size that it is nearly filled (not above shoulder) by sample. Vapor-tight g-s bottles or screw-cap bottles with Sn foil lined caps are satisfactory. Store samples at low temp.; cool to <18° before opening for analysis.

#### C. Apparatus

(**a**) *Gas chromatograph.*—With flame ionization or thermal conductivity detector. Typical operating conditions: Column temp., 110°; injection port temp., 200°; flow rate, 80 mL He/min.

(**b**) *Recorder.*—0.05–1.05 mv, full scale response. Integrator may be used.

(**c**) *Syringe.*—Hamilton Co. 10 μL No. 701N, or equiv.

(**d**) *Column.*—1.2 m (4′) stainless steel, $^1/_4$″ od, 0.194″ id, packed with reagent **966.05D(a)**. Max. temp. is 160°. Other columns can be used but chromatgc conditions and sample size must be adjusted in accordance with column requirements. One such column is: 3 m (10′) stainless, $^3/_{16}$″ od, 0.12″ id, packed with 20% by wt *N,N*-bis-(2-cyanoethyl) formamide on 80–100 mesh Chromosorb W, acid-washed. Columns are available from com. suppliers. Criterion for use is emergence of each component of mixt. of $CS_2$, $CCl_4$, $(CH_2)_2Cl_2$, and $(CH_2)_2Br_2$ as sep. peak.

#### D. Reagents

(**a**) *Column packing.*—30% by wt tricresyl phosphate on Chromosorb P, 30–60 mesh.

(**b**) *Carbon disulfide std.*—ACS.

(**c**) *Carbon tetrachloride std.*—ACS.

(**d**) *Ethylene dichloride std.*—Purified 1,2-dichloroethane, available from laboratory supply houses, or use center cut of fractionation of com. product.

(**e**) *Ethylene dibromide (1,2-dibromoethane) std.*— Purified or distd as in (**d**).

#### E. Preparation of Standards

Prep. fresh stds just before analysis which approximate expected composition, by wt, of fumigant mixt. Place proper wt of each component in 25 mL g-s vol. flask and mix well. Do not prep. by vol. Cool $CS_2$ to prevent loss. Adjust wt stds to detector response.

Carefully fill weighed 10 mL vol. flask to mark with prepd std and weigh. Use this wt to det. g/5 μL values for each component of std.

#### F. Determination

Purge column thoroly at 110° before use. Establish 0 baseline at full sensitivity. Inject 5 μL std fumigant mixt. into chromatograph. Attenuate successively so that each peak is at max. % of chart scale, adjusting sample size and attenuation, if necessary. Repeat injection. Detd area for each component, corrected for any baseline drift, should differ by ≤1%. Order of elution from column is: $CS_2$, $CCl_4$, $(CH_2)_2Cl_2$, and $(CH_2)_2Br_2$. Total analysis time is ca 21 min.

Inject 5 μL sample into chromatograph. Det. corrected area of each component from chromatogram, or note integrator reading.

g Component=$S \times C/B$, where $S$ = wt component in std, $B$ = area for component in std, and $C$ = area for component in sample. Perform calcn for each component in sample.

% Component = g component in sample × 100/ sum of g components in sample

Last equation is not applicable in presence of unmeasured contaminants.

Refs.: JAOAC **48,** 576(1965); **49,** 207(1966).

CAS-75-15-0 (carbon disulfide)
CAS-56-23-5 (carbon tetrachloride)
CAS-106-93-4 (ethylene dibromide)
CAS-107-06-2 (ethylene dichloride)

# PESTICIDES RELATED TO NATURAL PRODUCTS AND THEIR SYNERGISTS

## 953.05 Allethrin (Technical) and Pesticide Formulations

### Titrimetric Method

**First Action**

(*Caution:* See safety notes on on pesticides.)

#### A. Principle

Allethrin reacts quant. with ethylenediamine to form chrysanthemum monocarboxylic acid which is detd by titrn with std NaOMe in pyridine. Chrysanthemum monocarboxylic acid, anhydride, and acid chloride interfere quant. and are detd independently.

### B. Reagents

(**a**) *Absolute alcohol*.—SDF No. 2-B is satisfactory.

(**b**) *Methanolic hydrochloric acid std soln*.—0.1*N*. Dil. 17 mL HCl (1+1) to 1 L with anhyd. MeOH. Stdze against std 0.1*N* NaOH, using phthln. If used at temp., *T*, different from that at which stdzd, $T_0$, calc. corrected normality = $N[1-0.001(T - T_0)]$.

(**c**) *Sodium methylate std soln*.—0.1*N* in pyridine. Transfer 50 mL 2*N* NaOMe (*Caution*: See safety notes on sodium biphenyl, methylate, and ethylate.) to 1 L bottle contg 75 mL anhyd. MeOH and dil. to 1 L with redistd pyridine. Stdze against NBS benzoic acid, using pyridine as solv. and thymolphthalein, (**i**), as indicator. Dispense from 50 mL automatic buret with vents connected to Ascarite tubes. Stdze daily against std methanolic HCl, (**b**).

(**d**) *Methanolic potassium hydroxide std soln*.— 0.02*N*. Dissolve 1.12 g KOH in 1 L MeOH. Stdze as in **926.16D**.

(**e**) *Morpholine soln*.—Transfer 8.7 mL redistd morpholine to 1 L bottle and dil. to 1 L with anhyd. MeOH. Fit bottle with 2-hole rubber stopper; thru 1 hole insert 20 mL pipet so that tip extends below surface of liq., and thru other hole insert short piece of glass tubing to which is attached aspirator bulb.

(**f**) *Ethylenediamine*.—Redistd com. grade contg <3% $H_2O$. Dispense from automatic buret with vents connected to Ascarite tubes.

(**g**) *Dimethyl yellow-methylene blue mixed indicator*.—Dissolve 1 g dimethyl yellow (*p*-dimethylaminoazobenzene; *caution*: see safety notes on carcinogens) and 0.1 g methylene blue in 125 mL anhyd. MeOH.

(**h**) *α-Naphtholbenzein indicator*.—1% alc. soln.

(**i**) *Thymolphthalein indicator*.—1% pyridine soln.

### C. Determination of Chrysanthemum Monocarboxylic Acid Chloride

Add 8–10 drops mixed indicator, (**g**), to ca 150 mL anhyd. MeOH and add 0.1*N* HCl, (**b**), dropwise until soln appears reddish brown by transmitted light. Add 0.02*N* KOH, (**d**), dropwise until appearance of first green. Transfer 25 mL to each of three 125 mL g-s erlenmeyers, reserving 1 flask as ref. color for end point. Into each of other flasks add 1.5–2.5 g sample from weighing pipet, swirling flask while adding sample. Within 5 min, titr. with 0.02*N* KOH, (**d**), to first green end point, using blank as ref. color. Calc. milliequiv. chrysanthemum monocarboxylic acid chloride/g sample, $C = V \times N$/g sample, where $V$= mL *N* normal KOH required;

% Chrysanthemum monocarboxylic acid chloride = $C \times 18.67$

### D. Determination of Chrysanthemum Monocarboxylic Acid

Transfer 25 mL anhyd. alcohol to each of two 125 mL g-s erlenmeyers, add 8–9 drops α-naphtholbenzein indicator, and cool to 0° in ice bath. Neutze by adding 0.02*N* NaOH dropwise to bright green end point. To each flask add 1.5–2.5 g sample from weighing pipet. Immediately titr. with 0.02*N* NaOH, **936.16C**, to first bright green end point. Calc. milliequiv. chrysanthemum monocarboxylic acid and acid chloride/g sample: $D = X \times N$/g sample, where $X$ = mL *N* normal NaOH required; $(D - C) \times 16.82$ = % chrysanthemum monocarboxylic acid.

### E. Determination of Chrysanthemum Monocarboxylic Anhydride

Pipet 20 mL morpholine soln, (**e**), into each of four 250 mL erlenmeyers, using same pipet. Fill pipet by exerting pressure in bottle with aspirator bulb. Reserve 2 flasks for blanks; into each of other flasks add 1.5–2.5 g sample from weighing pipet. Swirl flasks and let samples and blanks stand 5 min at room temp. Add 4–5 drops mixed indicator, (**g**), to each flask and titr. with 0.1*N* HCl, (**b**), until color changes from green to faint red when viewed by transmitted light. Calc. milliequiv. chrysanthemum monocarboxylic anhydride/g sample: $E = (B - Y) \times N$/g sample, where $Y$ = mL *N* normal HCl required for sample, and $B$ = mL *N* normal HCl required for blank; $(E - 2C) \times 31.84$ = % chrysanthemum monocarboxylic anhydride.

### F. Determination of Allethrin

Add sample contg 0.8–1.1 g allethrin to each of two 250 mL erlenmeyers from weighing pipet. To each of 2 flasks as blanks and to samples add 25 mL ethylenediamine, (**f**), with swirling. Let samples and blanks stand 2 hr at 25±2°. Wash down sides of flasks with 50 mL redistd pyridine. To each flask add 6–10 drops thymolphthalein indicator, (**i**), and titr. with 0.1*N* NaOMe, (**c**), to first permanent blue-green end point. (With colorless samples, first blue end point may be used.) Calc. milliequiv. allethrin/g sample: $F = (Z - B) \times N$/g sample, where $Z$ = mL *N* normal NaOMe required for sample, and $B$ = av. mL *N* normal NaOMe required for blank; $(F + C - D - E) \times 30.24$ = % allethrin.

Refs.: Anal. Chem. **25**, 1207(1953). JAOAC **40**, 732(1957).

CAS-37-98-4 (allethrin)

## 973.12 *d-trans*-Allethrin in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1973**
**Final Action 1978**

(*Caution:* See safety notes on pesticides.)

### A. Principle

*d-trans*-Allethrin is dild in acetone contg dibutyl phthalate as internal std. Ratios of GC peak hts of *d-trans*-allethrin and dibutyl phthalate in sample and std are compared for quant. detn. Method is applicable to both tech. *d-trans*-allethrin and various formulations of it. Not applicable to formulations contg large amt MGK Repellent 874 (2-hydroxyethyl-*n*-octyl sulfide).

### B. Apparatus and Reagents

(**a**) *Gas chromatograph*.—Equipped with flame ionization detector and 1.2 m (4′) × 4 mm id glass column packed with 5% OV-1 (Analabs, Inc.) on 80–100 mesh Chromosorb W (HP). Operating conditions: temps (°)—column 165, injection port 230, detector 230; gas flows (mL/min)—N carrier gas 125, air 350–400, H 40–50; sensitivity—$10^{-9}$ amp full scale, attenuation 4× for tech. material, $10^{-9}$ amp full scale, attenuation 1 for formulations. Before use, condition column 2–3 hr at 275° with N flow 50 mL/min. If necessary, vary column temp. or gas flow to attain retention times of ca 4 and 7 min for internal std and *d-trans*-allethrin, resp. Also vary detector sensitivity or injection vol. to attain ≥100 mm peak ht for each compd (ca 16 μg *d-trans*-allethrin). Theoretical plates/ft must be >200.

Calc. theoretical plates/ft ($N$) as follows: $N = 16L^2/(M^2 \times F)$, where $L$ = retention of GC peak in mm; $M$ = peak baseline produced by drawing tangents to points of inflection of peak; and $F$ = length of column (ft).

(**b**) *Internal std soln*.—4.0 mg dibutyl phthalate/mL acetone.

(**c**) *d-trans-Allethrin std solns*.—(*1*) *Soln 1*.—Approx. 4 mg/mL. Accurately weigh ca 1.0 g *d-trans*-allethrin (available from McLaughlin Gormley King Co., 8810 Tenth Ave N, Minneapolis, MN 55427) into 50 mL vol. flask and dil. to vol. with acetone. Pipet 20 mL this soln into 100 mL vol. flask, add 50 mL internal std soln by pipet, and dil. to vol. with acetone. Use this soln for detn of tech. material. (2) *Soln 2*.—Approx. 1 mg/mL. Pipet 25 mL *Soln 1* into 100 mL vol. flask and dil. to vol. with acetone. Use this soln for detn of *d-trans*-allethrin in formulations.

### C. Preparation of Sample

(**a**) *Technical material*.—Accurately weigh sample contg ca 1.0 g *d-trans*-allethrin into 50 mL vol. flask and dil. to vol. with acetone. Pipet 20 mL aliquot into 100 mL vol. flask, add 50 mL internal std soln by pipet, and dil. to vol. with acetone.

(**b**) *Formulations*.—Accurately weigh sample contg ca 200 mg*d-trans*-allethrin into 50 mL vol. flask, add 25 mL internal std soln by pipet, and dil. to vol. with acetone. Pipet 25 mL aliquot into 100 mL vol. flask and dil. to vol. with acetone.

### D. Gas Chromatography

(**a**) *Technical material*.—Inject aliquots (ca 3 μL) std *Soln 1* until ratio of *d-trans*-allethrin:dibutyl phthalate peak hts varies <1% for successive injections. Repeat with sample soln, followed by duplicate injections of std soln. If peak ht ratios differ >±1% from previous std injections, repeat series of injections.

(**b**) *Formulations*.—Proceed as in (**a**), using std *Soln 2*. Repeat std injections after each series of 3 sample injections. If peak ht ratios differ >±1.5% from previous std injections, repeat injections.

### E. Calculations

(**a**) *Technical material*.—Calc. peak ht ratios for duplicate std injections before and after sample injections and average the 4 values. Calc. and average peak ht ratios for sample injections.

$$\% \ d\text{-}trans\text{-Allethrin} = (R/R') \times (W'/W) \times P$$

where $W'$ and $W$ = g std and sample, resp.; $P$ = % purity of std; and $R'$ and $R$ = peak ht ratios of std and sample, resp.

(**b**) *Formulations*.—Calc. av. for all std peak ht ratios and for sample peak ht ratios.

$$\% \ d\text{-}trans\text{-Allethrin} = 2\ (R/R') \times (W'/W) \times (P/5)$$

Ref.: JAOAC **55**, 907(1972).

CAS-137-98-4 (allethrin)

## 985.03 Cypermethrin in Pesticide Formulations

**Capillary Gas Chromatographic Method**

**First Action 1985**
**Final Action 1987**

(Method is suitable for tech. and formulated cypermethrin.)

### A. Principle

Sample is dissolved in $CH_2Cl_2$ contg dicyclohexyl phthalate, and 1.0 μL is injected into capillary GC in split mode, with flame ionization detection. Peak areas are measured for each cypermethrin isomer and dicyclohexyl phthalate and compared with those from std injection.

### B. Apparatus

(**a**) *Capillary gas chromatograph*.—With heated, glass-lined split mode injection port, flame ionization detector, and automatic sample injector. Temps (°)—column 240, injection port 250, detector 250; gas flows (mL/min)—He carrier gas 2.75, split vent 200 (split ratio 72.6:1), septum purge 0.5–1.0, He auxiliary gas to detector 30, H 60, air 240; column head pressure 15–20 psig; sample size 1.0 μL; retention times (min)—cypermethrin isomers: *cis* A, 11.18, *trans* C, 11.55, *cis* B, 11.85, *trans* D, 12.02; internal std, 5.58. Adjust parameters to assure sepn of 4 peaks and peak hts ca 60–80% full scale on chart at quoted retention times. Sepn of isomer peaks is critical to avoid inclusion of impurity peaks in active ingredient calcn.

(**b**) *Column*.—25 m × 0.32 mm (id) fused silica column with thick film OV-1 phase (Hewlett-Packard Co., Avondale Division, Cat. No. 19091-62025). Precondition 1 h at 260° before use.

### C. Reagents

(**a**) *Dicyclohexyl phthalate internal std soln*.—Weigh 0.9 g dicyclohexyl phthalate (Pfaltz and Bauer, 172 E Aurora St, Waterbury, CT 06708), dissolve in $CH_2Cl_2$, and dil. to 500 mL. Check internal std soln for interfering components by injecting 1.0 μL into chromatograph. Store in tightly capped bottles to avoid evapn.

(**b**) *Cypermethrin std soln*.—Accurately weigh ca 100 mg cypermethrin std of known purity (ICI Americas, Inc.) into scintillation vial. Pipet 10.0 mL internal std soln into vial, cap, and shake to dissolve. Store in tightly capped bottles to avoid evapn.

### D. Determination

(**a**) *Liquid and technical samples*.—Accurately weigh amt sample contg ca 100 mg cypermethrin into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake to dissolve.

(**b**) *Powder formulations*.—Accurately weigh amt sample contg ca 100 mg cypermethrin into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake on wrist-action shaker 10 min. Let insoluble inerts settle 10 min before analysis.

Inject 2 or more 1.0 μL aliquots of std soln to optimize instrument and integration parameters and to stabilize instrument. Monitor response factor until results agree ±2%. Inject 4 aliquots of std soln and 2 aliquots of sample in succession. Calc. response factor, $R$, for each injection; and take means for std and sample for calcn:

$R$ = total area of 4 cypermethrin isomer peaks/area internal std peak

$$\text{Cypermethrin, } \% = (R/R') \times (W'/W) \times \text{P}$$

where $R$ and $R'$ = response factors for sample and std solns, resp.; $W'$ and $W$ = mg std and sample, resp.; and $P$ = purity (%) of std.

Ref.: JAOAC **68**, 592(1985).

CAS-52315-07-8 (cypermethrin)

## 986.02 Cypermethrin in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1986**

***CIPAC-AOAC Method***

### A. Principle

Sample is dissolved in methyl isobutyl ketone contg di-(ethylhexyl) phthalate as internal std; 4 cypermethrin isomers, isolated as one peak, are detd by gas chromatgy with flame ionization detection.

### B. Apparatus and Reagents

(**a**) *Gas chromatograph with recorder and integrator*.—With flame ionization detector and 1.0 m × 4 mm (id) glass column packed with 3% OV-101 on 100–120 or 80–100 mesh Chromosorb WHP, capable of on-column injection. Condition newly packed column overnight at 260° with low N flow. Operating conditions: temps—inlet 250°, column 230–240°, detector 250°; carrier gas flow to elute internal std at ca 5.5 min and cypermethrin at ca 11.5 min with ≥ 30 mm between intercepts of tangents on baseline of std and internal std peaks; adjust H and air for detector as recommended by manuf.; adjust sensitivity to give peak hts 75% full scale.

(**b**) *Di-(2-ethythexyl) phthalate (DEHP; dioctyl phthalate).*—Fisher Scientific Reagent, or equiv.

(**c**) *Methyl isobutyl ketone (MIBK).*—GC quality (J.T. Baker Inc., no. 9212, or equiv.)

### *C. Preparation of Standards*

(**a**) *Internal std soln.*—20 mg DEHP/mL. Weigh ca 10 g DEHP into 500 mL vol. flask, dil. to vol. with MIBK, and mix (soln I). Concn may be varied to accommodate column and instrument differences. If necessary, adjust concn so that peak ht or area of DEHP closely matches peak ht or area of cypermethrin within 10%.

(**b**) *Cypermethrin std soln.*—4.0 mg/mL. Warm sealed bottle of cypermethrin std (ICI-Americas, Inc.) at 40–50° until no crystals remain; shake bottle. Accurately weigh, in duplicate, ca 0.2 g std into 50 mL vol. flask, and dissolve in 3–4 mL MIBK. Pipet 10.0 mL internal std soln into each flask, dil. to vol. with MIBK, and mix (solns CA, CB). Similarly, weigh ca 0.1 g cypermethrin std into 25 mL vol. flask, dil. to vol. with MIBK, and mix (soln CO).

### *D. Preparation of Sample*

(**a**) *Technical formulations.*—Proceed as above under cypermethrin std soln, using sample wt contg ca 0.2 g cypermethrin (solns SA, SB, SO).

(**b**) *Wettable powders.*—Accurately weigh, in duplicate, sample contg 0.2 g cypermethrin into 50 mL vol. flask, pipet in 10.0 mL internal std soln. and add sufficient MIBK to suspend powder. Thoroly shake flask 10 min, dil. to vol. with MIBK, and let powder settle or centrf. until clear. Similarly, prep. soln without internal std, using sample contg ca 0.1 g cypermethrin/25 mL MIBK.

(**c**) *Ultra-low volume formulations.*—Proceed as above under technical formulations, beginning "Accurately weigh. . .".

(**d**) *Emulsifiable concentrates.*—Proceed as above under wettable powders.

### *E. System Performance Check and Determination*

Using instrument conditions listed under *Apparatus and Reagents,* inject 1.5 μL portions of solns I, CO, and SO onto column and check for interfering peaks. On-column injection is necessary. Inject std soln CA and adjust parameters to give peak ht ca 75% full scale with peak quality and elution time specified.

Inject 1.5 μL portions of std solns CA and CB until response ratio (area cypermethrin peak/area internal std peak) varies <0.5% of mean. (Area measurements by digital electronic integration are preferred over other methods.) Carry out injections of std and sample solns in following sequence: $CA_1$, $SA_1$, $SA_2$, $CB_1$, $CA_2$, $SB_1$, $SB_2$, $CB_2$. Average response ratios for sample and stds that bracket each sample. Successive response ratios should agree ± 5% of their mean. If not, repeat analysis.

$$\text{Cypermethrin, } \% = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. peak area ratios for sample and std, resp.; $W'$ = g cypermethrin in std soln; $W$ = g sample extd for analysis; and $P$ = % purity of std.

Ref.: JAOAC **70,** 51(1987).

CAS-52315-07-8 (cypermethrin)

## 986.03 Permethrin in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1986**
**Final Action 1988**

***CIPAC-AOAC Method***

### *A. Principle*

Sample is dissolved in methyl isobutyl ketone contg *n*-octacosane as internal std, and permethrin is detd as total area of 2 isomer peaks by gas chromatgy with flame ionization detection.

### *B. Apparatus and Reagents*

(**a**) *Gas chromatograph with recorder and integrator.*—With flame ionization detector and 1.0 m × 4 mm (id) glass column packed with 3% OV-210 on 100–120 or 80–100 mesh Chromosorb WHP, capable of on-column injection. Condition newly packed column overnight at 275° with low N flow. Operating conditions: temps—inlet 260°, column 190–220°, detector 250°; carrier gas flow to elute internal std in ca 4.0 min and *trans*-permethrin at ca 9.5 min with ≥30 mm between intercepts of tangents on baseline of internal std and std *cis*- and *trans*-isomer peaks; adjust H and air for detector as recommended by manuf.; adjust sensitivity to give peak hts 75% full scale.

(**b**) *n-Octacosane std.*—With no peaks at retention times of permethrin isomers (Kodak Laboratory Chemicals, or equiv.).

(**c**) *Methyl isobutyl ketone (MIBK).*—GC quality (J.T. Baker Inc., no. 9212, or equiv.).

### *C. Preparation of Standards*

(**a**) *Internal std soln.*—1 mg *n*-octacosane/mL. Weigh ca 0.5 g *n*-octacosane into 500 mL vol. flask, dissolve in 300 mL MIBK, dil. to vol. with MIBK, and mix (soln I). Concn may be varied to accommodate column and instrument differences. If necessary, adjust concn so that peak ht of *n*-octacosane closely matches peak ht of permethrin isomers.

(**b**) *Permethrin std soln.*—4.0 mg/mL. Warm sealed bottle of permethrin std (ICI Americas, Inc.) at 40–50° until no crystals remain; shake bottle. Accurately weigh, in duplicate, ca 0.1 g std into 100 mL g-s erlenmeyer. Pipet 25.0 mL internal std soln into each flask and shake until permethrin is dissolved (solns CA, CB). Similarly, weigh ca 0.1 g permethrin std into 25 mL vol. flask, dissolve in 15 mL MIBK, dil. to vol. with MIBK, and mix (soln CO).

### *D. Preparation of Sample*

(**a**) *Technical formulations.*—Proceed as above under permethrin std soln, using sample wt contg ca 0.1 g permethrin (solns SA, SB, SO).

(**b**) *Wettable and dustable powders (suspendibility >50%).*—Accurately weigh, in duplicate, sample contg 0.1 g permethrin into 100 mL g-s erlenmeyer. Pipet 25.0 mL internal std soln into flask, stopper, and shake thoroly 10 min. Let settle, filter thru Whatman No. 54 paper into g-s flask, and use filtrate for analysis. Similarly, prep. soln without internal std, using sample contg ca 0.1 g permethrin/25 mL MIBK.

(**c**) *Emulsifiable concentrates.*—Proceed as above under wettable and dustable powders.

(**d**) *Water-dispersible granules.*—Grind ca 20 g sample to fine powder and thoroly mix. Accurately weigh, in duplicate, sample contg 0.1 g permethrin into 100 mL g-s erlenmeyer. Pipet 25 mL internal std soln into flask and place in ultrasonic bath 10 min. Proceed as above under wettable and dustable powders, beginning "Let settle. . .".

### *E. System Performance Check*

Using instrument conditions listed under *Apparatus and Reagents,* inject 3 or more 1.5 μL portions of soln CA onto column and adjust paramenters to give peak ht ca 75% full scale with peak quality and elution time specified. On-column injection is necessary. Inject 1.5 μL solns I, CO, and SO and check for interfering peaks.

### F. Determination

Inject 1.5 μL std solns CA and CB until response ratio (total area of *cis*- and *trans*-permethrin peaks/area internal std peak) varies <0.5% of mean. (Area measurements by digital electronic integration are preferred over other methods.) Carry out injection of std and sample solns in following sequence: $CA_1$, $SA_1$, $SA_2$, $CB_1$, $CA_2$, $SB_1$, $SB_2$, $CB_2$. Average response ratios for sample and stds that bracket each sample. Successive response ratios should agree ± 5% of their mean. If not, repeat analysis.

$$\text{Permethrin, \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. peak area ratios for sample and std, resp.; $W'$ = g permethrin in std soln; $W$ = g sample extd for analysis; and $P$ = % purity of std.

Ref.: JAOAC **70**, 53(1987).

CAS-52645-53-1 (permethrin)

## 938.01 Rotenone in Derris and Cubé Powder

**Crystallization Method**

**Final Action**

(*Caution:* See safety notes on carbon tetrachloride and toxic dusts.)

### A. Reagents

(**a**) *Purified rotenone.*—Dissolve rotenone in boiling $CCl_4$; cool in refrigerator or ice bath at 0–10° until pptn of rotenone-$CCl_4$ solvate stops. Filter thru buchner and wash once or twice with ice-cold $CCl_4$. Conc. filtrate, crystallize, and filter as before. Transfer cryst. residue to beaker, add ca twice their vol. alcohol, and heat nearly to boiling. (Crystals need not dissolve completely.) Cool to room temp., filter thru buchner, and draw air thru cryst. residue until most alcohol is removed. Remove rotenone from funnel, dry in air, and finally heat 1 hr at 105°. Mp, detd in Pyrex, of purified material should be 163–164°. (Mother liquors may be concd and rotenone-$CCl_4$ solvate allowed to crystallize. Cryst. material may be used for further purification, or kept for prepn of wash solns or for seeding to induce crystn in detn.)

(**b**) *Rotenone-$CCl_4$ solvate.*—Ppt rotenone from $CCl_4$ soln, filter by suction, and dry in air.

(**c**) *Rotenone-$CCl_4$ wash soln.*—Sat. $CCl_4$ at 0°, and keep at 0° during use.

(**d**) *Alcohol saturated with rotenone at room temp.*

(**e**) *Charcoal, activated.*—Norit-A neutral, or equiv.

### B. Preparation of Solution

(**a**) Weigh 30 g (if sample contains >7% rotenone, use amt to give 1.0–1.5 g rotenone in 200 mL aliquot) finely powd root and 10 g of the C, (**e**), into 500 mL g-s erlenmeyer. Add 300 mL $CHCl_3$, measured at known room temp.; fasten stopper securely and place flask on shaking machine. Agitate vigorously ≥4 hr, preferably interrupting shaking with overnight rest (or flask may be shaken continuously overnight). Rapidly filter mixt. into suitable flask, using fluted paper without suction and keeping funnel covered with watch glass to avoid evapn loss. Stopper flask and adjust temp. of filtrate to that of original $CHCl_3$.

(**b**) *Alternative extraction method.*—If sample has ratio of rotenone to total ext of >0.4, use amt contg 1.0–1.5 g rotenone and successively ext 4 times with $CHCl_3$, using 300 mL $CHCl_3$ and 4 hr agitation for first extn as in (**a**) and 200 mL and 2 hr each for other extns. Filter after each extn and return marc to flask for extn with fresh solv. Finally combine exts, evap. almost to dryness, and use entire ext to det. rotenone.

(**c**) *Extraction method for formulations containing 0.75–1.0% rotenone with or without sulfur and/or pyrethrins.*—Weigh two 50 g portions sample into sep. 500 mL g-s erlenmeyers. Add 5 g of the C and 300 mL $CHCl_3$, measured at known room temp., to each. Stopper and continue as in (**a**).

### C. Determination

(*Caution:* See safety notes on distillation, pipets, acetone, carbon tetrachloride, and chloroform.)

Pipet 200 mL soln, **938.01B** (or entire soln if alternative extn, (**b**), is used), into 500 mL Pyrex erlenmeyer and distil until ca 25 mL remains. (For formulations, **938.01B(c)**: In absence of S, combine the 2 exts in one of the erlenmeyers. In presence of S, remove all $CHCl_3$ on steam bath in air current, avoiding prolonged heating. Add 35 mL acetone to each residue and boil gently on steam bath to dissolve all resins. Remove from steam bath, stopper tightly, and hold 2 hr at 0–5°. Filter both acetone solns thru same 15 mL, medium porosity, fritted glass buchner into single 500 mL erlenmeyer. Rinse and wash with acetone at 5°. Remove acetone as $CHCl_3$ was removed above.)

Evap. almost to dryness on steam bath in current of air. Remove remainder of solv. under reduced pressure, heating cautiously on steam bath when necessary to hasten evapn. (Suction may be applied directly to flask if stopper with vent is used to release pressure, so that excessive vac. may be avoided. Use flasks with slightly convex bottoms; do not use flasks below av. wt.) Dissolve ext in 15 mL hot $CCl_4$ and again, in similar manner, remove all solv. Repeat with another 10–15 mL portion hot $CCl_4$. (This treatment removes all $CHCl_3$ from resins. $CHCl_3$ ext is usually completely sol. in $CCl_4$; if small amts of insol. material are present, purification described later will eliminate them.)

Dissolve residue in ca 10 mL $CCl_4$ and transfer quant. with hot $CCl_4$ to 50 mL erlenmeyer marked at 25 mL. Adjust vol. to 25 mL by evapg on steam bath or by adding $CCl_4$. Cool flask in ice bath several min, stopper flask, and swirl until crystn is apparent. Seed with few crystals of rotenone-$CCl_4$ solvate if necessary to induce crystn. If at this stage only small amt of cryst. material seps, add accurately weighed amt of purified rotenone, **938.01A(a)**, estd to be enough to assure that final result, expressed as pure rotenone, is ≥1 g. Then warm to dissolve completely, and again induce crystn. At same time prep. satd soln of rotenone in $CCl_4$, **938.01A(c)**, for washing. Place flasks contg ext and washing soln in ice bath capable of holding temp. at 0°, and let stand overnight. (Store ice bath in refrigerator to keep ice from melting too rapidly.)

After 17–18 hr in ice bath, rapidly filter ext thru weighed gooch fitted with filter paper disk, removing flask from ice bath only long enough to pour each fraction of ext into crucible. Rinse cryst. residue from flask and wash under suction once with the ice-cold satd rotenone-$CCl_4$ wash soln. (≤12–15 mL soln should be used for rinsing and washing.) Continue suction ca 5 min; then dry to const wt at 40° (ca 1 hr). Wt obtained is crude rotenone-$CCl_4$ solvate.

Break up contents of crucible with spatula, mix thoroly, and weigh 1.000 g into 50 mL erlenmeyer. Add 10 mL alcohol previously satd with rotenone at room temp., swirl flask few min, stopper tightly, and set aside ≥4 hr, preferably overnight, at same temp. Filter on weighed gooch fitted with filter paper disk. Rinse crystals from flask and wash under suction with alcohol satd with rotenone at temp. of recrystn (ca 10 mL usually required). Continue suction 3–5 min and then dry crucible at 105° to const wt (ca 1 hr).

Multiply g residue by g total crude rotenone-$CCl_4$ solvate, and add 0.07 g to product as correction for rotenone held in soln in the 25 mL $CCl_4$ used in crystn. If any pure rotenone was added, subtract its wt from value obtained. This gives wt pure rotenone contained in aliquot of ext.

*Note:* Most important precaution in using this method is to keep temp. of $CCl_4$-rotenone wash soln and crucibles as near 0° as possible. Keep wash soln surrounded by crushed ice except when actually being used. In warm weather keep crucibles in refrigerator until ready to use.

Refs.: Ind. Eng. Chem. Anal. Ed. **10,** 19(1938). JAOAC **21,** 148(1938); **22,** 408(1939); **24,** 70(1941); **43,** 376(1960).

CAS-83-79-4 (rotenone)

## 961.03 Rotenone in Derris and Cubé Powder

### Infrared Spectroscopic Method

### First Action

(Not applicable to derris products)

### A. Standardization

Prep. std solns of purified rotenone, **938.01A(a)**, in $CHCl_3$ at concns of 5, 10, 15, and 20 mg/mL. Scan each std soln from 7.0 to 8.0 μm at speed of 6 min/μm and scale of 10 cm/μm, using 0.1 mm cell and accurately matching cell filled with $CHCl_3$ as ref. Scan each in duplicate. Obtain av. $A$ of each concn, using 7.57 μm as base point and 7.65 μm as peak. Plot $A$ against concn.

### B. Determination

Weigh sample contg 250–300 mg rotenone into 25 × 200 mm culture tube. Add 1–2 g anhyd. $Na_2SO_4$, 2 g activated charcoal, and 50 mL $CHCl_3$ by pipet. Close securely with Teflon-lined screw cap and tumble end over end 1 hr at ca 35 rpm. Filter thru medium paper, avoiding evapn losses. Transfer 20 mL aliquot to 50 mL erlenmeyer and evap. on steam bath with current of air. Transfer residue to 10 mL g-s vol. flask and dil. to vol. with $CHCl_3$. Stopper, and mix thoroly.

Scan from 7.0 to 8.0 μm, using 0.1 mm cell and matched cell filled with $CHCl_3$ as ref. Det. $A$ by baseline method from 7.57 to 7.75 μm and peak at 7.65 μm, using same scanning speed and scale expansion as in stdzn.

Calc. % rotenone from std curve and wt sample in final diln.

Refs.: JAOAC **44,** 580(1961); **46,** 668(1963); **59,** 380(1976).

CAS-83-79-4 (rotenone)

## 940.04 Ether Extract of Derris and Cubé Powder

### Final Action

(*Caution*: See safety notes on monitoring equipment, flammable solvents, diethyl ether, and peroxides.)

Ext 5 g finely powd root with ether 48 hr in Soxhlet or other efficient extn app. Conc. ext and filter off any insol. material present. Receive filtrate in tared beaker, evap. ether on steam bath, and dry in oven at 105° to const wt.

## 983.06 Rotenone in Pesticide Formulations

### Liquid Chromatographic Method

### First Action 1983

### A. Principle

Sample is extd with dioxane, and rotenone is detd by reverse phase LC with UV detection at 280 nm.

### B. Apparatus and Reagents

(**a**) *Liquid chromatograph.*—M6000A pump, U6K injector, Model 450 variable UV detector (all Waters Associates, Inc.), and Omni-Scribe recorder (Houston Instrument, 8500 Cameron Rd, Austin, TX 78753), or equiv. system. Operating conditions: column ambient; flow rate 1.0 mL/min for Partisil column, 1.5 mL/min for Zorbax column, 1.2 mL/min for Bondapak column; injection vol. 5 μL for Partisil column and 10 μL for others; detector wavelength 280 nm; absorbance range 0.4 AUFS; chart speed 1 cm/min.

(**b**) *Chromatographic columns.*—Partisil 5 ODS-3, 5 μm particle size, stainless steel, 25 cm × 4.6 mm id (Whatman Inc.). Zorbax $C_8$, 10 μm particle size, stainless steel, 25 cm × 4.6 mm id (DuPont Co.). μBondapak $C_{18}$, 10 μm particle size, stainless steel, 30 cm × 3.9 mm id (Waters Associates, Inc.).

(**c**) *Mobile phases.*—Use LC grade org. solvs (Fisher Scientific Co.). Use glass-distd $H_2O$ treated to remove org. compds by passing thru $C_{18}$ column system (Millipore Corp.) or use LC grade $H_2O$. Use MeOH-$H_2O$ (75+25), (68+32), and (66+34) for Partisil, Zorbax, and Bondapak, resp. If necessary, adjust mobile phase to give adequate sepn of tephrosin, rotenone, and deguelin in test soln (Fig. **983.06**).

(**d**) *Test soln.*—Accurately weigh portion of well mixed sample of Noxfish Fish Toxicant or powd cubé root ext (Roussel BioCorp, 400 Sylvan Ave, PO Box 1077, Lyndhurst, NJ 07071) contg ca 20 mg rotenone into 125 mL g-s erlenmeyer. Add 50 mL dioxane, and mix.

(**e**) *Std soln.*—Accurately weigh ca 20 mg 99% pure rotenone (Penick Co.) into 50 mL vol. flask and dil. to vol. with

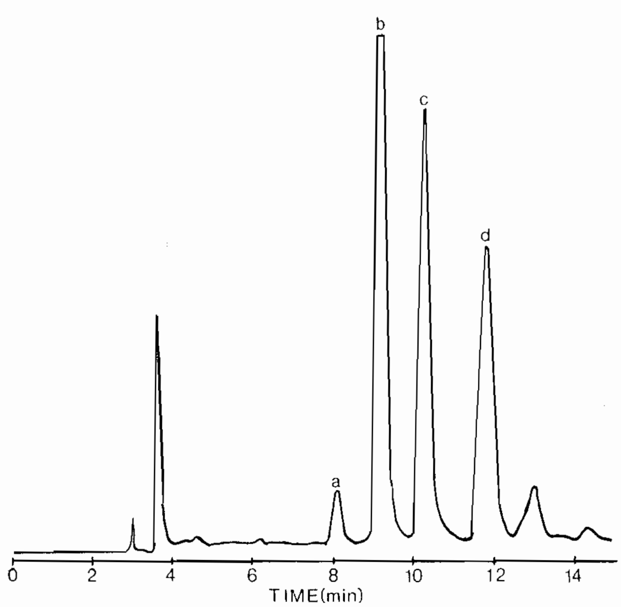


**FIG. 983.06—Liq. chromatogram of rotenone sample with Whatman column: a, rotenolone; b, tephrosin and an unknown; c, rotenone; d, deguelin**

dioxane (reagent grade). Keep rotenone from light or store in actinic glassware.

(**f**) *Sample extraction solvent*.—Reagent grade dioxane.

### C. Preparation of Sample

(**a**) *Solid formulations*.—Accurately weigh portion of well mixed sample contg ca 20 mg rotenone into 125 mL g-s erlenmeyer. Pipet in 50 mL dioxane, stopper, and shake 1½ hr on rotary shaker. Let settle and filter aliquot thru 0.45 μm organic filter (Millipore Corp.), or equiv.

(**b**) *Liquid formulations*.—Use same procedure as above, omitting rotary shaking and settling.

### D. Determination and Calculation

Inject std soln followed by 2 injections of sample soln and another injection of std soln. Measure peak hts, average, and calc. as follows:

$$\% \text{ Rotenone} = (PH/PH') \times (W'/W) \times \% \text{ purity of std}$$

where $PH$ and $PH'$ = av. peak hts of sample and std solns, resp.; $W'$ = g rotenone std/50 mL; and $W$ = g sample extd.

Ref.: JAOAC **66**, 796(1983).

CAS-83-79-4 (rotenone)

## 960.11 Piperonyl Butoxide in Pesticide Formulations

**Colorimetric Method**

**First Action 1960**
**Final Action 1961**

### A. Apparatus and Reagents

(**a**) *Photoelectric colorimeter*.—Equipped with narrow bandpass interference type filter with central wavelength 630 nm. (Filter is available from PTR Optics Corp, 145 Newton St, Waltham, MA 02154.) Spectrophtr set at wavelength in range 625–635 nm may also be used.

(**b**) *Purified tannic acid*.—Purify as follows: To 20 g tannic acid (USP reagent grade) add 100 mL EtOAc (99%) and stir mech. ca 1 hr. Filter by suction thru fritted glass funnel, and wash residue with three 5 mL portions EtOAc. To combined filtrate and washings add 2 g finely powd Darco G-60 (or equiv. decolorizing C), and stir mech. ca 0.5 hr. Filter by gravity thru double thickness Whatman No. 1, or equiv., paper into graduated dropping funnel. Wash residue several times with EtOAc until vol. filtrate and washings is ca 125 mL. Place dropping funnel over 1 L, 3-neck, r-b flask, equipped with mech. stirrer, and with vigorous agitation in flask, add filtrate dropwise to 5 times its vol. of toluene. Purified tannic acid is pptd immediately.

Filter by suction thru fritted glass funnel, and wash product thoroly with toluene, stirring solids with toluene to assure complete removal of EtOAc. Continue suction until practically all toluene is removed. Dry purified tannic acid in vac. oven at ca 40°, and place in tightly stoppered bottle.

(**c**) *Tannic acid reagent*.—Completely dissolve exactly 0.025 g purified tannic acid in 20 mL HOAc by shaking at room temp. Add 80 mL $H_3PO_4$ and mix thoroly. Prep. fresh daily. Store tightly stoppered, as it is hygroscopic.

(**d**) *Purified piperonyl butoxide*.—Purify by low pressure fractional distn of tech. product. (*Caution*: See safety notes on vacuum.) Also available from Fairfield American Corp., 3932 Salt Rd, Medina, NY 14103.

(**e**) *Piperonyl butoxide std soln*.—50 μg/0.1 mL. Weigh exactly 1.000 g purified piperonyl butoxide into 100 mL vol. flask. (Hypodermic syringe and needle are convenient for adding compd to flask.) Dil. to vol. with deodorized kerosene and mix well. Pipet 10 mL of this soln into 200 mL vol. flask. Dil. to vol. with deodorized kerosene and mix well. This soln is stable for several months. If std is to be used with sample contg pyrethrum, add enough pyrethrum ext to std before initial diln to give ratio piperonyl butoxide to pyrethrins similar to sample.

### B. Preparation of Sample

Accurately weigh sample contg 0.5–1.5 g piperonyl butoxide into tared 100 mL vol. flask, dil. to vol. with deodorized kerosene, and mix well. Pipet 10 mL into 200 mL vol. flask, dil. to vol. with deodorized kerosene, and mix well.

### C. Determination

Pipet 0.1 mL (from 1 mL pipet graduated in 0.1 mL) sample soln into 18 × 150 mm test tube. Add exactly 5 mL tannic acid reagent and shake vigorously 1 min. Treat std and blank, consisting of 0.1 mL deodorized kerosene, simultaneously in same manner.

Place test tubes in test-tube basket and place in vigorously boiling $H_2O$ bath 5 min. Remove basket and let tubes cool to room temp. Transfer solns to colorimeter tubes and read, against $H_2O$, using 625–635 nm filter or setting. (After cooling to room temp. there is no appreciable change in $A$ for several hr.)

Subtract $A_0$ of deodorized kerosene from readings of both sample, $A$, and std, $A'$.

$$\text{mg Piperonyl butoxide} = A \times 0.05/A'$$

Refs.: JAOAC **35**, 771(1952); **43**, 350(1960).

CAS-51-03-6 (piperonyl butoxide)

## 936.05 Pyrethrin in Pesticide Formulations

**Mercury Reduction Method (1)**

**Final Action**

(*Caution:* See safety notes on flammable solvents, diethyl ether, peroxides, and petroleum ether.)

### A. Reagents

(**a**) *Deniges reagent*.—Mix 5 g yellow HgO with 40 mL $H_2O$, and, while stirring, slowly add 20 mL $H_2SO_4$; then add addnl 40 mL $H_2O$ and stir until all dissolves. Test for absence of mercurous Hg by adding few drops of (**b**) to 10 mL and titrg with (**c**) as in **936.05C**, par. 2, beginning "Add 50 mL previously prepd and cooled dil. HCl . . ."

(**b**) *Iodine monochloride soln*.—Dissolve 10 g KI and 6.44 g $KIO_3$ in 75 mL $H_2O$ in g-s bottle; add 75 mL HCl and 5 mL $CHCl_3$, and adjust to faint I color (in $CHCl_3$) by adding dil. KI or $KIO_3$ soln. If much I is liberated, use stronger soln of $KIO_3$ than 0.01$M$ at first, making final adjustment with 0.01$M$ soln. Keep in dark and readjust when necessary. Do not store in refrigerator.

(**c**) *Potassium iodate std soln*.—0.01$M$. Dissolve 2.14 g pure $KIO_3$, previously dried at 105°, in $H_2O$ and dil. to 1 L. 1 mL = 0.0057 g pyrethrin I and needs no further stdzn.

(**d**) *Alcoholic sodium hydroxide soln*.—(*1*) *1.0N*.—Dissolve 40 g NaOH in alcohol and dil. to 1 L with alcohol. (*2*) *0.5N*—Dil. 1.0$N$ with alcohol (1+1).

(**e**) *Petroleum ether*.—Aromatic-free, bp range 30–60°.

(**f**) *Ethyl ether*.—Peroxide-free, reagent grade.

### B. Preparation

(**a**) *Pyrethrum powder*.—Ext sample contg 40–150 mg total pyrethrins in Soxhlet or other efficient extn app. 7 hr with pet ether. After extn is complete, evap. pet ether to ca 40 mL, stopper flask, and place in refrigerator at 0 ± 0.5° overnight. Filter cold ext thru cotton plug satd with cold pet ether, in stem of funnel, collecting filtrate in 250 mL erlenmeyer. Wash with three 15 mL portions cold pet ether. Evap. filtrate and washings on $H_2O$ bath, using air current, until <1 mL solv. remains.

Add 15–20 mL 0.5*N* alc. NaOH to evapd ext, connect to reflux condenser, and boil gently 1–1.5 hr. Transfer to 600 mL beaker and add enough $H_2O$ to bring vol. to 200 mL. Add few glass beads, or preferably use boiling tube, and boil down to 150 mL. Transfer to 250 mL vol. flask and add 1 g Filter-Cel and 10 mL *10% $BaCl_2$ soln*. Do not shake before dilg to vol. Dil. to vol., mix thoroly, filter off 200 mL, neutze with $H_2SO_4$ (1 + 4), using 1 drop phthln, and add 1 mL excess. (If necessary to hold soln overnight at this point, leave in alk. condition.)

**(b)** *Pyrethrum extracts in mineral oil*.—Weigh or measure sample contg 40–150 mg total pyrethrins, add 50 mL pet ether and 1 g Filter-Cel, and place in refrigerator at 0 ± 0.5° overnight. Filter thru gooch into 300 mL erlenmeyer and wash with three 15 mL portions cold pet ether. Evap. filtrate and washings on $H_2O$ bath, using air current, until <1 mL solv. remains.

Add 20 mL 1*N* alc. NaOH, or more if necessary, to ext, connect to reflux condenser, and boil gently 1–1.5 hr. Transfer to 600 mL beaker and add enough $H_2O$ to make aq. layer 200 mL. If >20 mL alc. NaOH soln was used, add enough $H_2O$ so that all alcohol is removed when vol. is reduced to 150 mL. Add few glass beads, or preferably use boiling tube, and boil aq. layer down to 150 mL. Transfer to 500 mL separator and drain aq. layer into 250 mL vol. flask. Wash oil layer once with $H_2O$ and add wash $H_2O$ to aq. portion. If slight emulsion still persists after draining aq. layer and washings, add 2–3 mL 10% $BaCl_2$ soln, but do not shake vigorously after adding $BaCl_2$ because reversed emulsion difficult to sep. may form. To aq. soln in 250 mL flask add 1 g Filter-Cel and ≥10 mL of the $BaCl_2$ soln. Swirl gently and let stand 30 min. Dil. to vol., mix thoroly, and filter off 200 mL. Test filtrate with $BaCl_2$ soln to see if enough has been added to obtain clear soln. Neutze with $H_2SO_4$ (1 + 4), using 1 drop phthln, and add 1 mL excess. (If necessary to hold soln overnight at this point, leave in alk. condition.)

### C. Determination of Pyrethrin I

Filter acid soln from **936.05B(a)** or **(b)** thru 7 cm paper, coated lightly with suspension of Filter-Cel in $H_2O$, on buchner, and wash with three 15 mL portions $H_2O$. Transfer to 500 mL g-s separator and ext with two 50 mL portions pet ether. Shake each ext ≥1 min, releasing pressure if necessary by inverting separator and carefully venting thru stopcock. Let layers sep. ≥5 min or until aq. layer is clear before draining and re-extn. Reserve aq. layer for pyrethrin II detn. Do not combine pet ether exts but wash each in sequence with same three 10 mL portions $H_2O$, and filter pet ether exts thru small cotton plug into clean 250 mL separator. Wash separators and cotton in sequence with 5 mL pet ether. Ext combined pet ether solns with 5 mL 0.1*N* NaOH, shaking vigorously ≥1 min. Let layers sep. ≥5 min before draining aq. layer into 100 mL beaker. Wash pet ether with addnl 5 mL portion 0.1*N* NaOH and with 5 mL $H_2O$, adding washings to beaker. Add 10 mL Deniges reagent and let stand in complete darkness 1 hr at 25 ± 2°.

Add 20 mL alcohol and ppt HgCl with 3 mL *satd NaCl soln*.Warm to ca 60° and let stand several min until ppt coagulates and settles. Filter thru small paper, transferring all ppt to paper, and wash with ≥10 mL hot alcohol. Wash with 2 or more 10 mL portions hot $CHCl_3$ and place paper and contents in 250 mL g-s erlenmeyer. Add 50 mL previously prepd and cooled dil. HCl (3 + 2). Add 5 mL $CHCl_3$ or $CCl_4$ and 1 mL freshly adjusted ICl soln, and titr. with 0.01*M* $KIO_3$ soln, shaking vigorously ≥30 sec after each addn, until no I color remains in $CHCl_3$ or $CCl_4$ layer. Take as end point when red color disappears from solv. layer and does not return within 1–3 min. From mL std $KIO_3$ soln used in titrn and blank on Deniges reagent, calc. % pyrethrin I.

(Reactions:

$$2Hg_2Cl_2 + 4ICl = 4HgCl_2 + 2I_2$$

$$2I_2 + KIO_3 + 6HCl = KCl + 5ICl + 3H_2O$$

Addn of ICl does not change vol. relationship between mercurous Hg and $KIO_3$ soln, and aids in detg end point in titrn of small amts of Hg.)

*Note:* Chrysanthemum monocarboxylic acid reacts with Deniges reagent to form series of colors beginning with phthln red, which gradually changes to purple, then to blue, and finally to bluish green. Color reaction is very distinct with 5 mg monocarboxylic acid, and amts as low as 1 mg can usually be detected. Therefore no pyrethrin I should be reported if color reaction is neg.

With samples contg much perfume or other saponifiable ingredients, it may be necessary to use as much as 50 mL 1*N* alc. NaOH. When lethanes are present, after washing HgCl ppt with alcohol and $CHCl_3$, wash once more with alcohol and then several times with hot $H_2O$.

### D. Determination of Pyrethrin II (2)

If necessary, filter aq. residue from pet ether extn thru gooch. Conc. filtrate to ca 50 mL and transfer to 500 mL g-s separator. Wash beaker with three 15 mL portions $H_2O$. Acidify with 10 mL HCl and sat. with NaCl. (Acidified aq. layer must contain visible NaCl crystals thruout following extns.)

Ext with 50 mL ether, drain aq. layer into second separator, and ext again with 50 mL ether. Continue extg and draining aq. layer, using 35 mL for third and fourth extns. Shake each ext ≥1 min, releasing pressure, if necessary, by inverting separator and carefully venting thru stopcock. Let layers sep. ≥5 min or until aq. layer is clear before subsequent draining and extn. Combine ether exts, drain, and wash with three 10 mL portions satd NaCl soln. Filter ether exts thru cotton plug into 500 mL erlenmeyer and wash separator and cotton with addnl 10 mL ether. Evap. ether on $H_2O$ bath, and remove any fumes of HCl with air current and continued heating ≤5 min. Dry 10 min at 100°.

**(a)** *For crude pyrethrum exts*.—Treat residue with 75 mL boiling $H_2O$ and filter thru 9–11 cm Whatman No. 1, or equiv., paper. Wash flask and paper with five 20 mL portions boiling $H_2O$ or until filtrate from final wash is neut. to litmus. Add 1–2 drops phthln and rapidly titr. with 0.02*N* NaOH (1 mL = 0.00374 g pyrethrin II). Check normality of 0.02*N* NaOH same day sample is titrd.

**(b)** *For refined pyrethrum exts*.—Add 2 mL neut. alcohol and 20 mL $H_2O$, and heat to dissolve acid. Cool, filter thru gooch if necessary, add 1–2 drops phthln, and titr. with 0.02*N* NaOH (1 mL = 0.00374 g pyrethrin II). Check normality of 0.02*N* NaOH same day as sample is titrd.

Refs.: (1) Contrib. Boyce Thompson Inst. **8,** No. 3, 175(1936). Ind. Eng. Chem. Anal. Ed. **10,** 5(1938). JAOAC **43,** 358(1960).
(2) Soap **10,** No. 5, 89(1934). JAOAC **43,** 354(1960); **46,** 664(1963); **56,** 915(1973).

CAS-121-21-1, 121-29-9 (pyrethrins)

## 982.02 Pyrethrins and Piperonyl Butoxide in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1982**
**Final Action 1983**

### A. Principle

Sample is dild with acetone contg dicyclohexyl phthalate internal std and detd by GC with flame ionization detection. Method is applicable to tech. piperonyl butoxide [80% butylcarbityl 6-propylpiperonyl ether and 20% related compds] and most formulations contg pyrethrins and piperonyl butoxide except shampoo products. Occasionally, an oil diluent will interfere with GC detn. Method may not be applicable to samples contg <0.1% pyrethrins. Variation in active constituents of pyrethrin ext may cause minor deviations from expected results.

### B. Apparatus and Reagents

(**a**) *Gas chromatograph*.—Equipped with flame ionization detector and 122 cm × 4 mm id glass column packed with 5% OV-101 or 5% OV-1 (Analabs, Inc.) on 80–100 mesh Chromosorb W(HP). Operating conditions: column 210°, injection port 250°, detector 250°; gas flows (mL/min)—N carrier gas flow 50, air 350–400, and H 40–50; sensitivity $10^{-10}$ AUFS. Adjust attenuation to maintain 50–75% FSD for 1.0–1.5 μg piperonyl butoxide. Before use, condition column 2–3 h at 275° with N flow 50 mL/min. If necessary, vary column temp. or gas flow to attain retention times of ca 13–15 min for internal std. Theoretical plates/ft must be >400, based on dicyclohexyl phthalate peak.

Calc. theoretical plates/ft ($N$) as follows: $N = 16 \times (L^2/M^2 \times F)$, where $L$ = retention of GC peak (mm); $M$ = peak baseline (mm) produced by drawing tangents to points of inflection of peak; and $F$ = length of column (ft).

(**b**) *Internal std soln*.—8.0 mg dicyclohexyl phthalate (Pfaltz and Bauer, Inc., 172 Aurora St., Waterbury, CT 06708)/mL acetone.

(**c**) *Std soln*.—(*1*) *Std soln A*.—0.5 mg piperonyl butoxide/mL. Accurately weigh ca 0.25 g piperonyl butoxide (available from McLaughlin Gormley King Co., 8810 Tenth Ave N, Minneapolis, MN 55427) into 50 mL vol. flask and dil. to vol. with acetone. Pipet 10 mL this soln into 100 mL vol. flask, add 5 mL internal std soln by pipet, and dil. to vol. with acetone. Use this soln for detn of tech. piperonyl butoxide. (*2*) *Std soln B*.—Accurately weigh ca 0.25 g piperonyl butoxide into 50 mL vol. flask. Add weighed amt of pyrethrins such that ratio of active ingredients closely resembles that which is expected in sample. Dil. to vol. with acetone. Pipet 10 mL of this soln into 100 mL vol. flask, add 5 mL internal std soln by pipet, and dil. to vol. with acetone. Use this soln for detn of pyrethrins and piperonyl butoxide in formulations.

### C. Preparation of Sample

(**a**) *Technical piperonyl butoxide*.—Accurately weigh ca 0.25 g sample into 50 mL vol. flask and dil. to vol. with acetone. Pipet 10 mL this soln into 100 mL vol. flask, add 5 mL internal std soln by pipet, and dil. to vol. with acetone.

(**b**) *Pyrethrins-piperonyl butoxide formulations*.—(*1*) *Liqs*.—Accurately weigh sample contg ca 0.05 g piperonyl butoxide into 100 mL vol. flask, add 5 mL internal std soln by pipet, and dil. to vol. with acetone. (*2*) *Aerosol formulations*.—*Caution:* Open aerosol behind safety shield and in hood. Weigh aerosol can to nearest 0.1 g ($G$). Puncture as *small a hole as possible* in top of can with sharp punch and hammer to allow propellant to release very slowly. (Best results can be obtained by allowing punctured can to stand overnight.) After hiss of escaping propellant is no longer evident, cut open top of can with hand can opener. Leave ca 1 cm attached to can and bend top open. Carefully warm can in beaker of warm tap $H_2O$ several minutes to ensure complete removal of propellant. Transfer aerosol nonvolatiles to vol. flask with aid of acetone. Rinse can thoroly, adding rinses to vol. flask. If aerosol is 8 oz, use 2 L ($V$) vol. flask. This vol. is necessary to ensure complete miscibility of oil phase of aerosol contents. Dil. to vol. with acetone and mix thoroly. Dry empty can and weigh ($T$). Transfer aliquot ($A$) (must be ≤90 mL), equiv. to 50 mg piperonyl butoxide, to 100 mL vol. flask, add 5 mL internal std soln by pipet, and dil. to vol. with acetone.

$$\text{Wt sample} = (G - T) \times (A/V)$$

### D. Gas Chromatography

Inject 2–3 μL aliquots of std soln until internal std ratios vary ≤2% for successive injections. Det. baseline by drawing straight line to min. on either side of peak of interest. For pyrethrins, use combined ht of cinerin I and pyrethrin I peaks for internal std ratio. Repeat injection procedure with sample soln, followed by injection of std soln. If std peak ratios differ ≥±2.0%, repeat series of injections. Injection vol. should not vary >±10%. Calc. peak ht ratios (sample peak ht/internal std peak ht) of std injections before and after sample injections and average std ratio preceding and following sample injections. Calc. av. peak ht ratios for sample injections. After elution of piperonyl butoxide, allow ca 7 min for elution of extraneous peaks.

$$\% \text{ Piperonyl butoxide or pyrethrins} = (R/R') \times (W'/W) \times P$$

where $W'$ = g std in final diln; $W$ = g sample in final diln; $P$ = % purity of std; $R'$ = ratio of std; and $R$ = ratio of sample.

Ref.: JAOAC **65,** 249(1982).

CAS-51-03-6 (piperonyl butoxide)
CAS-121-21-1, 121-29-9 (pyrethrins)

## 980.04 *N*-Octyl Bicycloheptene Dicarboximide in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1980**
**Final Action 1981**

(*Caution:* See safety notes on pesticides.)

### A. Principle

Sample is dild with acetone contg dibutyl phthalate as internal std. GC peak ht or area ratios of MGK 264 (*N*-octyl bicycloheptene dicarboximide) to dibutyl phthalate peak of sample and std are compared for quantitation. Method is applicable to technical MGK 264 and to several formulations. Not applicable to formulations contg Dursban® and isopropyl palmitate. Presence of large amts of MGK® Repellent 326 causes slightly high results.

### B. Apparatus and Reagents

(**a**) *Gas chromatograph*.—With flame ionization detector and 120 cm × 4 mm (id) glass column packed with 5% OV-1 (Analabs, Inc.) on 80–100 mesh Chromosorb W(HP). Operating conditions: temps (°)—column 170, injection port 250, detector 250; gas flows (mL/min)—N carrier gas 60, air 350–400, H 40–50; sensitivity—$10^{-10}$ amp full scale, attenuation 16×. Before use, condition column 2–3 hr at 275° with N flow 50 mL/min. If necessary, vary column temp. or gas flow to attain retention times of ca 6 and 8 min for internal std and MGK 264, resp. Also vary detector sensitivity or injection vol.

to attain >100 mm peak ht for each compd (ca 9 μg MGK 264). Theoretical plates/ft must be >300.

Calc. theoretical plates/ft ($N$) as follows: $N = 16\ L^2/M^2 \times F$), where $L$ = retention GC peak in mm; $M$ = peak baseline produced by drawing tangents to points of inflection of peak; and $F$ = length of column (ft).

(**b**) *Internal std soln.*—5.0 mg dibutyl phthalate (Monsanto Co., 98%)/mL acetone.

(**c**) *MGK 264 std soln.*—Accurately weigh ca 0.15 g MGK (available from McLaughlin Gormley King Co., 8810 Tenth Ave N, Minneapolis, MN 55427) into 50 mL vol. flask, add 10.0 mL internal std soln, and dil. to vol. with acetone.

### C. Determination

Accurately weigh sample contg ca 0.15 g MGK 264 into 50 mL vol. flask, add 10.0 mL internal std, soln, and dil. to vol. with acetone.

Inject aliquots (2–3 μL) std soln until ratio of MGK 264 to dibutyl phthalate peak hts (larger peak) or area (use area of both MGK 264 peaks) varies <1% for successive injections. Repeat with sample soln, followed by duplicate injections of std soln. If std peak ratios differ by more than ±1.5% repeat series of injections. Injection vols should not vary more than ±10%. (After elution of MGK 264, it is advantageous to increase column temp. to reduce retention time of subsequent peaks, such as pyrethrins and piperonyl butoxide.) Calc. peak ht or area ratios for duplicate std injections before and after sample injections and average the 4 values. Calc. and average peak ht or area ratios for sample injections.

$$\%\ \text{MGK } 264 = (R/R') \times (W'/W) \times P$$

where $W'$ = g std; $W$ = g sample; $P$ = purity of std; $R'$ = ratio of std; and $R$ = ratio of sample.

Ref.: JAOAC **63,** 128(1980).

CAS-113-48-4 ($N$-octyl bicycloheptene dicarboxïmide)

## 960.12 Sabadilla Alkaloids in Pesticide Formulations

### Gravimetric Method

### First Action 1960
### Final Action 1961

(In dust formulations)

### A. Determination

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Weigh 10 g mixed 50% sabadilla dust (or corresponding amt of lesser concn) into 500 mL g-s erlenmeyer. Add exactly 300 mL ether-$CHCl_3$ (3+1), and shake 5 min. Make alk. with 10 mL $NH_4OH$ and shake mech. 2 hr. Let stand overnight; then shake 1 hr.

Filter, avoiding evapn. Place 200 mL aliquot in 500 mL separator, acidify with $H_2SO_4$ (3+97), and shake; withdraw small amt aq. layer and test with litmus paper, returning soln to separator. Add 50 mL of the dil. $H_2SO_4$ and shake. Let sep. and transfer acid ext to second 500 mL separator. Add 50 mL pet ether to acid ext and shake. Let layers sep. and transfer acid ext to third separator. Repeat extn of soln in first separator with two 50 mL portions of the dil. $H_2SO_4$, using same 50 mL pet ether in second separator for washing. Collect acid exts in third separator.

Make acid exts alk. to phthln with $NH_4OH$. Ext with three 50 mL portions $CHCl_3$. Wash each $CHCl_3$ ext by shaking gently with same 100 mL portion $H_2O$ in fourth separator. (If emulsion forms, add small amt anhyd. $Na_2SO_4$.)

Filter each $CHCl_3$ ext thru cotton into weighed 250 mL flask. Evap. $CHCl_3$ on steam bath. Add few mL alcohol, and evap. again. Dry 1 hr at 100° and weigh sabadilla alkaloids. Calc. % total alkaloids.

### B. Qualitative Test

Add 1–2 mL $H_2SO_4$ to few mg of residue, **960.12A**. Presence of sabadilla alkaloids is indicated by yellow that gradually becomes intensely red with greenish fluorescence.

Ref.: JAOAC **43,** 374(1960).

CAS-8082-57-7 (sabadilla alkaloids)

## 920.35 Nicotine in Tobacco Products

### Silicotungstic Acid Method

### Final Action

(*Note:* Nicotine is very toxic. Avoid contact with skin.)
(Includes nornicotine)

### A. Reagent

*Silicotungstic acid soln.*—Dissolve 120 g silicotungstic acid ($4H_2O.SiO_2.12WO_3.22H_2O$ or $SiO_2.12WO_3.26H_2O$) in $H_2O$ and dil. to 1 L. (Acid should be white or pale yellow crystals, free from green color; soln should be free from cloudiness and green color. Of the several silicotungstic acids, $4H_2O.SiO_2.10WO_3.3H_2O$ and $4H_2O.SiO_2.12WO_3.20H_2O$ do not give cryst. ppts with nicotine and should not be used.)

### B. Determination

Weigh sample contg preferably 0.1–1.0 g nicotine. If sample contains very little nicotine (ca 0.1%), do not increase amt to point where it interferes with distn. Wash with $H_2O$ into 500 mL Kjeldahl flask, and if necessary add little paraffin to prevent frothing and few small pieces pumice to prevent bumping. Add 10 g NaCl and 10 mL *NaOH soln* (*30% by wt*), and close flask with rubber stopper thru which passes stem of trap bulb and inlet tube for steam. Connect trap bulb to well-cooled condenser, lower end of which dips below surface of 10 mL HCl (1+4) in suitable receiving flask. Steam distil rapidly. When distn is well under way, heat flask to reduce vol. of liq. as far as practicable without bumping or excessive sepn of insol. matter. Distil until few mL distillate shows no cloud or opalescence when treated with drop silicotungstic acid soln and drop HCl (1+4). Confirm alky of residue in distn flask with phthln.

Adjust distillate, which may total 1.0–1.5 L, to convenient exact vol. (soln may be concd on steam bath without loss of nicotine), mix well, and pass thru dry filter if not clear. Test distillate with Me orange to confirm acidity. Pipet aliquot contg ca 0.1 g nicotine into beaker. (If samples contain very small amts of nicotine, aliquot contg as little as 0.01 g nicotine may be used.) To each 100 mL liq., add 3 mL HCl (1+4) and 1 mL silicotungstic acid for each 0.01 g nicotine supposed to be present. Stir thoroly and let stand overnight at room temp. Before filtering, stir ppt to see that it settles quickly and is in cryst. form. Filter on either ashless paper or gooch and wash with HCl (1+1000) at room temp. Continue washing for 2 or 3 fillings of filter after no more opalescence appears when few mL fresh filtrate is tested with few drops nicotine distillate. With paper, transfer paper and ppt to weighed Pt crucible, dry carefully, and ignite until all C is destroyed. Finally heat over Meker burner ≤10 min. Wt residue × 0.1141 = wt nicotine in aliquot. With gooch, dry in oven 3 hr at 105° and weigh. Wt residue × 0.1012 = wt nicotine in aliquot.

Ref.: USDA Bur. Animal Ind. Bull. **133.**

CAS-54-11-5 (nicotine)

## ORGANOHALOGEN PESTICIDES

### 985.04 Alachlor in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1985**
**Final Action 1987**

(Method is suitable for formulated products, including emulsifiable concs and granulated formulations.)

#### A. Principle

Sample is dissolved in acetone contg di-*n*-pentyl phthalate as internal std, analyzed by gas chromatgy with flame ionization detector, and quantd by comparison with internal std.

#### B. Apparatus

(**a**) *Gas chromatograph.*—With flame ionization detector and on-column injection ports. Temps (°)—column oven 230, injection port 250, detector 260; gas flows (mL/min)—He carrier gas 35, H 30; air 250; sample size 1.0 µL; run time 15 min.

(**b**) *Column.*—6 ft × 2 mm (id) glass column (on-column configuration) packed with 10% SP-2250 on 100–120 mesh Supelcoport (Supelco, Inc., Cat. No. 1-2132), or equiv. SP-2250 is methyl-phenyl silicone (50 + 50). Precondition overnight at 250° before use. Retention times for alachlor and internal std are ca 5.5 and 11.2 min, resp.

#### C. Reagents

(**a**) *Acetone.*—Pesticide grade, Fisher, or equiv.

(**b**) *Di-n-pentyl phthalate internal std.*—(CTC Organics, PO Box 6933, Atlanta, GA 30315). Weigh 5.3 g di-*n*-pentyl phthalate into 1 L vol. flask. Dissolve in acetone and dil. to vol. with acetone.

(**c**) *Alachlor.*—Recrystallized from MeOH (Monsanto Co., Muscatine, IA 52761). Accurately weigh 0.2 g alachlor into small flask. Add by pipet 30.0 mL internal std soln and shake to dissolve.

#### D. Determination

Accurately weigh sample contg ca 0.2 g alachlor into small flask. Add by pipet 30.0 mL internal std soln and shake well to ext alachlor. For granular formulation, mix ≥5 min on mech. shaker.

Make replicate 1 µL injections of alachlor std soln and measure response ratios, $R$ (area alachlor peak/area internal std peak) for each injection. Repeat until consecutive response ratios $R$ agree within 0.5%.

Make duplicate injections of sample soln and det. av. $R$. Follow with injection of alachlor std soln; det. av. $R'$ for std before and after sample injection.

$$\% \text{ Alachlor} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std, resp.; $W$ and $W'$ = wt (g) of sample and std, resp.; $P$ = % purity of std.

Ref.: JAOAC **68,** 573(1985).

CAS-15972-60-8 (alachlor)

### 988.03 Alachlor in Microencapsulated Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1988**

***AOAC-CIPAC Method***

#### A. Principle

Sample is dissolved in acetone contg di-*n*-pentyl phthalate as internal std, analyzed by gas chromatgy with flame ionization detection, and quantitated by comparison of integrated peak areas.

#### B. Safety

$LD_{50}$ of alachlor has been found to be 930 mg/kg in rat acute oral studies (Monsanto Co., 1985, MSDS No. 015972608). Alachlor has been detd to produce tumors in laboratory animals. Wear protective clothing to avoid excessive exposure.

#### C. Apparatus

(**a**) *Gas chromatograph.*—With flame ionization detector and on-column injection ports. Operating conditions: temps—column oven 230°, injection port 250°, detector 260°; gas flows (mL/min)—He carrier gas 35, H 30, air 250; sample size 1.0 µL; run time 15 min.

(**b**) *Column.*—Glass, 6 ft × 2 mm id (on-column configuration), packed with 10% SP-2250 on 100–120 mesh Supelcoport (Supelco Inc.), or equiv. SP-2250 is methyl-phenyl silicone (50 + 50).

#### D. Reagents

(**a**) *Acetone.*—Pesticide grade (Fisher or equiv.).

(**b**) *Di-n-pentyl phthalate internal std soln.*—Weigh 5.3 g di-*n*-pentyl phthalate (CTC Organics, PO Box 6933, Atlanta, GA 30315) into 1 L vol. flask. Dissolve in acetone and dil. to vol. with acetone.

(**c**) *Alachlor std soln.*—Recrystallize alachlor (Monsanto Co., PO Box 473, Muscatine, IA 52761) from MeOH. Accurately weigh 0.2 g recrystd alachlor into small flask. Add by pipet 30.0 mL internal std soln and shake mixt. to dissolve.

#### E. Instrument Setup and Calibration

Condition chromatgc column overnight at 250° with He flow at 35 mL/min. Suggested conditions represent best compromise for sepn and quantitation of cmpds of interest. Some minor adjustments may be required in other instruments and columns. Column, when working properly, should generate 4000–5000 plates calcd as follows: $N = 16(x/y)^2$, where $N$ = no. of theoretical plates, $x$ = distance from point of injection to peak max., and $y$ = distance along baseline between intercept points of lines drawn tangent to slope of peak, with $x$ and $y$ measured in same units. Typical retention times for alachlor and internal std are ca 6 and 11.5 min, resp. Impurity in internal std (peak C), which elutes at ca 9.9 min, should be completely resolved from internal std peak at ca 11.5 min (Fig. **988.03**). Internal std contains another impurity that elutes slightly after internal std causing slight tail on that peak. Careful control of integrator conditions is required to integrate internal std peak.

After instrument equilibration, make ≥3 injections of std soln before calibration.

#### F. Determination

Accurately weigh, to nearest 0.1 mg, ca 0.45 g alachlor microencapsulated formulation into 2 oz sample bottle. Avoid spilling sample on inside wall or neck of bottle; entire sample should be on bottom of bottle.

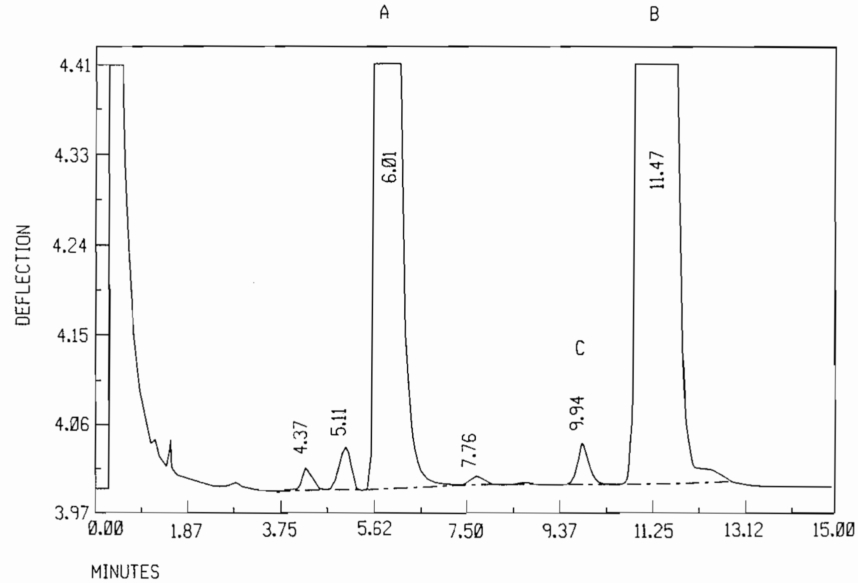


**FIG. 988.03—GC chromatogram of alachlor standard (A), internal standard di-*n*-pentyl phthalate (B), and unknown from internal standard (C)**

Pipel 30.0 mL internal std soln (**b**) into sample bottle. To reduce stirring time, use liq. stream from pipet to remove most of alachlor sample from bottom of bottle. Add mag. stirring bar (13 × 15 mm) and cap bottle with polyethylene-lined cap.

Mag. stir mixt. until sample is completely removed from inside wall and bottom of bottle. During stirring, aggregated sample turns fluffy and easily floats in acetone. For most samples, this requires ca 2–3 min moderately fast stirring. Then place bottles on shaker and shake 10 min at high speed.

Let solids settle and pipet off clear acetone soln.

Make replicate 1 μL injections of alachlor std soln and measure response ratio, $R$ (area alachlor peak/area internal std peak) for each injection. Repeat until consecutive response ratios agree within 0.5%.

Make duplicate injections of acetone sample soln and det. av. $R$. Follow with injection of alachlor std soln. Det. av. $R$ for std before and after sample injection.

$$\text{Alachlor, \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response for sample and std, resp.; $W$ and $W'$ = wt (g) of sample and std, resp.; $P$ = % purity of std.

Ref.: JAOAC **70,** 1056 (1987).

CAS-15972-60-8 (alachlor)

## 961.04★ Chlorine (Total) in Organohalogen Pesticide Formulations

### Sodium Biphenyl Reduction Method

**First Action 1961**
**Final Action 1962**
**Surplus 1978**

(Applicable to aldrin, dieldrin, and endrin)

*See* **6.177–6.181**, 13th ed.

## 961.05 Aldrin, Dieldrin, and Endrin in Pesticide Formulations

### Infrared Spectroscopic Method

**First Action 1961**
**Final Action 1962**

### *A. Principle*

Dieldrin and endrin in dusts, granules, wettable powders, emulsifiable concns, and solns are purified on adsorbent columns. Hexachloro-epoxy-octahydro-endo,exo-dimethanonaphthalene (HEOD) content of the purified dieldrin or of tech. dieldrin is detd by IR, using baseline technic, and dieldrin is calcd assuming 85% HEOD content. Endrin content of purified or tech. endrin is detd as hexachloro-epoxy-octa-hydro-endo,endo-dimethanonaphthalene similarly.

Aldrin is extd from dusts, wettable powders, and inorg. fertilizers on adsorbent column. Hexachloro-hexahydro-endo,exo-dimethanonaphthalene (HHDN) content of the ext or of tech. aldrin is detd by IR, using baseline technic, and aldrin is calcd assuming 95% HHDN content. Method is not applicable to emulsifiable concns or granules contg petroleum hydrocarbon solvs or to mixts contg other common pesticides or adjuvants that absorb in same wavelength region as HHDN.

### *B. Reagents and Apparatus*

(**a**) *Chromatographic solvent A*.—Mix 1 vol. $CHCl_3$ with 19 vols hexane.

(**b**) *Chromatographic solvent B*.—Mix 1.5 vols acetone with 98.5 vols chromtgc solvent A.

(**c**) *Extraction solvent*.—Mix 1 vol. acetone with 19 vols $CS_2$.

(**d**) *Infrared spectrophotometer*.—With sealed liq. cells with NaCl windows, having optical path length of ca 0.1 mm (dieldrin and endrin) and 0.2 mm (aldrin).

### *C. Preparation of Standard Solutions*

(**a**) *HEOD std soln for dieldrin*.—Accurately weigh ca 100, 200, 300, 400, 500, and 600 mg std hexachloro-epoxy-octahydro-endo,exo-dimethanonaphthalene (HEOD) into 10 mL vol. flasks, dissolve in $CS_2$, and dil. to vol. Concns will be 1, 2, 3, 4, 5, and 6 g/100 mL, resp.

(**b**) *Std soln for endrin.*—Accurately weigh ca 50, 100, 150, 200, 300, and 400 mg std hexachloro-epoxy-octahydro-endo,endo-dimethanonaphthalene (endrin) into 10 mL vol. flasks, dissolve in $CS_2$, and dil. to vol. Concns will be 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 g/100 mL, resp.

(**c**) *HHDN std soln for aldrin.*—Accurately weigh ca 100, 150, 200, 250, 300, and 350 mg std hexachloro-hexahydro-endo,exo-dimethanonaphthalene (HHDN) into 10 mL vol. flasks, dissolve in $CS_2$, and dil. to vol. Concns will be 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 g/100 mL, resp.

### D. Preparation of Standard Curve

Fill 0.1 mm cell (0.2 mm for aldrin) with most dil. of stds solns, using hypodermic syringe. Adjust spectrophtr to optimum settings for gain, slit width, response, speed, and drum drive. Make duplicate scans of $CS_2$ soln over scanning range indicated in Table **961.05** and repeat with each of other std solns at same instrument settings.

For each of scans of the 6 std solns of each compd, draw line between baseline points indicated in table. Draw perpendicular from zero radiation line thru absorption peak to baseline and measure distance from 0 line to peak, $P$, and to baseline $P_0$. Calc. $A$ (= log $P_0/P$) and plot as ordinate against concn in g/100 mL as abscissa.

Since std curve intersects abscissa at pos. concn value, method is not applicable to concns below this value.

Peak wavelengths given in table are characteristic for low concns and they shift at higher concns. $P$ is always detd as distance from 0 line to point of max. absorption.

### E. Preparation of Sample

(*Caution:* See safety notes on distillation, pesticides, acetone, chloroform, and hexane.)

(**a**) *Dusts and wettable powders.*—Transfer 3–20 g sample, depending on concn (75–0.5%), weighed to nearest 0.01 g, to chromatgc tube contg 25–50 mm (ca 5.5 g) Hyflo Super-Cel. (For finely divided dieldrin or endrin powder, use 3 g activated C instead of Super-Cel.) Tamp or vibrate column slightly to settle contents. Place 250 mL wide-mouth erlenmeyer or 500 mL evapg dish under tip of column.

Working in well ventilated hood, add 50 mL portions extn solv. to column (if S is present, ext with acetone instead of extn solv.), letting solv. percolate thru column between addns, until 150 mL ext collects. Rinse tip of column with addnl 10 mL extn solv.

Evap. solv. almost to dryness on steam bath under N. Dry HEOD or HHDN residues 15 min at 75°; dry endrin in vac. oven 15 min at 30° and 10 mm pressure. (Extd endrin may no longer be associated with its inhibitors. Residue must not be exposed to elevated temps and must be dissolved promptly to avoid decomposition.)

Cool residue and dissolve in few mL $CS_2$. Quant. transfer to vol. flask of such size (5–100 mL) as to give optimum concn of 3 g HEOD, 2 g endrin, or 2 g HHDN/100 mL, dil. to vol. with $CS_2$, and mix thoroly. If soln is cloudy from $H_2O$, add little NaCl, shake, and let settle.

(**b**) *Granules containing dieldrin or endrin.*—Slurry 40 g Florisil in 200 mL beaker with 100 mL hexane. Transfer to chromatgc column with stream of hexane from wash bottle. Eliminate any bubbles or voids by vibration or agitation. Let hexane drain until only 2–3 mm layer remains above surface of column. Add small layer of $Na_2SO_4$ to top of column.

Transfer 2–10 g finely ground sample, depending on concn (10–1%), to prepd column. Rinse down column walls with three 10 mL portions chromatgc solv. A, letting each portion enter column before adding next. Add 170 mL chromatgc solv. A, let percolate thru column, and discard.

Gently flow 10 mL chromatgc solv. B down walls of tube, avoiding disturbing surface of adsorbent. After solv. sinks into column, repeat washing with 2 addnl 10 mL portions. Add 220 mL chromatgc solv. B and let flow at rate of 2–5 mL/min, collecting eluate in 500 mL wide-mouth erlenmeyer or evapg dish. Evap. solv. to dryness on steam bath, avoiding spattering, and proceed as in (**a**), using 5–10 mL vol. flask.

(**c**) *Emulsifiable concentrates and solns.*—Weigh 1.5 g dieldrin conc. (1.5 lb/gal.), 1.0 g endrin conc. (1.6 lb/gal.), or 30.0 g 0.5% dieldrin soln, and add 5 mL hexane. Transfer to prepd column and proceed as in (**b**).

(**d**) *Technical materials.*—Transfer sample contg 1.75–4.00 g dieldrin, 1.50–3.00 g endrin, or 1.00–2.00 g aldrin, weighed to 0.01 g, to 100 mL vol. flask. Dissolve in $CS_2$ and dil. to vol. with $CS_2$.

### F. Determination

Fill same 0.1 mm cell (0.2 mm for aldrin) used for prepn of std curve with sample soln. Make duplicate scans, and calc. $A$ and mean $A$ as in prepn of std curve. From appropriate std curve, obtain g HEOD, endrin, or HHDN/100 mL sample soln, $W$.

$$\% \text{ dieldrin} = W \times V \times 1.175/S$$
$$\% \text{ endrin} = W \times V/S$$
$$\% \text{ aldrin} = W \times V \times 1.053/S$$

where $V$ = mL sample soln; $S$ = g sample; 1.175 and 1.053 = conversion factors HEOD to dieldrin and HHDN to aldrin, resp.

Ref.: JAOAC **44,** 595(1961).

CAS-309-00-2 (aldrin)
CAS-60-57-1 (dieldrin)
CAS-72-20-8 (endrin)

**Table 961.05 Characteristic Wavelength Points for Infrared Determination of Dieldrin, Endrin, and Aldrin, μm**

| Compound | Scanning Range | Baseline Points | Peak at Low Concn |
|---|---|---|---|
| HEOD | 11.59–12.18 | 11.64, 12.18 | 11.80 |
| Endrin | 11.43–12.04 | 11.50, 11.97 | 11.76 |
| HHDN | 11.79–12.24 | 11.85, 12.24 | 12.01 |

## 949.05 gamma-BHC (Lindane) in Pesticide Formulations (1)

**Partition Chromatographic Method**

**Final Action**

(*Caution:* See safety notes on monitoring equipment, distillation, flammable solvents, toxic solvents, pesticides, and diethyl ether.)

### A. Apparatus

(**a**) *Partition column.*—Column and O type reduction valve are shown in Fig. **949.05A**. Construct column of heavy-wall Pyrex tubing ca 3.5 mm thick, 90 cm long × 2.5 cm diam. Seal coarse porosity fritted glass disk in place and attach No. 18/9 ⯑ joint 5 cm below disk. Supply pressure from laboratory supply line. (Column available from Lurex Scientific, No. JC 1800-0104 constructed from heavy rather than std wall tubing.)

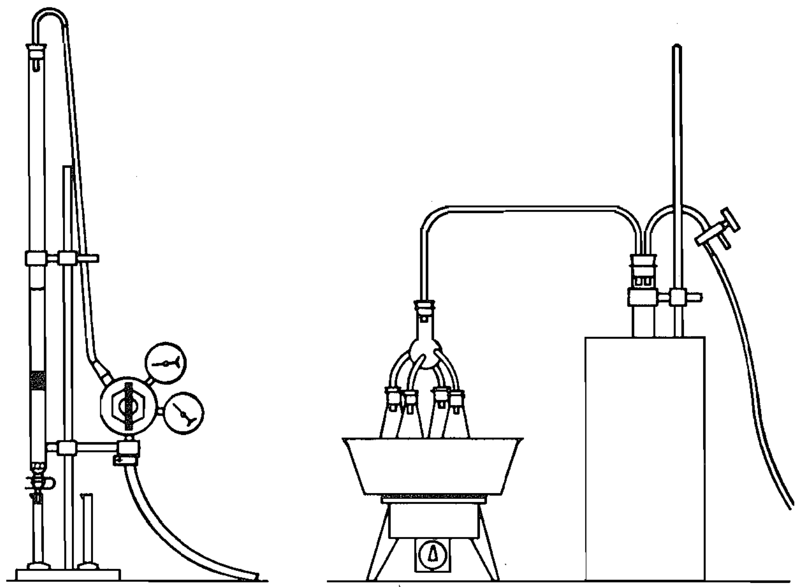

FIG. 949.05A—Partition column and solvent evaporator

(**b**) *Solvent evaporator*.—Fig. **949.05A**. Evap. fractions to dryness under reduced pressure at 60°, with aid of $H_2O$ pump. Recover solv. in trap consisting of Kjeldahl flask immersed in mixt. of NaCl and ice.

(**c**) *Melting point apparatus*.—Use Thiele mp app. equipped with mech. stirrer. App. shown in Fig. **949.05B**, or Hershberg modification (2) (available from Ace Glass, Inc., Cat No. 7686) is suitable.

(**d**) *Thermometer*.—Precision grade, meeting NIST specifications: partial immersion; range 90–120° in 0.2° subdivisions. Calibrated by NIST or against thermometer checked by NBS.

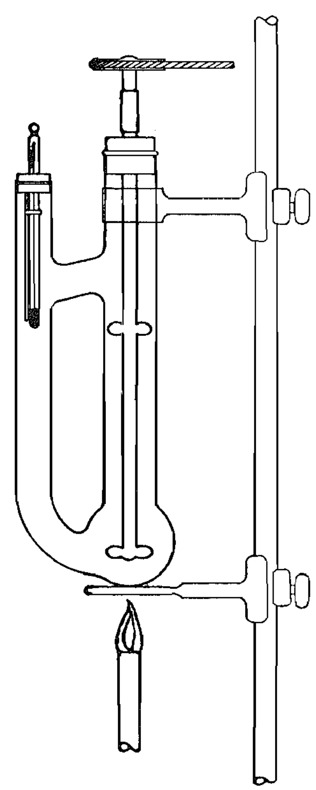

FIG. 949.05B—Melting point apparatus

(**e**) *Melting point tubes*.—1–2 mm capillary tubes of uniform wall thickness and diam.

### B. Reagents

(**a**) *n-Hexane*.—Com. grade, distd before use.

(**b**) *Nitromethane*.—Reflux com. grade material 4 hr and distil. No visible residue is left after evapn of 10 mL purified material.

(**c**) *Silicic acid*.—Use Mallinckrodt reagent grade (for chromatgy) which meets following requirements: When column prepd as in **949.05D** is used for detn on sample contg known amt of γ-isomer, flow rate and packing characteristics should be similar to those of an $H_2SIO_3$ known to be satisfactory, and recovery of γ-BHC should be within ±3% of the γ-BHC content.

(**d**) *Dye soln*.—Dissolve 25 mg D&C Violet No. 2 (1-hydroxy-4-*p*-toluidino-anthraquinone) in 50 mL mobile solv. and store in g-s bottle. (Available from Sigma Chemical Co.)

(**e**) *Mobile solvent*.—Satd soln nitromethane in *n*-hexane. Vigorously shake 2 L *n*-hexane with excess nitromethane in g-s bottle. Decant mobile solvent from nitromethane as needed.

### C. Preparation of Sample

(**a**) *Powders containing more than 10% γ-BHC*.—Crush and thoroly mix sample with mortar and pestle. Weigh enough sample into tared 125 mL erlenmeyer to provide ca 0.2 g γ-isomer after extg and aliquoting. Add 25 mL mobile solv., heat just to bp on steam bath, and cool to room temp., shaking occasionally. Decant ext thru buchner with ca 34 mm medium porosity fritted disk into 100 mL Kohlrausch flask, with gentle suction. Re-ext residue in flask, using 10 mL mobile solv. Wash residue and flask with five 10 mL portions cold mobile solv., decanting each wash thru buchner. Add 2 mL dye soln and dil. to vol. with mobile solv.

(**b**) *Dusts containing less than 10% γ-BHC*.—Weigh enough sample to provide 1.75–2.00 g γ-isomer. Transfer to Soxhlet extractor and ext overnight with ether. Evap. most of ether on steam bath and evap. remainder at room temp. under vac. Ext γ-isomer from residue with mobile solv. as in (**a**).

### D. Preparation of Column

(*Caution:* See safety notes on blenders and hexane.)

Transfer 100±0.5 g $H_2SiO_3$ to high-speed blender, add 300 mL mobile solv., and with mixing, add 55 mL nitromethane. Mix 15–30 sec; then pour into column thru glass funnel. Stir slurry with long glass stirring rod to displace air bubbles. Wash down sides of column with few mL mobile solv. and apply 5 lb pressure to pack column and force out excess solv.; tap column gently to aid packing. When boundary between solv. and $H_2SiO_3$ remains stationary, release pressure cautiously, pipet out most of excess solv., and reapply pressure until ca 3 mm solv. remains above adsorbent.

### E. Determination

(*Caution:* See safety notes on vacuum and pipets.)

Pipet 10 mL aliquot of sample soln onto column by letting it flow slowly down inside of column without disturbing $H_2SiO_3$ surface. Wash down side of column with 2 mL mobile solv. and force soln into column by applying 2–3 lb pressure, releasing pressure when all solv. has entered column. Add 10 mL mobile solv. and force into column. Release pressure and slowly add mobile solv. to within 7–12 cm from top of column. Apply enough pressure to force solv. thru column at 3–4 mL/min. Just before last trace of dye leaves column, begin to collect 10 mL fractions, alternately using two 10 mL graduates. Transfer each fraction to 125 mL erlenmeyer and evap. to dryness, using solv. evaporator. (Evap. fractions without boiling; if boiling begins, raise flask momentarily from $H_2O$ bath.)

Appearance of γ-isomer upon evapn is recognized by its tendency to cover bottom of flask as white residual film with typical crystal formation. When first residue of γ-isomer is recognized, begin to collect 10 mL fractions until all γ-isomer is obtained (usually ≤8 fractions). Dissolve residue in each flask with 5 mL *n*-hexane and transfer to weighed flask, rinsing flasks successively with 5 mL portions *n*-hexane. Evap. solv., using solv. evaporator. Evacuate flask ca 20 min at room temp. with vac. pump. (There is little danger in evacuating 125 mL erlenmeyer; larger size erlenmeyer, however, is likely to collapse under vac.) Release vac., wipe with clean, moist towel, and let stand 5 min. Weigh, and calc. % γ-benzene hexachloride in original sample.

### F. Melting Point Determination of the Gamma Fraction

Dissolve residue in min. amt acetone and transfer quant. to 10 mL beaker. Evap. acetone at 40°, using filtered air stream. Scrape residue from beaker for mp detn. (Beaker may be set on piece of solid $CO_2$ to ensure prepn of finely powd product.) Place material in agate mortar and mix thoroly with pestle.

Select 2 clean, dry capillary tubes and fill with sample. Be sure material is well packed into bottom of tube to ensure max. contact between sample and wall of tube. Insert tubes and thermometer bulb in Thiele tube so that samples and thermometer bulb touch. Start stirrer and heater, and adjust heating rate to 1°/min at 90°. Continue heating until sample melts or reaches 106°. Reduce heating rate to 0.5°/min and continue heating until sample melts.

Sample mp is corrected temp. of bath when last solid disappears into the clear melt. If mp is <108°, check result by IR method, **947.01**.

Refs.: (1) JAOAC **32,** 684(1949); **39,** 373(1956).
(2) Ind. Eng. Chem. **8,** 312(1936).

CAS-58-89-9 (γ-BHC)

## 953.06★ gamma-BHC in Pesticide Formulations

**Radioactive Tracer Method**
**First Action**
**Surplus 1970**

*See* **6.257–6.260**, 11th ed.

## 984.05 gamma-BHC in Technical BHC, Pesticide Formulations, and Lindane Shampoos and Lotions

**Gas Chromatographic Method**
**First Action 1984**
**Final Action 1986**
***CIPAC-AOAC Method***

(Applicable to tech., emulsifiable conc., and $H_2O$-dispersible powd. formulations and in lindane shampoo and lotion)

### A. Principle

Samples of tech. BHC (benzene hexachloride) and formulations are dissolved in EtOAc with dipropyl phthalate added as internal std. Lindane (γ-BHC) shampoo and lotion samples are extd with EtOAc-isooctane contg dipropyl phthalate internal std. EtOAc-isooctane layer is then extd with $CH_3CN$ to remove interferences. Std and sample solns are carried thru same extn procedures. γ-BHC content is detd by GC using flame ionization detector.

### B. Apparatus and Reagents

(**a**) *Gas chromatograph.*—Suitable for on-column injection; equipped with flame ionization detector.

(**b**) *Lindane lotion and shampoo.*—Accurately weigh ca 100 mg pure γ-BHC into 50 mL screw-cap centrf. tube. Add by pipet 10.0 mL internal std soln. Agitate to dissolve γ-BHC and add 20 mL $H_2O$. Accurately weigh 10 g 1% lindane lotion or shampoo into 50 mL screw-cap centrf. tube. Add 20 mL $H_2O$. Gently agitate contents by tapping tube with fingers to mix $H_2O$ and shampoo or lotion. Add by pipet 10.0 mL internal std soln. Vigorously shake std and samples by hand 1 min. Add 5 drops isoamyl alcohol to each tube and centrf. Transfer by pipet 3.0 mL each ext (top layer) to sep. 15 mL screw-cap centrf. tubes. Add by pipet 5.0 mL isooctane followed by 3.0 mL $CH_3CN$. Vigorously shake each tube by hand 30 s. After phases sep., withdraw by disposable pipet a portion of $CH_3CN$ phase (lower layer) for GC analysis.

### D. Analysis of Solutions

(**a**) *Technical BHC and formulations.*—Inject 1 μL portions of std soln of γ-BHC until response ratios (area or peak ht) for γ-BHC to internal std agree ±2%. Make duplicate injections of std soln followed by duplicate injections of sample solns. Recalibrate after not more than 4 injections of sample solns.

(**b**) *Lindane lotion and shampoo.*—Inject 1 μL portions of ext of std soln of γ-BHC until response ratios (area or peak ht) for γ-BHC to internal standard agree ±2%. Make duplicate injections of std soln followed by duplicate injections of unknown exts. Recalibrate after not more than 4 injections of unknown exts.

### E. Calculations

For each injection, calc. response ratio ($R$) = area (or peak ht) of γ-BHC peak to area (or peak ht) of internal std peak.

$$\gamma\text{-BHC, wt\%} = (R/R')(W'/W) \times P$$

where $R'$ and $R$ = av. response ratio for std and sample solns, resp.; $W'$ and $W$ = wt (mg) of $\gamma$-BHC in std and in sample, resp.; and $P$ = purity of $\gamma$-BHC std (%).

Ref.: JAOAC **67,** 834(1984).

CAS-58-89-9 ($\gamma$-BHC)

## 947.01 Benzene Hexachloride in Pesticide Formulations

### Infrared Spectrophotometric Method

### Final Action

(Applicable to tech. BHC. *Caution:* See safety notes on pipets, pesticides, and carbon disulfide.)

#### *A. Apparatus*

*Infrared spectrometer.*—With matched pair of liq. absorption cells, 0.5–1.1 mm thick.

#### *B. Calibration of Cells*

Det., in spectrometer, difference between deflections of the 2 cells filled with $CS_2$. Plainly mark one cell to be used as sample cell for reading $I$. Correct values of $I_0$ obtained with other cell by adding or subtracting difference between cells and refer to this as cell factor $F$. Check factor every 10–14 days.

#### *C. Preparation of Standards and Working Curves*

Obtain $\alpha$, $\beta$, $\gamma$, and $\delta$ isomers of BHC, either by fractional crystn from tech. material or as sepd materials, and recrystallize several times from solvs that have been redistd from all-glass app. Recrystallize from following solvs until mps by capillary tube method become const: $\alpha$ isomer from benzene followed by MeOH (mp ca 158°); $\beta$ isomer from toluene (mp ca 210.5°, sealed capillary); $\gamma$ isomer from MeOH (mp ca 113°); and $\delta$ isomer from $CCl_4$ followed by $CHCl_3$ (mp ca 138.5°).

Confirm purity of each isomer as follows: Evap. to dryness enough mother liquor from last crystn to yield $\geq$1 g dissolved solids, grind residue, and dry overnight in evacuated desiccator. Weigh and dissolve in enough $CS_2$ to make 4 g/100 mL soln. Prep. corresponding soln of recrystd isomer as std. Compare solns of residue and std in spectrometer at wavelength points used for analysis of other isomers. Consider purity of isomer satisfactory if $A$ of residue soln is not significantly greater than that of std at these points.

Prep. working curves of the isomers by detg $T$ of their solns in $CS_2$ at various concns as in **947.01D**. Calc. $A$ and plot against concn in g/L.

#### *D. Determination*

Reduce sample of tech. BHC to ca 2 g by grinding and quartering, and dry 24 hr *in vacuo* at room temp. Weigh 1.5000 g dried material into 50 mL vol. flask and dil. to vol. with $CS_2$ (equiv. to 30 g/L). Shake vigorously to dissolve ($\beta$ isomer is not completely sol. and will settle out). Pipet 25 mL of this sample soln into another 50 mL vol. flask and again dil. to vol. with $CS_2$ (equiv. to 15 g/L). Fill sample cell with the concd soln for reading $I$, and fill blank cell with $CS_2$, place in spectrometer, and read $T$ in duplicate at following wavelengths:

| Isomer | Wavelength, μm |
|---|---|
| Alpha | 12.58 |
| Beta | 13.46 |
| Gamma | 14.53 |
| Delta | 13.22 |
| Epsilon | 13.96 |

Average duplicates for calcns. Repeat readings with dil. soln (15 g/L) at $\alpha$ and $\gamma$ wavelengths. Calc. $A$ of each of isomers at the various wavelengths from $T$ measurements by equation:

$$\text{Log}\,\frac{(F \times I_b) - (F \times I_b \times \%\,Sct)}{I_s - (F \times I_b \times \%\,Sct)} = A$$

where $F$ = cell factor, $I_b$ = reading of blank cell, $\%\,Sct$ = % scatter, $I_s$ = reading of sample cell, and $A$ = absorbance.

Obtain approx. concns from working curves, **947.01C**. Correct $A$ at each wavelength for absorption of interfering components. (Altho $\beta$ isomer has low solubility in $CS_2$, this isomer interferes with $\delta$ analytical point; therefore det. $A$ of $\beta$ isomer in $CS_2$ at this point and apply as correction.) Since these new values are overcorrected, make repeated evaluations until successive values are const, within desired precision.

Refs.: Anal. Chem. **19,** 779(1947); Report No. 4760; May 15, 1949, Phys. Chem. Lab., Hooker Electrochemical Co., Niagara Falls, NY.

CAS-608-73-1 (benzene hexachloride)

## 973.13 Benfluralin or Trifluralin in Pesticide Formulations

### Ultraviolet Spectroscopic Method

### First Action 1973
### Final Action 1975

#### *A. Principle*

Trifluralin or benfluralin is extd from solid carrier or dissolved in *n*-hexane if liq., purified by chromatgy on Florisil, and detd by UV spectrometry at 376 nm.

#### *B. Reagents*

(**a**) *Florisil.*—100–200 mesh. Test elution characteristics of Florisil by adding 5 mL std soln to prepd column. Proceed as in **973.13E**. Elution vol. should be $\geq$80 mL but <100 mL. If elution vol. does not fall within this range, adjust $H_2O$ content of Florisil by trial and error to obtain proper elution (add $H_2O$ to decrease elution time; dry at 130° to increase it).

(**b**) *Std soln.*—1.25 mg/mL. Weigh 0.125 g trifluralin or benfluralin Ref. Std (Elanco Products Co.), into 100 mL vol. flask, dil. to vol. with *n*-hexane, and mix.

#### *C. Preparation of Column*

Insert glass wool plug in bottom of 25 × 400 mm glass tube with Teflon stopcock. Add, with const tapping of column, 5 g anhyd. $Na_2SO_4$, stdzd Florisil, (**a**), to ht of 50 mm, and 5 g anhyd. $Na_2SO_4$. With stopcock open, add 50 mL *n*-hexane and let drain to top of column. Close stopcock.

#### *D. Preparation of Sample*

(**a**) *Dry formulations* (*containing more than 1% trifluralin or benfluralin*).—Weigh sample contg 0.25 g trifluralin or benfluralin into Soxhlet extn thimble (33 × 80 mm), cover with glass wool, and ext with $CHCl_3$ 1 hr beyond time when no further color is extd. Quant. transfer ext to 200 mL vol. flask with $CHCl_3$, dil. to vol. with $CHCl_3$, and mix. Transfer 5 mL to r-b flask and evap. *just* to dryness on rotary evaporator.

(**b**) *Dry formulations* (*containing 1% or less trifluralin or benfluralin*).—Weigh sample contg 0.05 g trifluralin or benfluralin, ext, transfer to 200 mL vol. flask, and dil. as in (**a**). Transfer 25 mL to r-b flask and evap. *just* to dryness on rotary evaporator.

(**c**) *Liquid formulations.*—Weigh sample contg 0.12 g trifluralin or benfluralin into 100 mL vol. flask. Dil. to vol. with *n*-hexane and mix vigorously. Proceed as in **973.13E**.

### E. Determination

Transfer 5 mL soln from (**c**) or residue from (**a**) or (**b**), with aid of *n*-hexane, to Florisil column. Transfer 5 mL std soln to second Florisil column. Wash sample into column with small portions *n*-hexane. Let each portion drain to top of column before adding next. Fill column with *n*-hexane, discarding eluate until band has moved ca $^3/_4$ length of column. Collect eluate contg trifluralin or benfluralin band (first yellow-orange band to elute) in 100 mL vol. flask. (If band requires >100 mL vol. to elute, replace vol. flask with r-b flask, evap., and transfer quant. to 100 mL vol. flask.) Dil. to vol. with *n*-hexane and mix. Det. *A* of sample and std solns in 1 cm cells at 376 nm against *n*-hexane as ref.

### F. Calculations

% Trifluralin or benfluralin

$$= (A \times \text{g std} \times F \times P)/(A' \times \text{g sample})$$

where $A$ and $A'$ refer to sample and std solns, resp.; $P$ = % purity of std; and $F$ = 2, 0.4, or 1 for sample prepns (**a**), (**b**), or (**c**), resp.

Ref.: JAOAC **56**, 567(1973).

CAS-1861-40-1 (benfluralin)
CAS-1582-09-8 (trifluralin)

## 973.14 Benfluralin or Trifluralin in Pesticide Formulations
### Gas Chromatographic Method
### Final Action

### A. Principle

Trifluralin or benfluralin is extd from solid carrier, or dissolved in acetone if liq., and detd by GC.

### B. Reagents

(**a**) *Diisobutyl phthalate internal std soln.*—Weigh 0.625 g diisobutyl phthalate (Eastman Kodak Co.) into 250 mL vol. flask, dil. to vol. with acetone, and mix.

(**b**) *Std soln.*—1.6 mg/mL. Weigh 0.16 g trifluralin or benfluralin Ref. Std into 100 mL vol. flask, dil. to vol. with acetone, and mix.

### C. Apparatus

(**a**) *Gas chromatograph.*—Equipped with flame ionization detector; capable of programmed column temp. from 135 to 190° at 8°/min. Approx. instrumental conditions: inlet 205°, detector 275°, N carrier gas 60 mL/min.

(**b**) *Column.*—1.5 m (5′) × $^1/_8$ or $^1/_4$″ od, stainless steel or Pyrex glass tube packed with 5% DC 200, 12,500 cstokes (Analabs, Inc.) on 80–100 mesh Chromosorb W (HP). Condition newly prepd column at 230° overnight with N carrier gas.

### D. Preparation of Sample

(**a**) *Dry formulations* (*containing more than 1% trifluralin or benfluralin*).—Weigh sample contg 0.16 g trifluralin or benfluralin into Soxhlet extn thimble (33 × 80 mm), cover with glass wool, and ext with acetone 1 hr beyond time when no further color is extd. (*Caution:* See safety notes on distillation and acetone.) Evap. to ca 60 mL on steam bath with stream of air directed into flask. Transfer quant. to 100 mL vol. flask with acetone. Dil. to vol. with acetone and mix.

(**b**) *Dry formulations* (*containing 1% or less trifluralin or benfluralin*).—Weigh sample contg 0.04 g trifluralin or benfluralin, ext, and evap. as in (**a**). Transfer quant. to 100 mL vol. flask with acetone and proceed as in **975.14E** without dilg, beginning, ". . . add 10 mL internal std soln, . . ."

(**c**) *Liquid formulations.*—Weigh sample contg 0.16 g trifluralin or benfluralin into 100 mL vol. flask, dil. to vol. with acetone, and mix.

### E. Determination

Pipet 25 mL acetone soln, **973.14D(a)** or (**c**), and 25 mL std soln, (**b**), into sep. 100 mL vol. flasks, add 10 mL internal std soln, dil. to vol. with acetone, and mix.

Inject 2.5 μL trifluralin or benfluralin std soln and start temp. program to give symmetrical peak ca 70% scale deflection and retention time 5.5 min. Diisobutyl phthalate internal std peak appears ca 2 min after std peak. Repeat injection of std soln until ratio of trifluralin or benfluralin peak area to internal std peak area is reproducible.

Without changing conditions inject 2.5 μL sample soln.

Calc. areas of trifluralin or benfluralin and diisobutyl phthalate peaks. Divide area of trifluralin or benfluralin peak by area of diisobutyl phthalate internal std peak to det. ratio, $R$.

$$\% \text{ Trifluralin or Benfluralin} = (R/R') \times (W'/W) \times (P/F)$$

where $R$ and $R'$ = ratio for sample and std solns, resp.; $W$ and $W'$ = g sample and std, resp.; $P$ = % purity of std; and $F$ = 1, 1, or 4 for sample prepns (**a**), (**c**), *or* (**b**), resp.

Ref.: JAOAC **56,** 567(1973).

CAS-1861-40-1 (benfluralin)
CAS-1582-09-8 (trifluralin)

## 980.05 Bromoxynil Octanoate in Pesticide Formulations
### Gas Chromatographic Method
### First Action 1980
### Final Action 1981

### A. Principle

Bromoxynil octanoate formulations are dild with $CHCl_3$, with *n*-docosane as internal std, and ester is detd by GC with flame ionization detection.

(*Caution:* See safety notes on pesticides and chloroform.)

### B. Apparatus and Reagents

(**a**) *Gas chromatograph.*—Hewlett-Packard Model 5830, or equiv., with flame ionization detector and 1.8 m (6′) × 2 mm (id) glass column with 10% SP-2100 on 100–120 mesh Supelcoport (Supelco, Inc.). Operating conditions: temps (°): injection port 240, column 220, detector 300; carrier gas flow 40 mL He/min; sensitivity 10 × 64; injection vol. 2 μL with heated on-column injector. Retention times for *n*-docosane and bromoxynil octanoate are 10 and 14 min, resp. Theoretical plates of column for bromoxynil octanoate must be ≥3,000.

(**b**) *Bromoxynil octanoate.*—Anal. std purity (Rhône-Poulenc Ag Co., 2 T.W. Alexander Dr, PO Box 12014, Research Triangle Park, NC 27709).

(**c**) *n-Docosane.*—Aldrich Chemical Co., Inc.; Cat. No. 13445-7, or equiv.

(**d**) *Internal std soln.*—8.0 g *n*-Docosane dild to 1 L with $CHCl_3$.

### C. Preparation of Standard and Sample

(**a**) *Std soln.*—Weigh 0.500 g anal. std bromoxynil octanoate into 100 mL vol. flask. Pipet 25 mL internal std soln into flask, dil. to vol. with $CHCl_3$, and shake well to dissolve ester.

(**b**) *Sample soln.*—Weigh amt of formulation contg 0.500 g bromoxynil octanoate into 100 mL vol. flask, pipet in 25 mL internal std soln, dil. to 100 mL with $CHCl_3$, and shake well.

### D. Determination

Inject std and sample solns in duplicate. Response ratios for each set of duplicate injections must not differ by >1%.

$$\% \text{ Bromoxynil octanoate} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = response ratio (area of bromoxynil octanoate peak to internal std peak) for sample and std, resp.; $W$ and $W'$ = g sample and std, resp.; and $P$ = % purity of std.

Ref.: JAOAC **62,** 1215(1979).

CAS-1689-99-2 (bromoxynil octanoate)

## 986.04 Butachlor in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1986**
**Final Action 1988**

***AOAC-CIPAC Method***

### A. Principle

Sample is dissolved in acetone contg triphenyl phosphate as internal std, analyzed by gas chromatgy with flame ionization detection, and measured by comparison with internal std on the basis of integrated relative peak areas.

### B. Safety

$LD_{50}$ of butachlor has been found to be 4600 mg/kg in rat acute oral studies (Monsanto). Avoid excessive exposure by wearing protective clothing.

### C. Apparatus

(**a**) *Gas chromatograph*.—With flame ionization detector and on-column injection ports. Temps—column over 250°, injection port 280°, detector 300°; gas flows (mL/min)—He carrier gas 30, H 34, air 430; sample size 1.0 µL; run time 25 min.

(**b**) *Column*.—6 ft × 2 mm (id) glass column (on-column configuration) packed with 10% SP-2250 on 100–120 mesh Supelcoport (Supelco, Cat. No. 1-2132), or equiv. SP-2250 is methyl-phenyl silicone (50 + 50). Precondition overnight at 250° before use. Retention times of butachlor and internal std are ca 5.9 and 18.5 min, resp.

### D. Reagents

(**a**) *Acetone*.—Pesticide grade (Fisher Scientific Co., or equiv.).

(**b**) *Triphenyl phosphate internal std soln*.—Gold Label (Aldrich Chemical Co., Inc.). Weigh 6.4 g into 1 L vol. flask. Dissolve in and dil. to vol. with acetone.

(**c**) *Butachlor std soln*.—99.7% (recrystd from hexane at −40°; Monsanto Chemical Co.). Accurately weigh 0.2 g into small flask. Add by pipet 25 mL internal std soln and shake to dissolve.

### E. Determination

Accurately weigh sample contg ca 0.2 g butachlor into small flask. Add by pipet 25.0 mL internal std soln and shake ≥5 min to ext butachlor. For emulsifiable concs, use ca 0.3000 g.

Make replicate 1 µL injections of butachlor std soln and measure response ratio, $R$ (area butachlor peak/area internal std peak) for each injection. Repeat until consecutive response ratios agree ±0.5%.

Make duplicate injections of sample soln and det. av. $R$. Follow with injection of butachlor std soln; average $R'$ for std before and after sample injection.

$$\text{Butachlor, } \% = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std, resp.; $W$ and $W'$ = wt (g) of sample and std, resp.; $P$ = % purity of std.

Ref.: JAOAC **69,** 721(1986).

CAS-23184-66-9 (butachlor)

## 971.05★ Captan in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1971**
**Final Action 1982**
**Surplus 1984**

***AOAC-CIPAC Method***

*See* **6.247–6.251**, 14th ed.

## 980.06 Captan in Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1980**
**Final Action 1982**

***AOAC-CIPAC Method***

(Method is suitable for tech. captan and formulations with captan as only active ingredient.)

### A. Principle

Captan is extd from inerts with soln of diethyl phthalate in $CH_2Cl_2$. Soln is chromatgd on microparticulate silica gel column, using $CH_2Cl_2$ as mobile phase. Ratio of captan peak ht to diethyl phthalate peak ht is calcd from UV response and compared to std material for quantitation.

(*Caution:* See safety notes on pipets and pesticides.)

### B. Apparatus and Reagents

(**a**) *Liquid chromatograph*.—Able to generate over 1000 psi and measure $A$ at 254 nm.

(**b**) *Chromatographic column*.—Large bore column contg narrow-range (≤10 µm) porous silica gel particles. Partisil-10, 4.65 mm id × 25 cm is suitable. Available from Whatman, Inc.

(**c**) *Strip chart recorder*.—Range to match output of LC detector.

(**d**) *Diethyl phthalate*.—EM Science No. 1295.

(**e**) *Reference std captan*.—Chevron Chemical Co., PO Box 4010, Richmond, CA 94804.

(**f**) *Methylene chloride*.—Spectroscopic grade or distd in glass. (Burdick and Jackson Laboratories, Inc.).

(**g**) *Glass fiber filter paper*.—Whatman GF/A, or equiv.

### C. Preparation of Standard

(**a**) *Internal std soln*.—0.312 mg diethyl phthalate/mL. Weigh ca 156 mg diethyl phthalate and transfer to 500 mL vol. flask. Dil. to vol. with same $CH_2Cl_2$ to be used for mobile phase. Concn may be varied to accommodate column and instrument differences. If necessary, adjust concn so that peak ht of diethyl phthalate matches peak ht of captan within 20%.

(**b**) *Std soln*.—0.8 mg captan/mL, which is in the optimum linearity range. Accurately weigh ca 40 mg std captan into glass bottle. Pipet in 50 mL internal std soln, shake mech. 15 min, and filter thru glass fiber paper. Prep. fresh std daily.

### D. Preparation of Sample

Accurately weigh sample expected to contain 40 mg captan into glass bottle. Pipet in 50 mL internal std soln. Place on mech. shaker 15 min. Centrf. and filter supernate thru glass fiber paper. Prep. fresh sample daily.

### E. Determination

Adjust operating parameters to cause captan to elute in 4–6 min. Maintain all parameters const thruout analysis. Typical values are: flow rate, 2.5 mL $CH_2Cl_2$/min, max.; pressure, ca 800 psi; chart speed 0.2″/min; mobile phase, degassed $CH_2Cl_2$ equilibrated with room air; $A$ range, 0.04 AUFS; temp., ambient; injection vol., 20 μL.

Changes in $H_2O$ content of mobile phase affect retention time and peak hts of captan and internal std, so use same source of $CH_2Cl_2$ thruout analysis.

Adjust injection size and attenuation to give largest possible on-scale peaks. Make repetitive injections of std until response is stable and ratio of captan peak ht to diethyl phthalate peak for successive injections agree to within ±2% of their mean.

Inject sample. Peak ht ratio must be within 10% of peak ht ratio for std. If not, reweigh samples to match std. If within 10%, reinject sample. Peak ht ratios for 2 sample injections must agree to within ±2% of their mean. If not, repeat detn starting with std injections.

Reinject std twice. Av. peak ht ratios of 2 stds immediately preceding and following sample injections must agree to within ±2% of their mean. If not, repeat detn.

### F. Calculation

Measure peak hts to 3 significant figures, and calc. ratio for each injection. Average 4 std ratios, and the 2 sample ratios.

$$\% \text{ Captan} = (R/R') \times (W'/W) \times P$$

where $R$ = av. sample ratio (captan peak ht/diethyl phthalate peak ht); $R'$ = av. std ratio (captan peak ht/diethyl phthalate peak ht); $W$ = mg sample; $W'$ = mg std, and $P$ = % purity of std.

Ref.: JAOAC **63,** 1231(1980).

CAS-133-06-2 (captan)

## 971.06 Chloramben in Pesticide Formulations

**Spectrophotometric Method**
**First Action 1971**
**Final Action 1973**
***AOAC-CIPAC Method***

### A. Principle

Chloramben contains conjugated π electron system of benzene which absorbs strongly in UV. Absorption is measured quant. at 297 mm. (*Caution:* See safety notes on pipets and pesticides.)

### B. Apparatus and Reagents

(**a**) *Spectrophotometer.*—For use in UV, with 1 cm cells.
(**b**) *Shake-out flask.*—250 mL erlenmeyer, with screw cap.
(**c**) *Chloramben std solns.*—(*1*) *Stock soln.*—0.38 mg/mL. Accurately weigh 19±2 mg chloramben (Rhône-Poulenc Ag Co., 2 T.W. Alexander Dr, PO Box 12014, Research Triangle Park, NC 27709) into 50 mL vol. flask, add 25 mL 1% NaOH, agitate until dissolved, dil. to vol., and mix. (*2*) *Working soln.*—0.038 mg/mL. Pipet 5 mL stock soln into 50 mL vol. flask, dil. to vol. with 1% NaOH soln, and mix.

### C. Preparation of Sample

Mix 10 g granular sample on 12 × 12″ paper by lifting alternate corners.

### D. Determination

(**a**) *Dry granular formulations.*—Add amt solid material and 1% NaOH soln specified in Table **971.06** to 250 mL shake-out flask and shake 30 min. Filter, and transfer stated aliquot to vol. flask. Dil. to vol. with 1% NaOH soln and mix. Det. $A$ at 360 and 297 nm against 1% NaOH. Calc. $\Delta A = A_{297} - A_{360}$. Det. $\Delta A'$ of working std soln similarly.

$$\% \text{ Chloramben} = (\Delta A \times (\text{mg std/mL}) \times F)/(\Delta A' \times \text{g sample} \times 10)$$

where $F$ = factor in Table **971.06**.

(**b**) *Liquid formulations.*—Weigh amt liq. indicated in Table **971.06** into 100 mL vol. flask, dil. to vol. with 1% NaOH soln, and mix. Transfer 1 mL aliquot to 100 mL vol. flask, dil. to vol. with 1% NaOH, and mix. Proceed as in (**a**).

Ref.: JAOAC **53,** 1155(1970).

CAS-133-90-4 (chloramben)

## 962.05 Chlordane (Technical) and Pesticide Formulations

**Total Chlorine Method**
**First Action 1962**
**Final Action 1965**

(*Caution:* See safety notes on distillation, pipets, sodium biphenyl, flammable solvents, toxic solvents, pesticides, and benzene.)

### A. Preparation of Standard Solutions

(**a**) *Sodium chloride std soln.*—0.1*N*. Dissolve 5.845 g NaCl, previously dried 2 hr at 105°, in $H_2O$, and dil. to 1 L in vol. flask.

(**b**) *Silver nitrate std soln.*—0.1*N*. Prep. as in **941.18A**. To 250 mL g-s erlenmeyer add 15.00 mL 0.1*N* NaCl, (**a**), 50 mL $H_2O$, 10 mL $HNO_3$ (1+1), boiled to expel oxides of N, and 25.00 mL of the $AgNO_3$ soln. Add 3 mL nitrobenzene, stopper, and shake vigorously 15 sec. Add 5 mL ferric indicator, **929.04A(e)**, and back-titr. with 0.1*N* KSCN, (**c**), to reddish-brown end point. (Potentiometric titrn using Ag indicator electrode and Ag-AgCl or glass ref. electrode may be substituted for indicator method, but must be used in both stdzn and detn.)

(**c**) *Potassium thiocyanate std soln.*—0.1*N*. Prep. and titr. against $AgNO_3$ soln, (**b**), as in **941.18D(b)**. Calc. $F$ = mL $AgNO_3$ soln/mL KSCN soln.

$$\text{Normality } AgNO_3 \text{ soln} = \text{mL NaCl soln} \times 0.1000/(\text{mL } AgNO_3 \text{ soln} - \text{mL KSCN soln} \times F)$$

(**d**) *Sodium biphenyl reagent.*—30% w/w. (*Caution:* See safety notes on sodium metals.) Place 300 mL dry toluene and 58 g Na in dry 2 L 3-neck flask equipped with adjustable speed sealed stirrer, inlet for N, and reflux condenser. With stirrer off, and with slow stream of N passing thru flask, warm until refluxing begins and Na is entirely melted. Agitate vigorously until Na is finely dispersed; then cool to <10°. Remove reflux condenser and add 1.25 L anhyd. ethylene glycol dimethyl ether. Add 390 g biphenyl with moderate stirring and with slow stream of N passing thru flask. Reaction should begin within few min, indicated by blue or green color which gradually darkens to black. Maintain temp. at <30° with oil bath or other cooling medium not involving hazard should flask contg Na break. Reaction should be complete in 1 hr. Reagent protected from moisture and air has useful life of 1–2 months at 25°.

(Premixed reagent, packed in 15 mL vials, each enough for 1 detn, is available from Southwestern Analytical Chemicals, Inc., PO Box 485, Austin, TX 78767.)

### B. Preparation of Sample

(**a**) *Emulsifiable concentrate formulations.*—Accurately weigh sample contg 0.5±0.05 g tech. chlordane into 50 mL

**Table 971.06 Parameters for Sample Analysis**

| Sample | Chloramben, % | Sample Wt, g±0.1 | 1% NaOH, mL | Aliquot, mL | Final Diln | Factor (F) |
|---|---|---|---|---|---|---|
| Dry granular | 1.2 | 3.0 | 50 | 2 | 50 | 1,250 |
| Dry granular | 4 | 7.5 | 100 | 1 | 100 | 10,000 |
| Dry granular | 10 | 3.0 | 100 | 1 | 100 | 10,000 |
| Liquid | 21.6 | 1.8 | 100 | 1 | 100 | 10,000 |

vol. flask, dissolve, and dil. to vol. with toluene. Transfer 5 mL aliquot to 125 mL separator, add 15 mL or g Na biphenyl reagent, **962.05A(d)** above and then swirl. If soln is not dark green, add more reagent. Let stand 3 min and add 3–5 mL $H_2O$ dropwise. With stopper removed, swirl soln gently to decompose excess reagent. Add 25 mL $H_2O$, stopper, and mix with gentle rocking motion. (Do not shake vigorously.) Let layers sep. and drain lower aq. layer into 250 mL erlenmeyer. Re-ext solv. layer with two 25 mL portions 3*N* $HNO_3$ and combine aq. solns in erlenmeyer.

(**b**) *Dusts, granular impregnates, and wettable powders.*—Accurately weigh sample contg 0.5±0.05 g tech. chlordane into Soxhlet extn thimble. Ext with 80 mL benzene in Soxhlet app. 1 hr. Transfer to 100 mL vol. flask, washing with several 3 mL portions benzene. Dil. to vol. with benzene and transfer 10 mL aliquot to 125 mL separator. Proceed as in (**a**).

### C. Determination

Add 15.00 mL 0.1*N* $AgNO_3$ and 3 mL nitrobenzene to erlenmeyer, stopper, and shake vigorously 15 sec. Rinse stopper, add 5 mL ferric indicator, **929.04A(e)**, and back-titr. with 0.1*N* KSCN to reddish-brown end point. (Designate mL KSCN as *D*.)

Det. blank on reagents by pipetting 5 mL toluene into 125 mL separator, add 15 mL or g Na biphenyl reagent, and proceed as in **962.05B(a)**, thru combining aq. solns in erlenmeyer. Add 15.00 mL 0.1*N* NaCl, 25.00 mL 0.1*N* $AgNO_3$, and 3 mL nitrobenzene, and proceed as above. Calc. blank correction factor, $C$ = mL KSCN used in stdzn of $AgNO_3$ − mL KSCN used in blank detn.

$$\% \text{ Chlorine} = [15 - (C + D) \times F] \times \text{normality } AgNO_3 \times 3.545/\text{g sample}$$

$$\% \text{ Tech. chlordane} = \% \text{ Cl} \times 1.56$$

Ref.: JAOAC **45,** 513(1962).

CAS-57-74-9 (tech. chlordane)

## 965.14★ Chlordane (Technical) and Pesticide Formulations

**Colorimetric Method**

**First Action 1965**
**Final Action 1967**
**Surplus 1982**

*See* **6.266–6.270**, 14th ed.

## 966.06 Hexachlorocyclopentadiene in Technical Chlordane

**Spectrophotometric Methods**

**First Action 1966**
**Final Action 1967**

(Applicable to tech. chlordane, but not to formulations)

### A. Reagent

*Hexachlorocyclopentadiene (HEX) std solns.—Stock soln.*—0.1 g/100 mL. Weigh 0.1000 g hexachlorocyclopentadiene Ref. Std (available from Velsicol Chemical Corp., 5600 N. River Rd, Rosemont, IL 60018-5119) in 100 mL vol. flask, dil to vol. with MeOH, and shake to dissolve. *Std soln 1.*—0.005 g/100 mL. Dil. 5 mL stock soln to 100 mL with MeOH. *Std soln 2.*—0.002 g/100 mL. Dil. 2 mL stock soln to 100 mL with MeOH.

**Method I**

### B. Calibration

With MeOH in both ref. and sample cells (matched 1 cm silica), adjust 0 and 100% settings on UV spectrophtr at 324 nm. Empty sample cell, rinse several times with, and then fill with *std soln 1*, and read *A*. Empty sample cell, rinse with MeOH, then rinse and fill with *std soln 2*, and read *A*. Calc. *A* factor, *K*, for each std soln = (g std HEX/100 mL)/*A*. Average the two *K* values.

### C. Determination

Weigh 0.5 g sample in 100 mL vol. flask, dil. to vol. with MeOH, and shake to dissolve. Proceed as in **966.06B**, treating sample soln in same manner as stds.

$$\% \text{ HEX in sample} = (A \text{ of sample soln} \times 100 \times K)/(\text{g sample}/100 \text{ mL})$$

### D. Method II

(Includes corrections for other components of chlordane which absorb at 324 nm)

Proceed as in **966.06B** and **C**, except det. *A* of all solns at 300, 324, and 350 nm. Settings of 0 and 100% must be repeated at 300, 324, and 350 nm for *A* readings at those points. Calc. $K = (\text{g std HEX}/100 \text{ mL})/[A_{324} - 0.5(A_{300} + A_{350})]$.

$$\% \text{ HEX in sample} = [A_{324} - 0.5(A_{300} + A_{350})] \times 100 \times K/(\text{g sample}/100 \text{ mL})$$

Ref.: JAOAC **49,** 254(1966).

CAS-77-47-4 (hexachlorocyclopentadiene)

## 973.15★ Alpha and Gamma Isomers in AG Chlordane Technical

**Infrared Spectroscopic Method**

**Final Action 1974**
**Surplus 1982**

(Not applicable to tech. chlordane or its formulations)

*See* **6.275–6.278**, 14th ed.

## 973.16★ AG Chlordane in Granular Pesticide Formulations

### Infrared Spectroscopic Method

**First Action 1973**
**Final Action 1974**
**Surplus 1982**

(Not applicable to tech. chlordane or its formulations)

*See* **6.279–6.283**, 14th ed.

## 973.17★ Heptachlor in AG Chlordane

### Gas Chromatographic Method

**Final Action 1974**
**Surplus 1982**

(Not applicable to tech. chlordane or its formulations)

*See* **6.284–6.287**, 14th ed.

## 985.05 Chlordimeform in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1985**
**Final Action 1987**

***AOAC-CIPAC Method***

### *A. Principle*

Chlordimeform is extd with $CH_2Cl_2$ and detd by flame ionization gas chromatgy, using diethyl terephthalate as internal std. Identity is verified simultaneously by comparing retention times with std.

### *B. Apparatus*

(**a**) *Gas chromatograph.*—Capable of temp. program, preferably equipped with auto-injector, flame ionization detector, and integration capabilities.

(**b**) *Chromatographic column.*—2 mm id × 1.83 m (6 ft) glass column packed with 3% CBWX-20M on 80–100 mesh Gas-Chrom Q. Condition column ≥24 h at 225°, using carrier gas at ca 20 mL/min. Operating conditions: injector 250°; detector 250°; column 170° for 22 min, then to 225° at 20°/min, and hold 15 min; He carrier gas flow ca 25 mL/min. Retention times for internal std and chlordimeform are ca 11 and 14.8 min, resp.

### *C. Reagents*

(**a**) *Internal std soln.*—4 mg/mL. Dissolve 4.0 g diethyl terephthalate in $CH_2Cl_2$ and dil. to 1 L with $CH_2Cl_2$. Check internal std soln for interfering components by injecting an aliquot into chromatograph.

(**b**) *Chlordimeform std soln.*—Accurately weigh 100 mg chlordimeform std of known purity (Ciba-Geigy Corp., Production Technical Dept, PO Box 18300, Greensboro, NC 27419) into 4 oz bottle. Pipet 50.0 mL internal std soln into bottle, cap, and shake 30 min.

### *D. Preparation of Sample*

Accurately weigh amt of sample contg ca 100 mg chlordimeform into 4 oz bottle. Pipet 50.0 mL internal std soln into bottle, cap, shake 30 min to ext.

### *E. Determination*

Set integration parameters, and stabilize instrument by injecting 1–3 μL aliquots of std soln until area ratios of chlordimeform to internal std vary less than 2% for successive injections. Using same established injection vol. as for std, inject sample. Sample area ratio should be ±10% of std area ratio. Inject 2 aliquots of std and 2 aliquots of sample followed by 2 aliquots of 2nd sample and 2 aliquots of std. Repeat sequence until all samples are analyzed. Calc. response factor, $R$, for each injection:

$$R = \text{peak area (or ht) chlordimeform} / \text{peak area (or ht) internal std}$$

$$\text{Chlordimeform, \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response factor for sample and std solns, resp.; $W$ and $W'$ = mg sample and std, resp.; and $P$ = purity (%) of std.

Ref.: JAOAC **68,** 589(1985).

CAS-6164-98-3 (chlordimeform)

## 978.05 2,4-D in Pesticide Formulations

### Automated Liquid Chromatographic Method

**First Action 1978**

### *A. Principle*

Esters of 2,4-D are saponified *in situ*; amine salts are converted to $H_2O$-sol. K salt of 2,4-D. Ionic 2,4-D is protonated by pH 2.95 $CH_3CN$-$H_2O$ (1+4) eluant, and sepd from all known impurities and *p*-bromophenol internal std on reversed phase bonded microparticulate column. 2,4-D elutes between impurities 2,4- and 2,6-dichlorophenol.

### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Fitted with 5000 psi pressure gage, 280 nm UV detector, line filter in mobile phase reservoir, and 10 mv full scale deflection strip chart recorder. Automated sampling system and computing integrator are optional. Typical operating conditions: chart speed, 0.2 cm/min; mobile phase flow rate, 0.9–3.0 mL/min depending upon psi range of pump; detector sensitivity, 0.64 $A$ unit full scale; temp., ambient; injection valve vol., 10 μL.

(**b**) *Liquid chromatographic column.*—No. 316 stainless steel, 250 × 4.6 (id) mm, Partisil® 10 μm ODS column with 7 cm × 2.1 mm id CSKI guard column, Pellicular ODS (Nos. 4223-001 and 4390-413, resp., Whatman Inc.) Regenerate, if necessary, by pumping $CH_3CN$ thru column until baseline is stable. Repack first 5 mm of guard and main columns with Co:Pell ODS if peaks begin to "tail."

### *C. Reagents*

(**a**) *Mobile phase.*—pH 2.95. $CH_3CN$ (distd-in-glass)-$H_2O$ (deionized, 0.4 μm filtered) (1+4) contg NaOH added from (1+1) aq. soln of known normality, **936.16B(b)**, at final vol. concn of 0.3$M$. Add $H_3PO_4$ to adjust pH to 2.95.

(**b**) *Saponification-internal std soln.*—4 g *p*-Bromophenol/L 0.2$N$ KOH in isopropanol-$H_2O$ (2+1). Add KOH from (1+1) aq. soln of known normality.

(**c**) *2,4-D std soln.*—300 mg/25 mL. Accurately weigh ca 300 mg 2,4-D anal. ref. std (99+% isomer pure; available from Dow Chemical Co., Sample Coordinator), previously dried 15 min at 100°, into 1 or 2 oz glass vial with polyethylene-lined screw cap. Pipet in 25 mL saponification-internal std soln, and shake to dissolve. Prep. 2,4-D std soln and sample soln, **D**, at same time, using same pipet.

### *D. Preparation of Sample*

Accurately weigh sample contg ca 300 mg 2,4-D acid equiv. into 1 or 2 oz glass vial with polyethylene-lined screw cap. Pipet in 25 mL saponification-internal std soln, and shake 15 min, warming ester formulations to 50° several min before shaking. Filter prepd sample thru 9 cm Whatman glass microfiber filter GF/A, or equiv., collecting major portion of aq. phase for chromatography. Adjust isopropanol-$H_2O$ ratio, if necessary, to obtain complete dissoln of sample; e.g., amine formulations are best prepd with isopropanol-$H_2O$ (1+1).

### E. Determination

Transfer ca 1 mL portions of samples and stds to automated sampler vials, and cap. Place samples and stds in position, and start automatic sampler. With programmed integrator use following calcn program automatically:

$$\% \text{ 2,4-D} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = peak ht or area ratios of 2,4-D to internal std for sample and std, resp.; $W'$ = mg 2,4-D in std; $W$ = mg sample; and $P$ = % purity of std. If automated sampler and computing integrator are unavailable, inject 10 μL samples and stds and perform calcns manually. As check on calibration, place stds in sample sequence at beginning, middle, and end. Periodically confirm linearity by analyzing stds contg 200, 300, and 400 mg 99+% 2,4-D/25 mL saponification-internal std soln. Continuously recycle and mag. stir mobile phase. Replace mobile phase after ca 200 injections/L.

Refs.: JAOAC **61**, 1163(1978); **62**, 334(1979).

CAS-94-75-7 (2,4-D)

## 962.06 Dalapon (Sodium Salt) in Pesticide Formulations

**Titrimetric Method**

**First Action 1962**
**Final Action 1966**

(*Caution:* See safety notes on pesticides.)

### A. Apparatus

(**a**) *Reflux apparatus*.—250 mL erlenmeyer connected thru $ 35/25 ball joint to reflux condenser.

(**b**) *Filtering apparatus*.—60 mL, medium porosity fritted glass funnel attached to glass filter bell, 11 cm od, 18 cm high, with bottom gasket and slide valve.

### B. Reagents

(**a**) *Mercuric-cupric nitrate soln*.—(*Caution:* See safety notes on mercury.) Dissolve 100.0 g yellow HgO and 60 g $Cu(NO_3)_2.3H_2O$ in 500 mL 3.100±0.003$N$ $HNO_3$, measured from vol. flask, in 1 L vol. flask, dil. to vol. with $H_2O$, and filter.

(**b**) *Potassium iodide soln*.—Dissolve 150 g KI in $H_2O$, dil. to 1 L, and neutze to phthln.

### C. Determination

Accurately weigh sample contg 0.11–0.22 g Na salt of 2,2-dichloropropionic acid, transfer to erlenmeyer of reflux app., and add 100 mL Hg-Cu nitrate soln. Add some boiling chips, attach condenser, and reflux 15 min. Cool in $H_2O$ bath. Filter thru filtering app., washing flask and ppt acid-free with $H_2O$ from wash bottle. Discard filtrate and washings, and place 250 mL narrow-mouth erlenmeyer in filtering bell.

Add 50 mL KI soln to erlenmeyer to dissolve any remaining ppt, transfer to funnel, and stir until ppt dissolves. Draw soln into narrow-mouth erlenmeyer with vac. Wash flask and funnel with ≤50 mL KI soln from wash bottle, adding washings to filtrate. Add few boiling chips to filtrate and boil 1 min. Cool in $H_2O$ bath. Titr. immediately with 0.1$N$ HCl, using phthln.

$$\% \text{ Na salt 2,2-dichloropropionic acid} = \text{mL } 0.1N \text{ HCl} \times 0.004499 \times 100/\text{g sample}$$

Refs.: Anal. Chem. **31**, 418(1959). JAOAC **43**, 382(1960); **45**, 522(1962).

CAS-127-20-8 (dalapon sodium salt)

## 984.06 Dalapon (Magnesium and/or Sodium Salt) in Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1984**
**Final Action 1987**

### A. Principle

Dalapon is sepd from related compds on reverse phase LC column, using paired-ion eluant, and detected by UV spectrophtry.

### B. Apparatus

(**a**) *Liquid chromatograph*.—Fitted with 5000 psig pressure gage, 214 nm UV detector, line filter in eluant reservoir, 20 μL loop-type injection valve, and strip chart recorder. Computing integrator optional. Operating conditions: temp., 20–30°; eluant flow rate, 160 mL/h; detector sensitivity, 0.25 AUFS.

(**b**) *Liquid chromatographic columns*.—No. 316 stainless steel, 50 × 4.6 (id) mm Co:Pell ODS pellicular guard column (No. 6561-404, Whatman Inc.); 100 × 8 (id) mm C-18, 10 μm radial compression main column with RCM-100 column holder (Waters Associates, or equivalent (such as Whatman Partisil 10-25-ODS-3)). Columns slowly degrade in use. Replace when 30% loss of retention occurs and repack first 5 mm of guard column. Radial compression column recommended gives lower back-pressure and longer column life than std 316 stainless steel columns.

### C. Reagents

(**a**) *Eluant*.—Dilute mixt. of 200 mL $CH_3CN$ (UV grade), 1.6 mL $n$-octyl amine (Eastman P7588 or equivalent), and 2.4 g $(NH_4)_2HPO_4$ (J.T. Baker, Inc., No. 0784, or equivalent) to 1 L with $H_2O$ (0.4 μm filtered). Adjust pH of eluant to 7.0 with $H_3PO_4$. Eluant may be recycled if reservoir is mag. stirred and no more than 200 injections are made per L eluant.

(**b**) *Dalapon std soln*.—130 mg/50 mL. Accurately weigh ca 130 mg dalapon acid ref. std (99+% isomer pure; available from Dow Chemical Co.) into 50 mL vol. flask, add $H_2O$ to mark, and shake. Do not use std soln after 24 h. Det. % $H_2O$ in dalapon acid ref. std by Karl Fisher titrn. Labeled purity of ref. std is on anhyd. basis and must be appropriately reduced according to $H_2O$ content.

### D. Preparation of Sample

Accurately weigh sample contg ca 130 mg dalapon acid equiv. into 50 mL beaker. Quant. transfer to 50 mL vol. flask with $H_2O$. Fill to mark with $H_2O$ and shake. Do not use sample soln after 24 h. Dalapon acid and salts are hygroscopic; protect from moisture in air by storing in well sealed bottle. Duplicate sample and std prepn is recommended.

*Note:* Do not add mineral acids in sample prepn.

### E. Determination

Inject std soln, sample soln, and then std soln. Calc. results as

$$\% \text{ Dalapon acid equiv.} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = average peak ht or peak area of dalapon peak for sample and std solns, resp.; $W$ and $W'$ = mg sample and std, resp.; and $P$ = % purity of std. Note: Initial system stability may be poor. Before injecting sample soln, repeat injection of std soln to confirm system stability. Periodically confirm linearity by analyzing stds contg 100, 130, and 160 mg 99+% dalapon acid/50 mL $H_2O$. Flush injection loop with ≥1 mL sample or std soln before operating injection valve.

Refs.: JAOAC **66,** 1390(1983); **70,** 265(1987).

CAS-75-99-0 (dalapon)

## 970.05 DCPA in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1970**
**Final Action 1972**

(*Caution:* See safety notes on distillation, pipets, flammable solvents, toxic solvents, pesticides, benzene, acetone, and carbon disulfide.)

(Under conditions specified, other pesticides or ingredients may interfere with GC analysis, e.g., aldrin has same retention time as DCPA. Aldrin and DCPA may be sepd at 170° column temp.)

### *A. Apparatus*

*Gas chromatograph.*—1.8 m (6′) × $^1/_8''$ id stainless steel column contg 10% silicone UC-98 (Applied Science) on 80–100 mesh silanized Diatoport S (Hewlett-Packard Co., Avondale, Div.). Conditions (applicable to Hewlett-Packard F&M Model 5750)—temps(°): column 200, injection port 240, flame ionization detector 260; H, air, and He carrier flows, 115, 600, and 25 mL/min, resp.; chart speed 0.25″/min; attenuation 4×; range setting $10^2$ ($10^{-10}$ amp full scale).

### *B. Preparation of Standard Curve*

(**a**) *DCPA std solns.*—Weigh 0.5 g DCPA (available from Diamond Shamrock Corp., PO Box 348, Painesville, OH 44077) into 100 mL vol. flask, add ca 90 mL acetone (soln is rapid), and dil. to vol. Pipet 5, 10, and 15 mL into sep. 25 mL vol. flasks and dil. to vol. with acetone.

(**b**) *Hexachlorobenzene (HCB) std solns.*—Weigh 0.5 g ref. grade HCB into 100 mL vol. flask, add 90 mL benzene, and dil. to vol. with benzene. Pipet 1, 2, and 3 mL into sep. 25 mL vol. flasks and evap. to dryness with current of dry air. Add 20 mL acetone to each flask and dil. to vol. with acetone.

Inject 5 μL each dild HCB and DCPA std at least twice. Prep. curve of peak area or ht against concn for DCPA and peak ht against concn for HCB.

### *C. Determination*

(**a**) *Benzene extraction.*—Grind granular product. Weigh portion contg ca 300–400 mg DCPA into Whatman extn thimble (33 × 88 mm). Cover with glass wool. Place thimble in medium Soxhlet extractor; add 150–175 mL benzene and 3 glass beads. Ext 6 hr. Quant. transfer ext to 400 mL beaker and evap. to ca 5 mL on steam bath with dry air current; remove and evap. to dryness with air current. Add ca 150 mL acetone and let stand until soln is complete (white, flaky crystals may indicate incomplete soln; soln may be hastened by placing flask in ultrasonic cleaner). Filter soln thru glass wool into 200 mL vol. flask. Wash beaker with acetone, transfer washings to vol. flask, and dil. to vol.

(**b**) *Alternative acetone extraction.*—Substitute acetone for benzene in extn. Proceed as in (**a**) thru "Ext 6 hr." Continue with "Filter soln thru glass wool . . ."

Inject duplicate 5 μL sample soln into gas chromatograph. Compare peak ht or peak area to std curve to det. % hexachlorobenzene (HCB) and DCPA.

Ref.: JAOAC **52,** 1284(1969).

CAS-1861-32-1 (dimethyl tetrachloroterephthalate)

## 970.06 DCPA in Pesticide Formulations

### Infrared Spectroscopic Method

**First Action 1970**
**Final Action 1972**

### *A. Preparation of Sample*

Grind granular product. Weigh sample contg 200–500 mg DCPA into Whatman extn thimble. Proceed as in **970.05C(a)** thru ". . . evap. to dryness with air current." Add 25 mL $CS_2$, allow ca 30 min for complete soln, and transfer quant. to 50 mL vol. flask with $CS_2$, filtering sample thru glass wool. Dil. to vol.

### *B. Preparation of Standard Solution*

Weigh 1.25 g DCPA into 100 mL vol. flask. Add ca 90 mL $CS_2$ (soln may be hastened by placing flask in ultrasonic cleaner) and dil. to vol. Pipet 10, 15, and 20 mL into sep. 25 mL vol. flasks and dil. to vol.

### *C. Determination*

Set spectrophtr at optimum operating condition. Use 0.5 mm KBr (or NaCl) matched cells. Fill ref. cell with $CS_2$. Transfer dild stds to other cell and scan slowly from 1100 to 900 $cm^{-1}$. Repeat with samples. Construct baseline from 1030 to 925 $cm^{-1}$ and draw line from midpoint of max. $A$ at ca 964 $cm^{-1}$ to intersect baseline. Compute $\Delta A$ at 964 $cm^{-1}$ at point of intersection of stds and sample.

Prep. $\Delta A$-concn curve for std; Beer's law is obeyed over concn range 2–15 mg DCPA/mL. Calc. % DCPA from std curve.

Ref.: JAOAC **52,** 1284(1969).

CAS-1861-32-1 (dimethyl tetrachloroterephthalate)

## 947.02★ DDT in Pesticide Formulations

### Total Benzene-Soluble Chlorine Method

**Final Action**
**Surplus 1978**

(Applicable in absence of other org. Cl compds.)

*See* **6.254–6.256**, 13th ed.

## 960.13 DDT in Pesticide Formulations

### Infrared Spectroscopic Method

**First Action 1960**
**Final Action 1961**

(*Caution:* See safety notes on pesticides.)

### *A. Reagent*

*DDT std soln.*—Weigh 0.250 g tech. DDT into 50 mL vol. flask or g-s container and add exactly 25 mL $CS_2$. If sample to be analyzed contains S, add wt of S expected in portion of sample to be taken for analysis. Shake to dissolve and add small amt anhyd. $Na_2SO_4$. Centrf. portion of soln if it is not clear.

### *B. Determination*

Weigh sample contg ca 0.25 g DDT into 50 mL vol. flask and add exactly 25 mL $CS_2$ and small amt anhyd. $Na_2SO_4$. Let stand ≥30 min with occasional shaking. Transfer portion to g-s test tube and centrf. short time. Transfer to NaCl cell and scan with infrared spectrophtr, using 0.5 mm cell in region, 8.5–10.5 μm.

Scan std soln in same manner.

Measure *A* of DDT peak at 9.83 μm with baseline from 9.4 to 10.2 μm, and calc. % DDT.

Refs.: JAOAC **40,** 286(1957); **43,** 342(1960).

CAS-50-29-3 (*p,p'*-DDT)

## 969.07 Dicamba in Pesticide Formulations

**Infrared Spectrophotometric Method**

**First Action 1969**
**Final Action 1972**

### A. Reagents and Apparatus

(**a**) *Acetone*.—Spectral grade.

(**b**) *Dimethylamine* (*DMA*) *soln*.—60% (w/w).

(**c**) *Dicamba std*.—Ref. grade (Sandoz, Inc., 480 Camino del Rio South, Suite 204, San Diego, CA 92108).

(**d**) *Infrared spectrophotometer*.—With $BaF_2$ cells, 0.025 mm, and matched NaCl cells, 0.2 mm.

### B. Preparation of Sample

(Sample wts are for cell thicknesses specified. For other cells, adjust wts to yield peak between 30 and 60% *T*.)

(**a**) *Aqueous solns of DMA salt* (*4 lb/gal.*).—Pipet, using same pipet as for std, 5.00 mL sample into tared 25 mL vol. flask and weigh. Dil. to vol. with acetone. (Use this soln directly in 0.025 mm $BaF_2$ cell.)

(**b**) *Solns of DMA salt* (*other concentrations*).— Prep. as in (**a**), adjusting sample size to yield 2.4 g dicamba/25 mL.

(**c**) *Technical dicamba*.—Weigh 0.2±0.005 g sample into tared 25 mL vol. flask and dil. to vol. with $CS_2$.

### C. Preparation of Standard

(**a**) *Liquid formulations*.—(*1*) *Aqueous solns of DMA salt* (*4 lb/gal.*): Weigh 11.98±0.02 g dicamba std into tared 50 mL beaker. Add 5 mL $H_2O$ and 4 mL 60% DMA. Adjust pH to 7.0 by titrg with 60% DMA soln, using mag. stirrer and pH meter. (All solids should be dissolved at this time.) Rinse each pH electrode with two 1 mL $H_2O$ rinses (4 mL total), collecting rinses in the 50 mL beaker. Cool soln to room temp. and transfer to tared 25 mL vol. flask. Rinse beaker twice with $H_2O$, collecting rinses in flask. Dil. to vol. with $H_2O$ and mix thoroly. Weigh flask and contents to det. total wt of soln. Pipet 5.0 mL std formulation into tared 25 mL vol. flask, weigh, and dil. to vol. with acetone.

(*2*) *Aqueous solns of DMA salt* (*other concentrations*): Prep. as in (**a**)(*1*), adjusting dicamba content to required concn.

(**b**) *Technical dicamba*.—Weigh 0.2±0.005 g dicamba std into tared 25 mL vol. flask and dil. to vol. with $CS_2$.

### D. Determination

(**a**) *Liquid formulations*.—Record spectra of std and sample between 1070 and 930 $cm^{-1}$ (9.3–10.7 μm), using $BaF_2$ cell. Use air in ref. beam. Obtain $\Delta A$ and $\Delta A'$ for sample and std, resp., at 1012 $cm^{-1}$ (9.89 μm) from horizontal baseline tangent to min. between 1020 and 1070 $cm^{-1}$ (9.4–9.7 μm).

(**b**) *Technical dicamba*.—Record spectra of std and sample from 1100 to 930 $cm^{-1}$ (9.1–10.7 μm), using NaCl cells. Use $CS_2$ in ref. cell. Obtain $\Delta A$ and $\Delta A'$ for sample and std, resp., at 1012 $cm^{-1}$ (9.89 μm) from horizontal baseline tangent to min. between 1075 and 1035 $cm^{-1}$ (9.3–9.66 μm).

### E. Calculations

(**a**) *Liquid formulations*.—Dicamba, lb/gal. = $\Delta A \times C/\Delta A'$, where $C$ = lb std/gal. = (g std × % purity of std × 8.35)/25.

% Dicamba by wt = ($\Delta A \times F$)/(g sample/25 mL), where $F$ = [(g std/25 mL) × % purity of std]/$\Delta A'$.

(**b**) *Technical dicamba*.—% Dicamba by wt = $\Delta A \times F$/g sample, where $F$ = (g std × % purity of std)/$\Delta A'$.

Ref.: JAOAC **51,** 1301(1968).

CAS-1918-00-9 (dicamba)

## 971.07 Dicamba–MCPA and Dicamba–2,4-D in Pesticide Formulations

**Infrared Spectrophotometric Method**

**First Action 1971**
**Final Action 1973**

***AOAC-CIPAC Method***

(*Caution:* See safety notes on pesticides.)

### A. Principle

Method is applicable to aq. dimethylamine (DMA) salt formulations of dicamba and 2-methyl- 4-chlorophenoxyacetic acid (MCPA) or 2,4-D (2,4-dichlorophenoxyacetic acid). Active ingredients are pptd by HCl and extd with $CHCl_3$. Solv. is evapd, residue dissolved in acetone, and *A* measured at characteristic IR wavelengths.

### B. Preparation of Standard Solutions

(**a**) *Dicamba–MCPA*.—Accurately weigh 0.20±0.02 g dicamba and 0.60±0.02 g MCPA into tared weighing bottle. Pipet in 25 mL acetone and swirl until completely dissolved. If cells other than 0.2 mm are used, adjust wts to give *A* of 0.2–0.5 (30–65% *T*) for both std and sample solns.

(**b**) *Dicamba–2,4-D*.—Prep. as in (**a**), using 0.20±0.02 g dicamba and 0.40±0.02 g 2,4-D.

### C. Preparation of Sample

(**a**) *Dicamba–MCPA*.—Accurately weigh sample contg 0.20±0.02 g dicamba and 0.60±0.02 g MCPA into tared weighing bottle. Add 5 mL $H_2O$ and transfer quant. to 125 mL separator with 5–10 mL $H_2O$.

(**b**) *Dicamba–2,4-D*.—Prep. as in (**a**), using 0.20±0.02 g dicamba and 0.40±0.02 g 2,4-D.

### D. Determination

To soln add HCl dropwise with const swirling to pH 1; then add 5 drops excess. Pipet in 25 mL $CHCl_3$ and shake to dissolve ppt. Drain $CHCl_3$ ext into 125 mL erlenmeyer and re-ext with two 15 mL portions $CHCl_3$. Add boiling chips to combined ext and evap. on steam bath to dryness. Let dry in hood overnight at room temp. (Do not dry in air or vac. oven.) Pipet in 25 mL acetone and swirl to completely dissolve residue. Add few g granular anhyd. $Na_2SO_4$ if any $H_2O$ is present.

Record IR spectrum and measure $\Delta A$ in matched 0.2 mm NaCl cells with acetone in ref. cell at following wavelengths:

(**a**) *Dicamba–MCPA*.—Range, 1135–930 $cm^{-1}$ (8.8–10.75 μm); dicamba peak, 1012 $cm^{-1}$ (9.89 μm); MCPA peak, 1070 $cm^{-1}$ (9.35 μm); baseline, horizontal tangent to min. at 970–965 $cm^{-1}$ (10.3–10.4 μm) for both constituents.

(**b**) *Dicamba–2,4-D*.—Range, 1130–945 $cm^{-1}$ (8.85–10.6 μm); dicamba peak, 1012 $cm^{-1}$ (9.89 μm); 2,4-D peak, 1080 $cm^{-1}$ (9.26 μm); baseline, horizontal tangent to min. at 970–960 $cm^{-1}$ (10.3–10.4 μm) for both constituents.

### E. Calculations

% by wt of constituent = $(\Delta A/W)(W' \times P/\Delta A')$, where $\Delta A$ and $\Delta A'$ = absorbance of constituent in sample and std solns, resp.; $W$ and $W'$ = g constituent in sample and std solns, resp.; and $P$ = % purity of constituent in ref. std.

$$\text{lb/gal.} = \text{\% by wt} \times \text{sp gr} \times 8.345$$

Ref.: JAOAC **54,** 706(1971).

CAS-94-75-7 (2,4-D)
CAS-1918-00-9 (dicamba)
CAS-94-76-6 (MCPA)

## 984.07 Dicamba, 2,4-D, and MCPP in Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1984**

### A. Principle

Combinations of dicamba, 2,4-D, and MCPP in liq. formulations as their salts are detd in any combination, using binary mobile phase 22% and 33% $CH_3CN$–$H_2O$, phosphate buffered, pH 2.68–2.70, on reverse phase bonded microparticulate column. Specified column seps known impurities, analytes, and internal stds salicylic acid and butyrophenone.

### B. Apparatus

(**a**) *Liquid chromatograph.*—Fitted with 5000 psi pressure gage, 280 nm detector, 10 μL injector, eluant reservoir line filter, and 10 mV FS strip chart recorder; flow rate 2.0 mL/min.

(**b**) *Liquid chromatographic column.*—250 × 4.6 (id) mm Partisil 10–25 ODS-3 with Co:Pell ODS pellicular guard column (Whatman Inc.).

(**c**) *Operating conditions.*—Flow rate 2.0 mL/min; detector 280 nm; injection vol. 10 μL; temp. ambient (≤76°F); setting 0.2 AUFS for dicamba and 1.0 AUFS for 2,4-D and MCPP; chart speed 0.5 cm/min.

(**d**) *Performance characteristics.*—10 μL injection of 0.95 g salicylic acid/L causes ca 50% recorder deflection at 1.0 AUFS. Optimum conditions for column sepn are obtained when *o*-chlorophenoxyacetic acid impurity, from technical 2,4-D, is sepd between salicylic acid and dicamba in 33% eluant and immediately after dicamba in 22% eluant.

### C. Reagents

Prep. each eluant in 1 L erlenmeyer and re-circulate to conserve mobile phase.

(**a**) *Eluant I.*—22% mobile phase: 725 mL $H_2O$ (0.4 μm-filtered), 220 mL $CH_3CN$ (distd in glass), 16.9 mL NaOH (ca 17.7N, 1 + 1, **936.16B(b)**); add $H_3PO_4$ to pH 2.69 ± 0.01 (note vol.) and dil. to 1 L with $H_2O$.

(**b**) *Eluant II.*—33% mobile phase: 610 mL $H_2O$, 330 mL $CH_3CN$, 16.9 mL NaOH (ca 17.7N, 1 + 1, **936.16B(b)**); add $H_3PO_4$ to pH 2.69 ± 0.01 (note vol.) and dil. to 1 L with $H_2O$.

(**c**) *Diluting solvent.*—Isopropanol–$H_2O$ (2 + 1).

(**d**) *Internal std soln.*—0.95 g salicylic acid (Aldrich Chemical Co.) + 9.00 mL butyrophenone (Aldrich Chemical Co.); dil. to 1 L with dilg solv.

(**e**) *Std soln.*—Governing factor is highest peak. For expected 2,4-D in highest concn, weigh 0.150 g; for expected MCPPA in highest concn, weigh 0.225 g. Weigh stds according to expected sample composition and ratio of herbicides to each other. Weigh into graduated, conical centrf. tube (Corning, Cat. No. 25330-50) or other stoppered, leak-proof container marked at 25 mL. Pipet 20.0 mL internal std soln; dil. to 25.0 mL with dilg solv.

### D. Preparation of Sample Solution

Use same container as std soln and accurately weigh sample equiv. to 0.150 g 2,4-D or 0.225 g MCPP according to which is expected to be in highest concn in formulation. For sample having vol. >5 mL, dil. to 50 mL after addn of 20 mL internal std soln. If sample is dild to 50 mL, also dil. internal std soln to approx. same concn. Increase sensitivity to obtain approx. same peak ht. Filter samples and stds thru Whatman 9.0 cm glass fiber GF/C, or equiv. Use same pipet for adding internal std soln to stds and samples.

### E. Determination

Flush LC column of previous solv. with $H_2O$ for several minutes to stable baseline.

*Dicamba:* With eluant I flowing at 2.0 mL/min, inject 10 μL salicylic acid (0.95 g/L in dilg solv.) to condition column. Set detector to maximize peak (ca 0.2 AUFS), inject 10 μL std soln, and, when pen returns to baseline after elution of dicamba, inject 10 μL sample soln.

2,4-D and/or MCPP must be flushed from column after sample soln injection by the sequence: $H_2O$ 2 min, 50% $CH_3CN$ until pen returns to stable baseline. Elution order is salicylic acid first and dicamba second.

*2,4-D and MCPP:* With eluant II flowing at 2.0 mL/min, and attenuation at 1.0 AUFS, inject 10.0 μL std soln. When pen returns to baseline after elution of butyrophenone, inject 10.0 μL sample soln. Elution order is salicylic acid, dicamba, 2,4-D, MCPP, and butyrophenone.

### F. Calculation

Use salicylic acid as internal std for calc. of dicamba; use butyrophenone for calcg 2,4-D, MCPP. Either peak ht or computer-integrated areas may be used.

$$\text{\% Compd} = (W'/W) \times (R/R') \times P$$

where $W'$ = mg std; $W$ = mg sample; $P$ = % purity of std; $R$ and $R'$ = peak ht or area ratios of compd to internal std for sample and std, resp.

Ref.: JAOAC **67,** 837(1984).

CAS-94-75-7 (2,4-D)
CAS-1918-00-9 (dicamba)

## 979.03 Dichlobenil in Pesticide Formulations

**Gas Chromatographic Method**

**Final Action**

***CIPAC-AOAC Method***

(*Caution:* See safety notes on pesticides.)

### A. Reagents and Apparatus

(**a**) *Dichlobenil.*—≥99.5% purity (Duphar B.V., Box 2, 1380 AA Weesp, The Netherlands), or equiv.

(**b**) *Methyl myristate.*—Fluka AG Cat. No. 70129, ≥99.5% purity (Fluka Chemie, AG, Industriestrasse 25, CH-9470, Switzerland), or equiv.

(**c**) *Mixed solvent soln.*—1,2-Dichloroethane-ether (1+1).

(**d**) *Internal std soln.*—Dissolve 0.80 g Me myristate, (**b**), in 100 mL mixed solv. soln, (**c**).

(**e**) *Calibration soln.*—Accurately weigh ca 0.10 g dichlobenil, (**a**), into conical flask, pipet in 5 mL internal std soln, (**d**), and add 45 mL solv. soln, (**c**).

(**f**) *Gas chromatograph.*—With on-column injection, flame ionization detector, injection port heating, and, preferably, detector heating. Pyrex column 1.8 m × 3 mm id, packed with

10% Carbowax 20M on 100–120 mesh Chromosorb P, acid washed, dimethyldichlorosilane treated (available from Analabs, Inc., Cat. No. GCP-009D). Operating temps (°): oven 200, injection port 210, detector 210. Carrier gas (N) flow rate 25 mL/min. Approx. retention times 7 and 12 min for Me myristate and dichlobenil, resp.

### B. Preparation of Sample

(**a**) *Technical dichlobenil.*—Accurately weigh ca 2.0 g dichlobenil into 100 mL vol. flask. Dissolve in mixed solv. soln, (**c**), and dil. to vol. Pipet 5 mL aliquot into 100 mL conical flask, add 5.00 mL internal std soln, (**d**), and dil. to 50 mL with mixed solv., (**c**).

(**b**) *Wettable powders.*—Accurately weigh sample contg ca 1.0 g dichlobenil into 100 mL vol. flask, add few mL mixed solv., (**c**), swirl, and dil. to vol. with mixed solv. Let settle, pipet 10 mL clear supernate into 100 mL conical flask, and continue as in (**a**).

(**c**) *Granules.*—Accurately weigh ca 6.0 g sample into 100 mL conical flask. Add 20 mL dichloroethane and stir 10 min on mag. stirrer. Filter with vac. thru glass filter paper (No. G8, Fisher Scientific Co., or equiv.), supported on fritted glass filter. Wash granules 5 times with 5 mL dichloroethane, collect filtrate in 100 mL vol. flask, and dil. to vol. with ether. Pipet aliquot of this soln, contg ca 0.1 g dichlobenil (10 mL for 20% granules, 25 mL for 7% granules), into 100 mL conical flask, add 5.00 mL internal std soln, (**d**), and dil. to 50 mL with mixed solv., (**c**).

### C. Determination

Inject 2 μL portions of calibration soln, (**e**), until response factor varies <1% for successive injections. Inject duplicate 2 μL portions of sample soln, followed by 2 μL portions of calibration soln, (**e**). Measure peak areas of dichlobenil and Me myristate, either by multiplying peak ht by retention time, or by digital integration. Use av. of duplicate values.

### D. Calculation

$$p = (I_q \times r \times 20)/(I_r \times q)$$

where $p$ = response factor, $I_q$ and $I_r$ = peak areas of internal std and dichlobenil, resp., $q$ = g internal std, $r$ = g dichlobenil in calibration soln. (Response factor is ca 1.5.)

$$\% \text{ Dichlobenil} = (I_d \times q \times p \times F \times 100)/(I_m \times W \times 20)$$

where $I_d$ and $I_m$ = peak areas of dichlobenil and internal std, resp., $p$ = response factor, $F$ = diln factor for sample (100/$x$, where $x$ = mL taken to obtain final soln), and $W$ = g sample.

Ref.: JAOAC **62,** 8(1979).

CAS-1194-65-6 (dichlobenil)

## 976.02 Dicofol in Pesticide Formulations
### Potentiometric Method
### First Action 1976

### A. Principle

Dicofol is hydrolyzed in alc. KOH under reflux, and hydrolyzable org. Cl is converted to ionizable Cl which is titrd potentiometrically with std $AgNO_3$.

### B. Apparatus

(**a**) *Condenser.*—$ 24/40 Pyrex condenser, water cooled, 400 mm long with drip tip.

(**b**) *Potentiometer.*—Fisher Accumet Model 320 (new model 325) expanded scale pH meter, or equiv., with 50 mL buret graduated in 0.1 mL, Ag billet indicating electrode (Fisher No. 13-639-122), and Ag-AgCl ref. electrode (Fisher No. 13-639-53). Keep Ag electrode free from tarnish by polishing with aq. $NaHCO_3$-$CaCO_3$ (1 + 1) paste. Before each analysis, rinse Ag electrode with $NH_4OH$ (1 + 1) followed by $H_2O$.

### C. Reagents

(Use deionized $H_2O$ thruout.)

(**a**) *Alcoholic potassium hydroxide soln.*—0.5*N*. Dissolve 28.1 g KOH pellets in ca 600 mL alcohol and dil. to 1 L with alcohol.

(**b**) *Potassium chloride std soln.*—0.1*N*. Dissolve 7.456 g KCl in $H_2O$ and dil. to 1 L with $H_2O$.

(**c**) *Silver nitrate std soln.*—0.1*N*. Dissolve 17.00 g $AgNO_3$ in 100 mL $H_2O$, add 1.7 mL $HNO_3$, and dil. to 1 L with $H_2O$. To stdze, dil. 25 mL 0.1*N* KCl to 200 mL with $H_2O$ in 400 mL beaker. Adjust pH to 2.0±0.2, using $NH_4OH$ (1 + 4) and/or $HNO_3$ (1+4), and dil. to 300 mL. Titr., using potentiometer as in **F**. Plot mv against vol. 0.1*N* KCl and det. mL 0.1*N* $AgNO_3$ at end point. Calc. normality of $AgNO_3$ std soln. Stdze $AgNO_3$ std soln daily. (Equiv. wt dicofol = 370.5/3 = 123.5.)

(**d**) *Thymol blue indicator soln.*—0.1%. Dissolve 100 mg thymol blue in 100 mL alcohol (1 + 1).

### D. Preparation of Sample

(**a**) *Dicofol technical.*—Fuse sample in loosely capped jar in 100° oven and mix thoroly with glass rod. Accurately weigh ca 4–6 g molten sample into 150 mL beaker. Add 50–75 mL isopropanol and heat with occasional swirling until sample dissolves. Transfer quant. to 500 mL vol. flask, let cool to 25°, and dil. to vol. with isopropanol. Pipet 25 mL sample soln into 300 mL $ 24/40 Pyrex erlenmeyer. (*Caution:* See safety notes on pipets.)

(**b**) *Dicofol formulations.*—(*1*) *Dicofol MF and dicofol 35.*—Accurately weigh ca 1 g sample into 300 mL $ 24/40 Pyrex erlenmeyer. (*2*) *Dicofol EC.*—Proceed as in (*1*), using ca 2 g sample.

### E. Hydrolysis

Transfer 50 mL alc. KOH soln to erlenmeyer contg sample. Attach condenser, seal with 2–3 drops alcohol, and reflux gently on hot plate 1.5 hr. Let cool, and rinse condenser and tip with 25 mL alcohol. Quant. transfer soln to 400 mL beaker, using 50 mL alcohol and 100 mL $H_2O$. Rinse erlenmeyer with addnl portions $H_2O$ to total vol. of 250 mL.

Add 10 drops thymol blue indicator soln to beaker and, with stirring, add $HNO_3$ (1 + 1) dropwise to first pink color of indicator. Adjust pH to 2 ± 0.2, using $NH_4OH$ (1 + 4) and/or $HNO_3$ (1 + 4). Adjust total vol. to 300 mL with $H_2O$.

### F. Determination

Place sample beaker on mag. stirrer, and adjust to rapid stirring. Titr. with $AgNO_3$ std soln to same mv end point used for stdzg $AgNO_3$ std soln. Titr. blank (unhydrolyzed sample).

$$\% \text{ Active ingredient in tech. dicofol} = \{[(V_s/S_a) - (V_b/S_b)] \times N \times V_t \times 0.1235 \times 100\}/V$$

$$\% \text{ Active ingredient in dicofol formulations} = [(V_s/S_a) - (V_b/S_b)] \times N \times 0.1235 \times 100$$

where $V_s$ and $V_b$ = mL $AgNO_3$ std soln required to titr. sample and blank, resp.; $N$ = normality of $AgNO_3$ std soln; $S_a$ *and* $S_b$ = g sample taken for hydrolysis and blank, resp.; $V_t$ = total vol. sample soln = 500 mL; and $V$ = aliquot vol. sample soln = 25 mL.

Ref.: JAOAC **59,** 1109(1976).

CAS-115-32-2 (dicofol)

## 986.06 Dicofol in Pesticide Formulations
### Liquid Chromatographic Method
### First Action 1986

#### *A. Principle*

Dicofol is dissolved in MeOH, sepd by liq. chromatgy, and detd by comparison of peak hts of stds and samples.

#### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Provided with pulseless, const flow pump and 15 μL sample loop or auto-injector. Operating conditions: mobile phase flow rate 2.0 mL/min; detector sensitivity 0.5 AUFS; temp., 30°.

(**b**) *Detector*—UV spectrophtr or fixed wavelength UV detector at 254 nm.

(**c**) *Recorder.*—Range to match output of LC detector.

(**d**) *Liquid chromatographic columns.*—Analytical: stainless steel, 250 × 4.6 mm, packed with Zorbax C8, 6 μm spherical particles (DuPont Co., Instruments Div., Concord Plaza, Wilmington, DE 19898). Guard: stainless steel, 50 × 4.6 mm, packed with LiChrosorb RP-18, 10 μm particle size (Merck, EM Science) (replacement model from EM Science is a cartridge system: LiChrospher RP-18, 5 μm, prepacked guard column No. 50803 and universal guard column holder No. 16217).

(**e**) *Filter.*—Millex-HV disposable filter assembly, 0.45 μm (No. SLHV025NS, Millipore Corp., or equiv.).

#### *C. Reagents*

(**a**) *Mobile phase.*—MeOH-$H_2O$-HOAc (75 + 25 + 0.2), components individually filtered thru 0.45 μm filter. Mix 750 mL MeOH, 250 mL $H_2O$, and 2 mL glacial HOAc, and degas.

(**b**) *Dicofol std soln.*—Accurately weigh ca 35 mg pure 1,1-bis(4-chlorophenyl)-2,2,2-trichloroethanol and ca 7.5 mg pure 1-(2-chlorophenyl)-1-(4-chlorophenyl)-2,2,2-trichloroethanol into 1 oz vial and add 20 mL MeOH by pipet.

#### *D. System Performance Check*

Dissolve, in 1 oz vial, ca 50 mg Kelthane tech. in 20 mL MeOH. Sample is very plastic; heat at 60° to liquefy.

Inject 15 μL onto liq. chromatgc column and det. sepn factor (α) for dicofol and *p,p'*- and *o,p'*-DDE isomers as follows:

$$\alpha = k'2/k'1$$

where $k'2$ and $k'1$ are column capacity ratios of isomer pairs, defined as follows:

$$k' = (t_r - t_o)/t_o$$

where $t_r$ = elution time of retained component and $t_o$ = dead vol. elution time. Sepn factors are 1.62 for dicofol *o,p'*- and *p,p'*-isomers, and 1.38 for DDE isomers. Sepn factors of 1.55 for dicofol pair and 1.25 for DDE pair would be approx. limits for proper performance. Performance check should also confirm that key components are adequately resolved. DDT isomer pairs should be resolved from DDE isomers and should fit between DDE isomers. Example chromatogram is shown in Fig. **986.06A**. Fig. **986.06B** is example of unacceptable resolution where DDT isomer pair is not resolved from DDE isomers.

#### *E. Preparation of Sample*

(**a**) *Kelthane MF.*—Accurately weigh ca 100 mg MF formulation in 1 oz vial and add 20 mL MeOH by pipet. Shake to dissolve. Filter thru 0.45 μm filter.

(**b**) *Kelthane EC.*—Accurately weigh ca 250 mg EC formulation in 1 oz vial and add 20 mL MeOH by pipet. Shake to dissolve. Filter thru 0.45 μm filter.

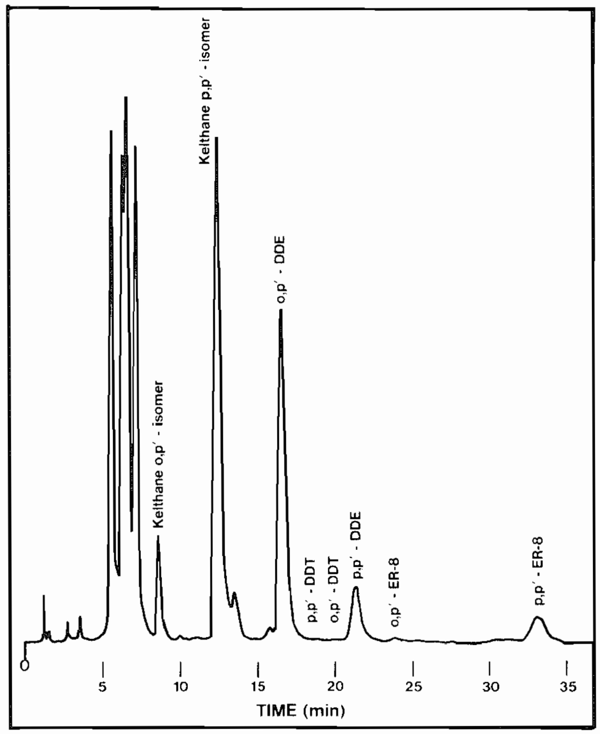


**Fig. 986.06A—LC chromatogram of Kelthane technical**

#### *F. Determination*

Inject 15 μL dicofol std soln. Det. peak hts of *p,p'*- and *o,p'*-dicofol. Inject 15 μL sample soln.

$$\%\ p,p'\text{-dicofol} = (PH/PH') \times (W'/W) \times 100$$
$$\%\ o,p'\text{-dicofol} = (PH/PH') \times (W'/W) \times 100$$
$$\%\ \text{Active ingredient} = \%\ p,p'\text{-dicofol} + \%\ o,p'\text{-dicofol}$$

where $PH$ and $PH'$ = peak ht of isomer in sample and std solns, resp.; $W$ and $W'$ = wt of sample and std injected, resp. Wt sample injected is calcd by wt = 15 × $W_v$/20, where $W_v$ = wt of sample in vial, μg.

Ref.: JAOAC **69,** 714(1986).

CAS-115-32-2 (dicofol)

## 983.07 Diflubenzuron in Pesticide Formulations
### Liquid Chromatographic Method
### First Action 1983
### Final Action 1984
### *CIPAC-AOAC Method*

#### *A. Principle*

Diflubenzuron is dissolved in 1,4-dioxane, sepd by LC, and detd from peak areas vs linuron internal std. Identity is confirmed by retention time.

#### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Provided with const flow pump and 20 μL sample loop. Operating conditions: mobile phase flow rate, 1.3 mL/min; detector sensitivity, 128 × $10^{-3}$ AUFS; temp., ambient, should not fluctuate >2°; retention time of diflubenzuron relative to internal std, ca 1.36.

(**b**) *Detector.*—UV spectrophtr or fixed wavelength UV detector at 254 nm.

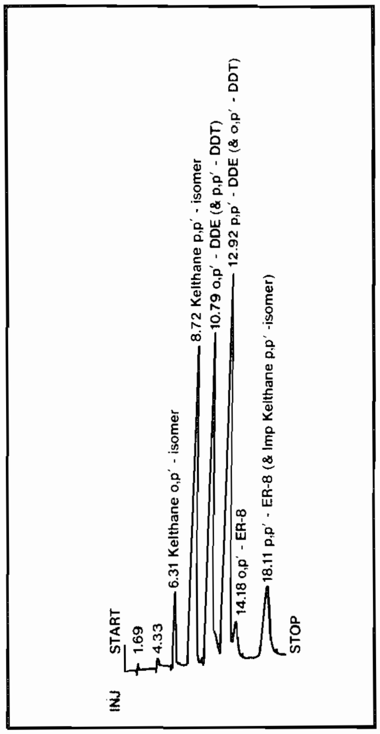


**Fig. 986.06B—LC chromatogram of Kelthane standards**

(**c**) *Recorder*.—Range to match output of LC detector.

(**d**) *Liquid chromatography column*.—Stainless steel, 250 × 4.6 mm, packed with Zorbax BP-$C_8$ (E.I. DuPont de Nemours & Co.), or equiv. (e.g., μBondapak $C_{18}$, 10 μm, Waters Associates, Inc.; Spherisorb ODS, 5 μm, Phase Separations Ltd, Deeside Industrial Estate, Queensferry Clwyd, UK; Zorbax BP-ODS, 7 μm, E.I. DuPont de Nemours).

(**e**) *Filter*.—Acrodisc disposable filter assembly, 1.2 μm (Gelman Sciences, Inc.) or equiv.

### C. Reagents

(**a**) *Mobile phase*.—Acetonitrile–$H_2O$–1,4-dioxane (45 + 45 + 10). Mix 450 mL acetonitrile, 450 mL $H_2O$, and 100 mL 1,4-dioxane and degas.

(**b**) *Solvent mixture*.—Acetonitrile–$H_2O$ (45 + 55).

(**c**) *Internal std soln*.—Accurately weigh 25 mg linuron (No. P14-04, Office of Reference Materials, Laboratory of the Government Chemist, Teddington, Middlesex, TW11 OLY, UK) into 100 mL vol. flask, dil. to vol. with acetonitrile, and mix.

(**d**) *Diflubenzuron std soln*.—Accurately weigh ca 50 mg pure diflubenzuron (≥99.5% Duphar B.V., PO Box 2, 1380 AA Weesp, The Netherlands) into 100 mL vol. flask. Add 50 mL dioxane and dissolve by heating 30 min in 80° $H_2O$ bath. Swirl occasionally. Add 40 mL dioxane, cool, and dil. to vol. with dioxane. Pipet 5 mL into 50 mL vol. flask, add 5.00 mL internal std soln, (**c**), dil. to vol. with solv. mixt., (**b**), and mix.

(**e**) *1,3-Di(4-chlorophenyl)urea*.—Duphar B.V.

### D. System Performance Check

Dissolve, in 100 mL vol. flask, 4.2 mg diflubenzuron and 1.8 mg 1,3-di(4-chlorophenyl)urea in 20 mL dioxane, dil. to vol. with solv. mixt., (**b**), and mix. Filter thru 1.2 μm filter before injection.

Inject 20 μL onto column and det. resolution ($R$) by following formula:

$$R = 2\,d/(W_l + W_2)$$

where $R$ = resolution; $d$ = distance between peak maxima; $W_1$ and $W_2$ = peak width at baseline of diflubenzuron and 1,3-di(4-chlorophenyl)urea, resp. Resolution should be >1. If necessary, resolution can be improved by slightly increasing $H_2O$ content of mobile phase.

### E. Preparation of Sample

(**a**) *Diflubenzuron pre-concentrate*.—Accurately weigh sample contg 1.0 g diflubenzuron into 200 mL vol. flask. Add 150 mL dioxane and heat 30 min in 80° $H_2O$ bath. Swirl occasionally. Add 40 mL dioxane, cool, and dil. to vol. with dioxane. Pipet 10 mL into 100 mL vol. flask and dil. to vol. with dioxane. Pipet 5 mL dild soln into 50 mL vol. flask, add 5.00 mL internal std soln, (**c**), dil. to vol. with solv. mixture (**b**), and mix. Filter thru 1.2 μm filter.

(**b**) *Water dispersible powder*.—Accurately weigh sample contg 0.5 g diflubenzuron into 200 mL vol. flask. Add 150 mL dioxane and heat 30 min in 80° $H_2O$ bath. Swirl occasionally. Add 40 mL dioxane, cool, and dil. to vol. with dioxane. Immediately pipet 20 mL homogeneous suspension into 100 mL vol. flask and dil. to vol. with dioxane. Pipet 5 mL dild soln into 50 mL vol. flask, add 5.00 mL internal std soln, (**c**), dil. to vol. with solv. mixture, (**b**), and mix. Filter thru 1.2 μm filter.

### F. Determination

Inject 20 μL diflubenzuron std soln, (**d**). Det. peak areas (or peak hts × retention times) of diflubenzuron and internal std. Repeat injections until response ratio (area diflubenzuron peak/area internal std peak) varies <1% for successive injections. Inject 20 μL sample soln.

### G. Calculation

$$\% \text{ Diflubenzuron} = (R/R') \times (W'/W) \times V \times 100$$

where $R$ and $R'$ = ratio of area of diflubenzuron peak to area of internal std peak for sample and std, resp.; $W$ and $W'$ = g sample and std, resp.; $V$ = diln factor (= 20 and 10 for preconc. and $H_2O$-dispersible powder, resp.).

Ref.: JAOAC **66**, 312(1983).

CAS-35367-38-5 (diflubenzuron)

## 983.08 Endosulfan in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1983**
**Final Action 1984**

***CIPAC-AOAC Method***

### A. Principle

Sample is extd with toluene and α- and β-endosulfan isomers are detd sep. by thermal conductivity or flame ionization GC, using di(2-ethylhexyl)phthalate as internal std.

### B. Apparatus

(**a**) *Gas chromatograph*.—Suitable for on-column injection; equipped with thermal conductivity detector. Flame ionization detector may be used with proper diln of samples and stds.

(**b**) *Chromatographic column*.—3 mm id × 1.5 m (5 ft) glass column packed with 10% OV-210 on 80–100 mesh Chromosorb W-HP. Condition column ≥16 h, at 250°, using carrier gas at ca 25 mL/min. Operating conditions: injector 300°, detector 250°, column 230°, He carrier gas flow ca 60 mL/min.

### C. Reagents

(a) *Toluene*.—GC quality.

(b) *Internal std soln*.—15 mg/mL. Dissolve 15 g di(2-ethylhexyl)phthalate, 99%+, in toluene and dil. to 1 L with toluene.

(c) *Endosulfan std soln*.—Accurately weigh 0.300 g endosulfan of known α- and β-isomer content (Riedel de Haen Co., D-3016 Hannover-Seelze 1, GFR; U.S. and Canadian distributor, Crescent Chemical Co., Inc., 1324 Motor Pkwy, Hauppauge, NY 11788) into 25 mL g-s flask. Pipet in 10 mL internal std soln, (**b**), mix, and store ≥20 min at 60° to ensure dissolution.

### D. Preparation of Sample

(a) *Technical endosulfan*.—Accurately weigh 0.300 g sample into 25 mL g-s flask, pipet in 10 mL internal std soln, (**b**), mix, and store ≥20 min at 60°.

(b) *Emulsifiable concentrates*.—Prep. as in (**a**), using wt equiv. to 0.300 g endosulfan.

(c) *Wettable powder*.—Accurately weigh sample contg 0.300 g endosulfan into 25 mL g-s flask, pipet in 10 mL internal std soln, (**b**) mix, store ≥20 min at 60°, and centrf.

### E. Determination

Inject 2 μL portions of std soln, (**c**), until response factor for each component varies <1% for successive injections. Inject duplicate 2 μL portions of sample soln followed by 2 μL portions of std soln. For each injection, calc. response ratio α-isomer peak to internal std peak, and ratio of β-isomer peak to internal std peak, either as peak ht or digital integrator count for area. Retention time for each isomer and internal std should be the same for sample and std solns.

### F. Calculation

$$\%\ \alpha\text{-isomer} = C_\alpha = (R_\alpha/R'_\alpha) \times (W'/W) \times P_\alpha$$
$$\%\ \beta\text{-isomer} = C_\beta = (R_\beta/R'_\beta) \times (W'/W) \times P_\beta$$

where $R_\alpha$ and $R'_\alpha$ = response ratios of α-isomer peaks to internal std peaks for sample and std soln, resp.; $R_\beta$ and $R'_\beta$ = response of β-isomer peaks to internal std peaks for sample and std soln, resp.; $W$ and $W'$ = wt (g) of sample and endosulfan std, resp.; $P_\alpha$ and $P_\beta$ = % α- and β-isomers in std, resp.

Ref.: JAOAC **66**, 999(1983).

CAS-115-29-7 (endosulfan)

## 984.08 Fluazifop-Butyl in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1984**
**Final Action 1985**

(Method is suitable for tech. and formulated fluazifop-butyl.)

### A. Principle

Sample is dissolved in $CH_2Cl_2$ contg dibenzyl succinate as internal std, and 1 μL is injected into GC for flame ionization detection. Peak areas are measured for fluazifop-butyl and dibenzyl succinate and compared with those from std injection.

### B. Apparatus

(a) *Gas chromatograph*.—With heated, glass-lined injection port and flame ionization detector. Temps (°)—column 230, injection port 250, detector 270; gas flows (mL/min)—N carrier gas 30, H 60, air 240; sample size 1.0 μL; retention times (min)—fluazifop-butyl 4.3, isomeric impurity 4.9, internal std 8.0. Adjust parameters to assure complete sepn of peaks, particularly isomeric impurity from fluazifop-butyl, and peak hts ca 60–80% full scale on chart at quoted retention times.

(b) *Column*.—1.8 m (6 ft) × 2 mm (id) glass column packed with 3% OV-17 on 100–120 mesh Chromosorb WHP (Supelco Inc., Cat. No. 1-1757). Precondition overnight at 250° before use.

### C. Reagents

(a) *Dibenzyl succinate internal std soln*.—Weigh 7 g dibenzyl succinate (ICN Pharmaceuticals, Inc., Cat. No. 13686), dissolve in $CH_2Cl_2$, and dilute to 1 L. Check internal std soln for interfering components by injecting 1 μL into chromatograph. Store in tightly capped bottles to avoid evapn.

(b) *Fluazifop-butyl std soln*.—Accurately weigh ca 100 mg fluazifop-butyl std of known purity (ICI Americas Inc.) into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake to dissolve. Store in tightly capped bottles to avoid evapn.

### D. Determination

(a) *Liquid and technical samples*.—Accurately weigh amt sample contg ca 100 mg fluazifop-butyl into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake to dissolve.

(b) *Granular formulations*.—Accurately weigh amt sample contg ca 100 mg fluazifop-butyl into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake on a wrist-action shaker 10 min. Let insoluble inerts settle 10 min before analysis.

Inject 2 or more aliquots of std soln to set integration parameters and stabilize instrument. Monitor response factor until results agree within 2%. Inject 4 aliquots of std soln and 2 aliquots of sample soln in succession. Calc response factor, $R$, for each injection:

$$R = \text{area fluazifop-butyl peak/area internal std peak}$$

$$\text{Fluazifop-butyl, }\% = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response factor for sample and std solns; $W$ and $W'$ = mg sample and std; and $P$ = purity (%) of std.

Ref.: JAOAC **67**, 499(1984).

CAS-69806-50-4 (fluazifop-butyl)

## 977.03 Folpet in Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1977**
**Final Action 1979**

***AOAC-CIPAC Method***

(Applicable to dry formulations contg folpet as only active ingredient and to folpet combination formulations except those contg propargite or Me parathion. Compds insol. in $CH_2Cl_2$, e.g., maneb or inorg. salts, do not interfere.)

(*Caution*: See safety notes on pesticides.)

### A. Apparatus

(a) *Liquid chromatograph*.—Equipped with 254 nm UV detector. Typical operating conditions: chart speed, 0.2″/min; flow rate, 2 mL/min (ca 800 psi); detector sensitivity, 0.16 *A* unit full scale; temp., ambient; valve injection vol., 20 μL. Adjust operating conditions to elute folpet peak in 4±1 min. Factors such as different $H_2O$ content in $CH_2Cl_2$ mobile phase can change retention times. Folpet peak must be completely resolved from dibutyl phthalate peak which normally elutes in ca 7 min.

(b) *Liquid chromatographic column*.—Stainless steel, 300

× 4 mm id, packed with 10 μm diam. silica gel particles (Waters Associates, Inc., No. 27477, or equiv.).

### B. Reagents

(**a**) *Mobile phase*.—Degassed $CH_2Cl_2$.

(**b**) *Internal std soln*.—Accurately weigh ca 0.5 g dibutyl phthalate (EM Science No. DX0605) into 200 mL vol. flask. Dil. to vol. with $CH_2Cl_2$ and mix.

(**c**) *Folpet std soln*.—(100 μg folpet + 250 μg dibutyl phthalate)/mL. Accurately weigh ca 20 mg folpet ref. std, 99+% pure (Chevron Chemical Co., PO Box 4010, Richmond, CA 94804) into glass vial, pipet 20 mL internal std soln into vial, and shake to dissolve. Pipet 1 mL into 10 mL vol. flask. Dil. to vol. with $CH_2Cl_2$.

### C. Preparation of Sample

Accurately weigh sample contg 20 mg folpet into vial. Pipet 20 mL internal std soln into vial and shake 30 min. Centrf. to ppt solids. Pipet 1 mL supernate into 10 mL vol. flask, dil. to vol. with $CH_2Cl_2$, and mix. Sample contains ca (100 μg folpet + 250 μg dibutyl phthalate)/mL.

### D. Determination

Inject 20 μL folpet std soln onto column thru sampling valve and adjust operating conditions to give largest possible on-scale peaks with retention time of 4±1 min for folpet. Repeat injections until ratio of folpet to dibutyl phthalate peak hts is within ±1% of previous injection. Without changing conditions, inject sample soln until its ratio is within ±1% of previous ratio for sample. Average last 2 peak ht ratios for sample and for std, resp., and calc. % folpet.

$$\% \text{ Folpet} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. peak ht ratios for sample and std, resp.; $W'$ = mg folpet in std soln (ca 20 mg); $W$ = mg sample extd for analysis; and $P$ = % purity of std.

Ref.: JAOAC **60,** 1157(1977); **61,** 384(1978).

CAS-133-07-3 (folpet)

## 962.07 Heptachlor in Pesticide Formulations

**Active Chlorine Method**

**First Action 1962**
**Final Action 1965**

### A. Reagents

(**a**) *Dilute acetic acid*.—80%. Dil. 800 mL HOAc to 1 L with $H_2O$.

(**b**) *Silver nitrate-acetic acid std soln*.—Dissolve 17 g $AgNO_3$ in 200 mL $H_2O$, add 56 mL $HNO_3$ (1+1), and dil. to 1 L with HOAc. Stdze potentiometrically by adding 25 mL of this soln to 600 mL beaker contg 250 mL 80% HOAc. Immerse glass and Ag electrodes in soln and stir with mag. stirrer. Titr. with 0.1$N$ NaCl soln, **962.05A(a)**, to end point (max. change in mv/mL NaCl soln). Normality $AgNO_3$ = mL NaCl × normality NaCl/mL $AgNO_3$.

### B. Preparation of Sample

(**a**) *Emulsifiable concentrate formulations*.—Accurately weigh sample contg 0.3±0.05 g heptachlor in 250 mL erlenmeyer. Dissolve in 50 mL HOAc, and pipet in 25 mL 0.1$N$ $AgNO_3$, (**b**). Attach reflux condenser and reflux 1 hr.

(**b**) *Granular and dust formulations*.—(*Caution:* See safety notes on flammable solvents, pesticides, and pentane.) Accurately weigh sample contg 0.3±0.05 g heptachlor into 80 × 25 mm Soxhlet extn thimble. Ext 2 hr with *pentane* and transfer ext to 250 mL erlenmeyer. Attach short reflux column such as 3-ball Snyder or 12″ (30 cm) Vigreux to flask and evap. to dryness on steam bath. (Results will be low if reflux column is not used.) Rinse down column with 50 mL HOAc, pipet in 25 mL 0.1$N$ $AgNO_3$, (**b**), attach reflux condenser, and reflux 1 hr.

(**c**) *Technical*.—Accurately weigh 0.40±0.05 g heptachlor and proceed as in (**a**).

### C. Determination

Rinse tip of condenser or column with $H_2O$ and cool soln to room temp. Transfer quant. to 600 mL beaker, rinsing with four 10 mL portions 80% HOAc. Immerse glass and Ag electrodes in soln and stir with mag. stirrer. Titr. with 0.1$N$ NaCl soln, **962.05A(a)**, to end point.

$$\% \text{ Heptachlor} = 37.33 \times (25 \times \text{normality } AgNO_3 \text{ soln} - \text{mL NaCl soln} \times \text{normality NaCl soln})/\text{g sample}$$

Ref.: JAOAC **45,** 513(1962); **57,** 424(1974).

CAS-76-44-8 (heptachlor)

## 968.04 Heptachlor in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1968**
**Final Action 1969**

### A. Apparatus

(**a**) *Gas chromatograph*.—Equipped with H flame ionization detector; capable of accepting glass column and glass-lined sample introduction system or on-column injection. Use following conditions: Temps (°): column 175, detector 175–190, sample inlet 190; N carrier gas pressure 30 psig; recorder chart speed 2.5 cm/min.

(**b**) *Glass-stoppered tubes*.—Approx. 25 and 75 mL capacity.

(**c**) *Microliter syringe*.—10 μL, Hamilton Co., 701-N.

### B. Reagents

(**a**) *Heptachlor*.—Ref. grade (Velsicol Chemical Corp. 5600 N. River Rd, Rosemont, IL 60018–5119).

(**b**) *Aldrin*.—Ref. grade (Sandoz, Inc., 480 Camino del Rio South, Suite 204, San Diego, CA 92108).

### C. Preparation of Column

To 9.5 g 100–120 mesh Gas Chrom Q in vac. flask add 0.50 g silicone GE Versilube F-50 (available from Applied Science) dissolved in 50 mL $CH_2Cl_2$. Shake slurry well to wet solid thoroly. Connect flask to $H_2O$ aspirator and evap. solv. with frequent shaking. When solids appear dry, complete drying by placing flask in steam bath and connecting to vac. pump until ca 4 mm pressure is attained. Remove flask from steam bath and let cool under vac.

Fill 1.5 m (5′) × 1/8″ od (0.067″ id) Pyrex glass tube with this packing, using vac. pump and gentle tapping. Plug ends of column with glass wool. Condition column 24 hr in 190° oven while purging with N. Let column cool while still purging with N; then install in chromatograph.

### D. Preparation of Sample

(**a**) *Liquids*.—Weigh sample contg ca 750 mg heptachlor into 75 mL g-s vial and add 500 mg ref. grade aldrin. Add 75 mL fresh $CS_2$, stopper, and shake vigorously 2 min.

(**b**) *Solids*.—Transfer weighed sample contg ca 750 mg heptachlor to Soxhlet and ext 2 hr with 75 mL pentane. Let cool, add 500 mg ref. grade aldrin to soln, and swirl.

### *E. Calibration*

Weigh 0.2500 g ref. grade heptachlor and 0.1670 g ref. grade aldrin into 25 mL g-s flask. Dissolve in 25 mL $CS_2$. Chromatograph this soln under conditions given in **968.04A(a)** 5 times to obtain accurate response correction factor. (On new column, it is sometimes desirable to inject several 5 μL aliquots of std soln to condition column before use.)

### *F. Determination*

Let instrument equilibrate as in **968.04A(a)**. Inject ca 1 μL sample soln at sensitivity setting such that ht of heptachlor peak is ca $^3/_4$ full scale. For each analysis, allow 10–12 min for heptachlor related components to elute. Components and approx. retention times in min are: heptachlor 4.5, aldrin 5.9, chlordene 3.1, and γ-chlordane 9.9.

### *G. Calculations*

Calc. area of heptachlor and aldrin peaks by multiplying peak ht in mm by width of peak at half ht in mm. Alternatively, use integrator. Calc. response correction factor (*f*, ca 0.82) for each of the 5 std injections as follows:

$$f = (\text{area of heptachlor peak} \times \text{mg aldrin} \times \text{purity of aldrin})/(\text{area of aldrin peak} \times \text{mg heptachlor} \times \text{purity of heptachlor})$$

Average 5 replicates and use av. to calc. % heptachlor in samples.

$$\%\ \text{Heptachlor} = (\text{area of heptachlor peak} \times \text{mg aldrin} \times \text{purity of aldrin} \times 100)/(\text{area of aldrin peak} \times \text{mg sample} \times f)$$

Ref.: JAOAC **51,** 565(1968).

CAS-76-44-8 (heptachlor)

## 980.07 MCPA Ester and Salt in Pesticide Formulations
### Liquid Chromatographic Method
### First Action 1980

(*Caution:* See safety notes on sodium hydroxide, and acetonitrile.)

### *A. Principle*

Esters of MCPA are saponified *in situ* and amine salts are converted to $H_2O$-sol. K salt of MCPA. Ion suppression in reverse phase bonded microparticulate column seps isomers and impurities. Ionic MCPA moiety is protonated by acidic mobile solv., forming nonionic MCPA moiety, which greatly increases partitioning into stationary phase. Small changes in mobile solv. pH significantly affects retention time. Using pH 2.83 and flow rate of 3 mL/min, MCPA at 16.3 min retention time will elute between impurities 2-Me-4-chlorophenol at 14.5 min and 2,6-diMe-4-chlorophenoxyacetic acid at 23.0 min.

### *B. Apparatus*

*See* **978.05B**. Do *not* use column other than that specified.

### *C. Reagents*

(**a**) *Saponification-internal std soln* (*SISS*).—Partly fill vol. flask with isopropanol-$H_2O$ (2+1). Dissolve salicylic acid in the aq. isopropanol to produce final concn of 0.6 g/L. Add KOH (1+1) of known normality (ca 10.7*N*) to produce final soln of 0.2*N* KOH. Dil. to final vol., adjusting concn of salicylic acid according to detector response to obtain peak ht approx. equal to that of MCPA, avoiding off scale peak. Inject different strength solns of salicylic acid in aq. isopropanol (2+1) to det. appropriate concn.

(**b**) *Eluant*.—pH 2.83. Mix $CH_3CN$ (distd in glass, Burdick & Jackson Laboratories, Inc., or equiv.) with $H_2O$ (deionized, 0.4 μm filtered) (15+85) contg NaOH added from (1+1) aq. soln of known normality, **936.16B(b)**, to concn in final vol. of 0.3*M*. Adjust to pH 2.83 with $H_3PO_4$.

(**c**) *MCPA std soln*.—300 mg/25 mL SISS. Accurately weigh ca 300 mg MCPA anal. ref. std (99+% isomer pure; available from Dow Chemical Co.), previously dried 15 min at 95°, into 1 or 2 oz (30 or 60 mL) glass vial with polyethylene-lined screw cap. Pipet in 25 mL SISS, and shake to dissolve. Prep. MCPA std and sample solns at same time, using same pipet.

### *D. Preparation of Sample*

Proceed as in **978.05D**, using sample contg ca 300 mg MCPA and 25 mL SISS, but filter thru microfiber filter GF/C, or equiv. For ester formulations, to eliminate baseline interference from aromatic solvents, transfer saponified sample to 30 mL separator. Add ca 5 mL hexane, shake 1 min, let sep., and collect major portion of aq. phase for chromatgy.

### *E. Determination*

Proceed as in **978.05E**, substituting MCPA samples and stds for 2,4-D.

Ref.: JAOAC **63,** 873(1980).

CAS-94-74-6 (MCPA)

## 982.03 Methazole in Pesticide Formulations
### Infrared Spectrophotometric Method
### First Action 1982

(Applicable to wettable powder contg methazole as only active ingredient)

### *A. Apparatus and Reagents*

(**a**) *Infrared spectrophotometer*.—Capable of measuring *A* from 700 to 900 $cm^{-1}$, with matched 0.5 mm NaCl or KBr cells.

(**b**) *Methazole std soln*.—Weigh, to nearest mg, 0.48–0.52 g ref. std methazole (available from Velsicol Chemical Corp., 5600 N. River Rd, Rosemont, IL 60018–5119) into 4 oz polyethylene screw-cap bottle, pipet in 50.0 mL acetone, and mech. shake 15 min to dissolve.

(**c**) *Acetone*.—Anal. reagent grade (Mallinckrodt, or equiv.).

### *B. Determination*

Weigh, to nearest mg, 0.63–0.67 g sample into 4 oz polyethylene screw-cap bottle, pipet in 50.0 mL acetone, and mech. shake 1 h. Centrfg. 30 min to obtain clear supernate.

Fill both cells of spectrophtr with acetone, and place in instrument. Optimize gain; set 100% adjust to give 95–98% *T* at 755 $cm^{-1}$. Set slit in program or manual mode for optimum sensitivity and resolution. Fill sample cell with std soln, and scan region from 860 to 700 $cm^{-1}$ ($A'$). Using same conditions, fill same cell with sample soln and scan twice ($A$). Measure $A$ and $A'$ at 755 $cm^{-1}$, using min. at 845 $cm^{-1}$ as baseline.

### *C. Calculation*

$$\%\ \text{Methazole} = (W' \times A \times P \times 100)/(W \times A')$$

where $W$ and $W'$ = g sample and std, resp.; and $P$ = % purity of std.

Ref.: JAOAC **64,** 1185(1981).

CAS-20354-26-1 (methazole)

## 985.06 Metolachlor in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1985**
**Final Action 1988**

***AOAC-CIPAC Method***

(Method is suitable for formulations where metolachlor is only active ingredient.)

#### A. Principle

Metolachlor is extd with acetone and detd by flame ionization gas chromatgy, using dipentyl phthalate as internal std. Identity is verified simultaneously by comparing retention times with std.

#### B. Apparatus

(**a**) *Gas chromatograph.*—Equipped with flame ionization detector.

(**b**) *Chromatographic column.*—2 mm id × 1.83 m (6 ft) glass column packed with 3% OV-101 on 80–100 mesh Gas-Chrom Q, or equiv. Condition column ≥24 h at 240°, using carrier gas at ca 20 mL/min. Operating conditions: injector 250°, detector 250°, column 180° ± 10°, He carrier gas flow ca 25 mL/min. Retention times for metolachlor and internal std are ca 8.8 and 15.6 min, resp.

#### C. Reagents

(**a**) *Internal std soln.*—4 mg/mL. Dissolve 4.0 g dipentyl phthalate in acetone and dil. to 1 L with acetone. Check internal std for interfering components by injecting aliquot into chromatograph.

(**b**) *Metolachlor std soln.*—Accurately weigh 200 mg metolachlor std of known purity (Ciba-Geigy Corp., Production Technical Dept, PO Box 18300, Greensboro, NC 27419) into 4 oz bottle. Pipet 50.0 mL internal std soln into bottle, cap, and shake 10 min.

#### D. Preparation of Sample

Accurately weigh amt sample contg ca 200 mg metolachlor into 4 oz bottle. Pipet 50.0 mL internal std soln into bottle, cap, and shake 10 min to ext.

#### E. Determination

Set integration parameters and stabilize instrument by injecting 1–3 μL aliquots of std soln until area ratios of metolachlor to internal std vary <2% for successive injections. Using same established injection vol. as for std, inject sample. Sample area ratio should be ±10% of std area ratio. Inject 2 aliquots of std and 2 aliquots of sample followed by 2 aliquots of second sample and 2 aliquots of std. Repeat sequence until all samples are analyzed. Calc. response factor, $R$, for each injection:

$$R = \text{peak area (or ht) metolachlor/peak area (or ht) int. std}$$

$$\text{Metolachlor, \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response factor for sample and std solns, resp.; $W$ and $W'$ = mg sample and std, resp.; and $P$ = purity (%) of std.

Ref.: JAOAC **68,** 570(1985).

CAS-51218-45-2 (metolachlor)

## 982.04 PCNB in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1982**
**Final Action 1983**

(*Caution:* See safety notes on pesticides.)

#### A. Principle

Sample is dissolved in $CHCl_3$, *o*-terphenyl is added as internal std, and PCNB is detd by GC with flame ionization detection.

#### B. Apparatus and Reagents

(**a**) *Gas chromatograph with recorder.*—With flame ionization detector and 1.8 m × 4 mm (id) glass column packed with 5% SE-30 on 80–100 mesh Chromosorb W (dimethylchlorosilane-treated) (Analabs, Inc.). Condition newly packed column 24 h at 285° with low N flow. Operating conditions: temps (°)—inlet 200, column 175–180, detector 250; carrier gas flow to elute PCNB at ca 4.5 min; adjust H and air as recommended for detector by manufacturer; sensitivity to give peak hts 60–80% full scale.

(**b**) *PCNB std soln.*—2.0 mg/mL $CHCl_3$. Accurately weigh ca 0.2 g PCNB (Olin Corp., Agriculture Products Dept., PO Box 991, Little Rock, AR 72203) into 100 mL vol. flask and dil. to vol. with $CHCl_3$.

(**c**) *Internal std soln.*—0.8 mg/mL $CHCl_3$. Accurately weigh ca 0.2 g *o*-terphenyl into 250 mL vol. flask and dil. to vol. with $CHCl_3$.

(**d**) *Mixed std soln.*—1.0 mg PCNB + 0.4 mg *o*-terphenyl/mL. Pipet 25 mL each of PCNB and internal std solns into vial and mix.

#### C. Preparation of Sample

(**a**) *Solid formulations.*—Grind 100 g well mixed sample to pass 1 mm sieve. Accurately weigh portion of well mixed, ground sample contg ca 0.2 g PCNB into 250 mL g-s erlenmeyer and add 100 mL $CHCl_3$; stopper and shake 2 h on rotary shaker. Let insol. matter settle.

(**b**) *Wettable powders.*—Accurately weigh portion of well mixed sample contg ca 0.2 g PCNB into 250 mL g-s erlenmeyer and proceed as for solids.

(**c**) *Liquids.*—Accurately weigh portion of well mixed sample contg ca 0.2 g PCNB into 100 mL vol. flask and dil. to vol. with $CHCl_3$.

(**d**) *Soln for analysis.*—Pipet 10 mL sample ext above and 10 mL internal std soln into vial, cap, and mix.

#### D. Determination and Calculation

Inject 4 μL aliquots of mixed std soln until variation in response ratio (area or peak ht) for PCNB (first peak) to *o*-terphenyl (second peak) is ca 1%. Inject mixed std, inject sample twice, and repeat injection of mixed std. Retention times must be the same for sample and std. Calc. av. ratios of PCNB to *o*-terphenyl for the 2 mixed std and sample injections, and calc. % PCNB.

$$\text{\% PCNB} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and mixed std, resp.; $W'$ = g PCNB/100 mL std soln; $W$ = g sample extd; and $P$ = purity (%) of PCNB std.

Refs.: JAOAC **65,** 110(1982); **66,** 410(1983).

CAS-82-68-8 (pentachloronitrobenzene)

## 976.03 Picloram and 2,4-D in Pesticide Formulations

### Liquid Chromatographic Method

**First Action 1976**
**Final Action 1978**

### *A. Apparatus*

(**a**) *Liquid chromatograph.*—Equipped with 280 nm UV detector and injection valve. Alternatively, septum injection head may be used; however, stop-flow injection is recommended. Operating conditions: flow rate, 0.7 mL/min (ca 1000 psi); detector sensitivity, 0.08 *A* unit full scale; temp., ambient, but within ±2.5°.

(**b**) *Liquid chromatographic column.*—No. 316 stainless steel, 1000 × 2.1 mm id, with Varian Instrument Group, No. 96-000075-00 reducing union ($^1/_8$" × $^1/_{16}$") contg 2 μm frit (regular reducing union packed with glass wool may be used instead) packed with DuPont No. 820960005 Zipax® SAX (strong anion exchange) resin. Preclean column with few mL each of $CHCl_3$, acetone, and MeOH, and vac.-dry. Pack in small increments over 40 min period while tapping column on hard surface.

### *B. Reagents*

(**a**) *Mobile phases.*—Prep. sep. solns of 0.01*M* $Na_2B_4O_7.10H_2O$ (3.8 g/L) and 0.002*M* $NaClO_4.H_2O$ (0.28 g/L) in previously boiled and cooled deionized $H_2O$.

(**b**) *Salicylic acid internal std soln.*—Accurately weigh ca 3.6 g USP Ref. Std Salicylic Acid into 1 L vol. flask, dil. to vol. with 0.05*N* NaOH in isopropanol-$H_2O$ (1+1), and mix.

(**c**) *Picloram-2,4-D std soln.*—(4 mg picloram + 12 mg 2,4-D + 3.6 mg salicylic acid)/mL. Accurately weigh ca 100 mg picloram ref. std, 99+% pure (Dow Chemical Co.), and ca 300 mg 2,4-D ref. std, 99+% pure (Dow Chemical Co.), into glass vial, pipet in 25 mL salicylic acid internal std soln, and shake to dissolve.

### *C. Preparation of Sample*

Accurately weigh ca 1.6 g sample into ca 10 dram glass vial, pipet in 25 mL salicyclic acid internal std soln, and shake to dissolve.

### *D. Determination*

Inject 2 μL picloram-2,4-D std soln onto column and adjust attenuation to give largest possible on-scale peaks. Repeat injections until peak ht ratios of herbicide:internal std vary ≤1% for successive injections. Without changing conditions, inject 2 μL aliquots sample soln until peak ht ratios vary ≤1%. Average last 2 peak ht ratios for picloram and 2,4-D and calc. % herbicide.

$$\% \text{ Herbicide} = (R/R') \times (W'/W) \times P$$

where *R* and *R'* = av. peak ht ratios of each herbicide to the internal std for sample and std, resp.; *W'* = mg herbicide in std; *W* = mg sample; and *P* = % purity of std.

Ref.: JAOAC **59,** 748(1976).

CAS-94-75-7 (2,4-D)
CAS-1918-02-1 (picloram)

## 986.05 Propachlor in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1986**
**Final Action 1988**

***AOAC-CIPAC Method***

### *A. Principle*

Sample is dissolved in acetone contg diisobutyl phthalate as internal std, analyzed by gas chromatgy with flame ionization detection, and measured by comparison with internal std on basis of integrated peak areas.

### *B. Safety*

$LD_{50}$ of propachlor has been found to be 1800 mg/kg in rat acute oral studies (Monsanto). Material is classified as slightly toxic. Avoid excessive exposure; wear protective clothing.

### *C. Apparatus*

(**a**) *Gas chromatograph.*—With flame ionization detector and on-column injection ports. Temps—column oven 200° (isothermal), injection port 250°, detector 250°; gas flows (mL/min)—He carrier gas 30, H 34, air 430; sample size 1.0 μL; run time 25 min.

(**b**) *Column.*—6 ft × 2 mm (id) glass column (on-column configuration) packed with 10% SP-2250 on 100–120 mesh Supelcoport (Supelco, Cat No. 1-2132), or equiv. SP-2250 is methyl-phenyl silicone (50 + 50). Precondition overnight at 250° before use. Retention times for propachlor and internal std are ca 5.29 and 11.51 min, resp.

### *D. Reagents*

(**a**) *Acetone.*—Pesticide grade (Fisher Scientific Co., or equiv.).

(**b**) *Diisobutyl phthalate internal std soln.*—Accurately weigh 6.4 g diisobutyl phthalate (Eastman Kodak Co.) into 1 L vol. flask. Dissolve in and dil. to vol. with acetone.

(**c**) *Propachlor std soln.*—Accurately weigh 0.2 g propachlor (recrystd from MeOH; Monsanto Chemical Co.) into small flask. Add by pipet 25 mL internal std soln and shake to dissolve.

### *E. Determination*

Accurately weigh sample contg ca 0.2 g propachlor into small flask. Add by pipet 25.0 mL internal std soln and shake ≥5 min to ext propachlor. For flowable formulations, use 0.5 g sample; for Ramrod®/Atrazine flowable formulation, use 0.6 g sample; for granular formulations (20 G), use 1.0 g sample. Thoroly mix solns by mech. shaking. Vigorously mix granular samples 15 min.

Make replicate 1 μL injections of propachlor std soln and measure response ratio, *R* (area propachlor peak/area internal std peak), for each injection. Repeat until consecutive response ratios agree ±0.5%.

Make duplicate injections of sample soln and det. av. *R*. Follow with injection of propachlor std soln; average *R'* for std before and after sample injection

$$\text{Propachlor, } \% = (R/R') \times (W'/W) \times P$$

where *R* and *R'* = av. response ratios for sample and std, resp.; *W* and *W'* = wt (g) of sample and std, resp.; *P* = % purity of std.

Ref.: JAOAC **69,** 723(1986).

CAS-1918-16-7 (propachlor)

## 980.08 2,4,5-T in Pesticide Formulations

### Liquid Chromatographic Method

**First Action 1980**

(Method is modification of LC method for 2,4,-D, **978.05**.)

(*Caution:* See safety notes on pesticides, and acetonitrile.)

### *A. Apparatus*

*See* **978.05B**.

### *B. Reagents*

(**a**) *Eluant.*—pH 2.95. 18% $CH_3CN$-82% $H_2O$, v/v, contg NaOH added from (1 + 1) aq. soln of known normality,

**936.16B(b)**, ca 17.7*N*, to concn in final vol. of 0.3*M*. Adjust to pH 2.95 with $H_3PO_4$. Use std buffers at pH 3.0 or 4.0 as ref.

(**b**) *Saponification-internal std soln.—See* **978.05C(b)**.

(**c**) *2,4,5-T std soln.*—240 mg/25 mL. Prep. as in **978.05C(c)**, using 240 mg 2,4,5-T instead of 300 mg 2,4-D.

### C. Preparation of Sample

Prep. sample as in **978.05D**, using sample contg ca 240 mg 2,4,5-T, and using GF/C filter. Ext with hexane as in **980.07D**.

### D. Determination

Proceed as in **978.05E**, substituting 2,4,5-T samples and stds for 2,4-D.

Ref.: JAOAC **63,** 873(1980).

CAS-93-76-5 (2,4,5-T)

## 981.02 Tetradifon (Technical) and Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1981**
**Final Action 1982**

***CIPAC-AOAC Method***

### A. Principle

Tetradifon is detd by flame ionization GC, using *n*-hexacosane as internal std.

### B. Apparatus

(**a**) *Gas chromatograph.*—Suitable for on-column injection; equipped with flame ionization detector.

(**b**) *Gas chromatographic column.*—3 mm id × 6 ft glass column packed with 3% SE-52 on 100–120 mesh Chromosorb W-HP. Operating conditions: injector and detector 250°, column 230°, N or He carrier gas flow ca 35 mL/min. Approx. retention times for tetradifon and *n*-hexacosane = 8.6 and 10 min, resp.

### C. Reagents

(**a**) *Internal std soln.*—Accurately weigh 0.30 g pure *n*-hexacosane into 100 mL vol. flask. Dil. to vol. with 1,2-dichloroethane and mix.

(**b**) *Tetradifon std soln.*—Accurately weigh 0.100 g pure tetradifon (≥99.5%, Duphar B.V., PO Box 2, 1380 Weesp, The Netherlands; or Chemical and Biological Investigations, Environmental Protection Agency, Beltsville, MD 20705) into 100 mL vol. flask, pipet 20.0 mL internal std soln, (**a**), into flask, dil. to vol. with dichloroethane, and mix.

### D. Preparation of Sample

(**a**) *Technical tetradifon.*—Accurately weigh 100 mg sample into 100 mL vol. flask. Pipet 20.0 mL internal std soln, (**a**), into flask, dil. to vol. with dichloroethane, and mix thoroly.

(**b**) *Wettable powder.*—Accurately weigh sample contg 100 mg tetradifon into 250 mL g-s flask. Pipet in 20.0 mL internal std soln, (**a**), and 80.0 mL dichloroethane. Heat on $H_2O$ bath 5 min, cool, and mix thoroly. Transfer ca 40 mL soln to centrif. tube, and centrif. 10 min.

(**c**) *Emulsifiable concentrates.*—Accurately weigh sample contg 100 mg tetradifon into 100 mL vol. flask. Pipet 20.0 mL internal std soln, (**a**), into flask. Dil. to vol. with dichloroethane and mix thoroly.

### E. Determination

Adjust chromatographic conditions to give ca $^1/_2$ FSD for 2 μg tetradifon. Inject 2 μL portions of std soln, (**b**), until response factor ($F$) varies <1% for successive injections. Inject 2 μL sample soln, and measure peak hts and retention times for both std and sample.

### F. Calculation

$$F = (I' \times T \times P \times 5)/(I \times S \times 100)$$

where $I'$ and $I$ = peak ht × retention time of internal std and tetradifon, resp.; $T$ = g tetradifon in calibration soln; $P$ = % purity of std; $S$ = g internal std. (Response factor is ca 1.5.)

$$\%\ \text{Tetradifon} = (I \times S \times F \times 100)/(I' \times W \times 5)$$

where $W$ = g sample.

Ref.: JAOAC **64,** 829(1981).

CAS-116-29-0 (tetradifon)

## 962.08 Sodium TCA in Pesticide Formulations

**Titrimetric Method**

**First Action 1962**
**Final Action 1966**

(*Caution:* See safety notes on distillation, flammable solvents, pesticides, and peroxides.)

### A. Apparatus and Reagent

(**a**) *Reflux apparatus.*—250 mL erlenmeyer attached thru ₸ 24/40 joint to 50 cm water-cooled condenser.

(**b**) *Dioxane.*—Freshly distd.

### B. Determination

Dissolve 25 g sample in $H_2O$ and dil. to 100.0 mL. Pipet aliquot (usually 10 mL), titrg ca half that of blank, into 250 mL refluxing flask, add 1 drop Me red, and neutze with ca 1*N* $H_2SO_4$ to distinct orange-pink. pH is 5.3–5.5; usually <0.15 mL is required. If soln is acid, titr. with ca 1*N* NaOH. Add 25.00 mL 1*N* $H_2SO_4$, 35 mL dioxane, and few glass beads. Boil vigorously under reflux ≥60 min. Cool, add 2 drops Me red, and titr. with std 1*N* NaOH to sharp change from orange to yellow end point. Perform blank detn, omitting sample.

$$\%\ \text{Na trichloroacetate} = \text{Net mL } 1N \text{ acid} \times 0.1854 \times 100/\text{g sample in aliquot}$$

Refs.: Anal. Chem. **27,** 1774(1955). JAOAC **43,** 382(1960); **45,** 522(1962).

CAS-650-51-1 (sodium TCA)

## Trifluralin

**Final Action**

*See* **973.13**.

# THIOPHOSPHORUS AND OTHER ORGANOPHOSPHORUS PESTICIDES

## 980.09 Azinphos-Methyl in Pesticide Formulations

**Infrared Spectrophotometric Method**

**First Action 1980**
**Final Action 1981**

(Applicable to 50% wettable powders and 2 lb/gal. liq. concns where azinphos-methyl is the only active ingredient.)

(*Caution:* See safety notes on pesticides.)

### A. Apparatus

(**a**) *Infrared spectrophotometer*.—Capable of making measurements in 600–700 $cm^{-1}$ region, such as Perkin-Elmer Corp., Models 421 and 521.
(**b**) *Cells*.—Perkin-Elmer sealed cells, 0.5 mm KBr for 2 lb/gal. liq. concs and 0.2 mm KBr for 50% wettable powders.
(**c**) *Disposable pipets*.—Scientific Products Inc., No. P5205-1 with bulb No. P2515, or equiv.
(**d**) *Mechanical shaker*.—Eberbach Model 6000 with 6040 carrier tray, or equiv.
(**e**) *Centrifuge*.—International centrifuge Model UV, or equiv., with head for 15 mL tubes.

### B. Reagents

(**a**) *Azinphos-methyl*.—Purified material, available from Mobay Corp.,
(**b**) *Azinphos-methyl std soln*.—Weigh, to nearest 0.1 mg, 0.18–0.19 g purified azinphos-methyl (for 2 lb/gal. liq. concs) into 50 mL vol. flask or 0.245–0.255 g (for 50% wettable powder) into 25 mL vol. flask. Dil. to vol. with 1.2-dimethoxyethane and shake well.
(**c**) *1,2-Dimethoxyethane*—Bp 83–85° (Eastman 4639, bp 83–85°, has been found satisfactory).

### C. Preparation of Sample

Weigh, to nearest 0.1 mg, 0.77–0.79 g 2 lb/gal. liq. conc. or 0.85–0.95 g 50% wettable powder sample into 50 mL vol. flask. Add 25 mL 1,2-dimethoxyethane into flasks and shake on mech. shaker 10 min. Dil. to vol. with 1,2-dimethoxyethane. Mix thoroly. Let wettable powder samples stand 15 min to allow clay to settle. *Do not* mix again before analyzing. If soln remains turbid, centrf. 10 mL aliquot in stoppered tube 15 min at 650–700 × g.

### D. Determination

Fill appropriate KBr cell with corresponding std soln from clean disposable pipet. Adjust gain to optimum setting (3–4) at 654 $cm^{-1}$. Set 100% adjust at 690 $cm^{-1}$ to give 95–98% $T$, auto suppression—0, scale change selector—linear, scan gear—A-18, B-72, scan time—1.5–1.8 min, slit program 950 × 2 (421), 750 manual (521), and speed change knob—fast gear. Scan std soln from 700 to 630 $cm^{-1}$ using air as blank. Using same instrument settings, scan sample soln twice, filling cell each time using clean pipet, in same manner as std. Repeat scan with std soln. Draw baseline thru minima near 690 and 630 $cm^{-1}$ and measure ht of 654 $cm^{-1}$ peak above this line in $A$ units. Record sample as $A$ and std as $A'$. If $A$ of sample and std differ by >0.030, repeat analysis using adjusted sample wt ($W_1$), where

$$W_1 = W \times A'/A$$

If difference between replicate std $A'$ or replicate sample $A$ is >2% of their average, repeat analysis:
(**a**) For 2 lb/gal. liq. concs:

$$K = W_I \times P/A'$$

(**b**) For 50% wettable powder:

$$K = W_I \times P \times 2/A'$$

$$\% \text{ (Azinphos-methyl)} = K \times A/W \text{ (or } W_1)$$

where $W$ (or $W_1$) and $W'$ refer to wts sample and std, resp.; and $P$ = % purity of std.

Ref.: JAOAC **64,** 628(1981), corr. 1269.

CAS-86-50-0 (azinphos-methyl)

## 989.01 Azinphos-Methyl in Pesticide Formulations
### Liquid Chromatographic Method
### First Action 1989
### *AOAC-CIPAC Method*

(Applicable to wettable powder and liq. formulated products contg azinphos-methyl as only active ingredient)

Method Performance
50% wettable powder:

$s_r = 0.19$; $s_R = 0.53$; $RSD_r = 0.40\%$; $RSD_R = 1.11\%$

2L emulsifiable conc. (2 lb/gal.):

$s_r = 0.41$; $s_R = 0.56$; $RSD_r = 1.79\%$; $RSD_R = 2.47\%$

2S emulsifiable conc. (2 lb/gal.):

$s_r = 0.17$; $s_R = 0.25$; $RSD_r = 0.79\%$; $RSD_R = 1.17\%$

### A. Principle

Azinphos-methyl is detd by liq. chromatgy. Peak hts of sample and anal. std are compared using *n*-butyrophenone as internal std.

### B. Apparatus

(**a**) *Liquid chromatograph*.—Able to generate >7 MPa (>1000 psi). Equipped with spectrophtr to measure $A$ at 285 nm and peak ht integrator or recorder. Operating conditions: column temp. ambient; flow rate ca 1.5 mL/min (ca 1000 psi); recorder speed 0.5 cm/min; recorder range 10 mV; injection vol. ca 10 μL; $A$ range 0.16 AUFS. Retention times: azinphos-methyl ca 4.0 min, internal std ca 4.8 min. Pump LC mobile phase thru column until system is equilibrated (flat baseline). After each injection, allow 10 min after internal std for elution of formulation excipients.
(**b**) *Chromatographic column*.—250 × 4.7 mm id packed with ≤10 μm $C_{18}$ bonded silica gel (Du Pont Zorbax ODS, or equiv.).
(**c**) *Filters*.—0.45 μm porosity (Gelman Acrodisc-CR, or equiv.)

### C. Reagents

(**a**) *Acetonitrile*.—LC grade or distd in glass (Burdick & Jackson, or equiv.)
(**b**) *Water*.—LC grade or distd in glass (Burdick & Jackson, or equiv.)
(**c**) *LC mobile phase*.—$CH_3CN$–$H_2O$ (65 + 35).
(**d**) *n-Butyrophenone internal std soln*.—10% *n*-butyrophenone (Aldrich No. 12,433-8, or equiv.) (v/v) in $CH_3CN$.
(**e**) *Azinphos-methyl reference std*.—(Mobay Corp., Agricultural Chemicals Div., PO Box 4913, Hawthorne Rd, Kansas City, MO 64120-0013). Store ref. std at refrigeration temp. (4–8°).
(**f**) *Azinphos-methyl std soln*.—Accurately weigh ca 220 mg azinphos-methyl ref. std into 100 mL vol. flask. Pipet 10.0 mL internal std soln into flask, dil. to vol. with $CH_3CN$, and mix thoroly. Filter portion of final soln and hold for LC analysis.

### D. Preparation of Samples

Accurately weigh amt sample contg ca 220 mg azinphos-methyl into 100 mL vol. flask. Pipet 10.0 mL internal std soln into flask, dil. to vol. with $CH_3CN$, and mix thoroly. Filter portion of final soln and hold for LC analysis.

### E. Determination

Adjust operating parameters so that azinphos-methyl elutes in 3.8–4.2 min. Adjust injection vol. and attenuation to give largest possible on-scale peaks. If peaks cannot be brought on scale at 0.32 AUFS setting with 10 μL injection, further dil. std and sample solns by pipetting 10 mL of each into 100 mL vol. flasks, dilg to vol. with $CH_3CN$, and mixing thoroly. Readjust injection vol. and attenuation to give largest possible on-scale peaks.

Make repetitive injections of std and calc. response ratios by dividing azinphos-methyl peak ht by internal std peak ht (area measurements are not acceptable). Response ratios must agree within ±1% before continuing. Inject duplicate aliquots of each sample soln (no more than 2 samples [4 injections] between std injections). Response ratios of sample injections must agree within ±1%. If not, repeat detn, starting with std injections. Reinject std soln. Average response ratios of std injections immediately preceding and following sample injections. These must agree within ±1%. If not, repeat detn.

### F. Calculation

For each injection: Response ratio ($R$) = (azinphos-methyl peak ht/internal std peak ht).

$$\text{Azinphos-methyl, wt } \% = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std solns, resp.; $W'$ and $W$ = wt (mg) of azinphos-methyl in std and sample solns, resp.; $P$ = % purity of azinphos-methyl std.

Ref.: JAOAC **71,** 988(1988).

CAS-86-50-0 (azinphos-methyl)

## 981.03 Chlorpyrifos in Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1981**
**Final Action 1982**

(*Caution:* See safety notes on acetic acid and acetonitrile.)

### A. Principle

$CH_3CN$ contg internal std is added to solid or liq. sample to ext chlorpyrifos. Aliquot is subjected to reverse phase LC. Small amt of HOAc is added to mobile phase to suppress non-reproducible ionization of 3,5,6-trichloro-2-pyridinol, which might otherwise interfere.

### B. Apparatus

(**a**) *Liquid chromatograph.*—Modular apparatus, LC-55, variable wavelength detector (LC-75 can be substituted) (Perkin-Elmer Corp.), or equiv.; Altex 100 pump (replacement Model 116 Programmable Solvent Module, Beckman Instruments, Inc., 2350 Camino Ramon, PO Box 5101, San Ramon, CA 97583-0701), or equiv.; Model 728 Autosampler (Alcott Chromatography, Inc., One Micromeritics Dr); column heating unit, LC-22A temperature controller, and LC-23A column heater (Bioanalytical Systems, Inc., 2701 Kent Ave, West Lafayette, IN 47906), or equiv. For manual injections, Model 7125 valve is recommended (Rheodyne, Inc., PO Box 996, Cotati, CA 94928).

Operating conditions: Flow rate, 2 mL/min (1100 psi); wavelength, 300 nm; 1.0 AUFS; injection volume, 10 μL; temp., ambient (if temp. control is available, 30° is recommended).

(**b**) *Liquid chromatographic column.*—Zorbax® ODS, 4.6 mm × 25 cm (E. I. DuPont de Nemours & Co., Instrument Products Div.); 2 μm column filter, Cat. No. 7302 (Rheodyne, Inc.).

### C. Reagents

(**a**) *Eluant.*—$CH_3CN$–$H_2O$–HOAc (82 + 17.5 + 0.5). Mix 820 mL $CH_3CN$, 175 mL $H_2O$, and 5 mL glacial HOAc and degas.

(**b**) *Internal std soln.*—37.5 mg/25 mL. Weigh 1.5 ± 0.1 g 1,4-dibromonaphthalene (Eastman Organic Chemicals P7595) into 1 L vol. flask, dil. to vol. with $CH_3CN$, and mix.

(**c**) *Chlorpyrifos std soln.*—Accurately weigh ca 80 mg chlorpyrifos ref. std, 99.7+% pure (Dow Chemical Co.), into glass vial, pipet in 25.0 mL 1,4-dibromonaphthalene internal std soln, and mix.

### D. Preparation of Sample

(**a**) *Liquid formulations.*—Accurately weigh into glass vial amt sample contg ca 80 mg chlorpyrifos. Pipet in 25.0 mL 1,4-dibromonaphthalene internal std soln and mix.

(**b**) *Solid formulations.*—Accurately weigh into glass vial amt sample contg ca 80 mg chlorpyrifos. Pipet in 25.0 mL 1,4-dibromonaphthalene internal std soln and place on wrist-action shaker 5 min. (For Lorsban 15G insecticide, use 1.2 g sample and 50 mL internal std soln.) Filter sample thru 1 μm filter before injection.

### E. Determination

Inject 10 μL chlorpyrifos std soln onto column and adjust attenuation to give largest possible on-scale peaks (ca 1.0 AUFS). Repeat injections until ratio of chlorpyrifos peak ht (or area) to internal std peak ht (or area) varies ≤0.5% ($R'$). Without changing conditions, inject 10 μL aliquots of sample soln until ratio varies ≤0.5%. Average last 2 ratios for sample soln ($R$) and calc. % chlorpyrifos.

$$\% \text{ Chlorpyrifos} = (R/R') \times (W'/W) \times P$$

where $W'$ = mg chlorpyrifos ref. std (= 80.0 mg); $W$ = mg sample; and $P$ = % purity of std.

Ref.: JAOAC **64,** 628(1981); corr. 1269.

CAS-2921-88-2 (chlorpyrifos)

## 964.04 DDVP in Pesticide Formulations

**Infrared Spectrophotometric Method**

**First Action 1964**

***Method I***

(Applicable to sand/sugar base fly bait contg ca 0.5% and 4 lb/gal. DDVP emulsifiable concs.)

### A. Apparatus and Reagent

(**a**) *Infrared spectrophotometer.*—Capable of recording in region 2–15 μm. Slit width must be adjustable to give signal-to-noise ratio of ca 100:1; with sealed liq. absorption cell, NaCl windows, and 0.2 mm path length.

(**b**) *Hypodermic syringe.*—Luer type, glass, 1.0 mL. Use 18 gage (Stubbs), 2″ slip-on needle.

(**c**) *2,2-Dichlorovinyl dimethyl phosphate.*—Use std DDVP of known purity.

### B. Calibration of Apparatus

Into each of five 10 mL vol. flasks, weigh, to nearest 0.1 mg, 25, 75, 100, 150, and 200 mg DDVP std, and dil. to vol. with $CHCl_3$. Calibration solns contain ca 2.5, 7.5, 10, 15, and 20 g DDVP/L.

Fill sealed liq. absorption cell with $CHCl_3$, adjust spectrophtr to optimum settings, and scan over 10.7–9.9 μm. Without changing settings, fill cell in turn with each of prepd calibration solns, starting with most dil., and scan each soln over 10.7–9.9 μm.

For each scan, construct baseline thru absorption min. at ca 10.0 μm parallel to 0 radiation line. Draw perpendicular to 0 radiation line thru absorption max. of calibration soln at ca 10.2 μm and measure radiant power $P_0$ (at 10.0 μm) and P (at 10.2 μm), in any convenient units but keeping same units thruout. Calc. $A$ as $\log(P_0/P)$. Repeat calcns, using absorption min. at ca 10.5 μm as ref. point.

Subtract $A$ of cell and $CHCl_3$ obtained above from $A$ of cell and calibration solns. Plot $\Delta A$ of DDVP as ordinate against g/L DDVP as abscissa for each ref. point (10.0 and 10.5 μm).

### C. Preparation of Sample Solution

(**a**) *Sand/sugar base fly baits.*—Prep. 25 × 400 mm extn column by adding enough diat. earth (Hyflo Super-Cel) to make layer 5 cm high when gently packed. Place 250 mL vol. flask under outlet. Accurately weigh sample contg 0.2–1.0 g DDVP. Transfer sample to extn column with $CHCl_3$, and rinse sample container with $CHCl_3$.

Working in well-ventilated hood, add 50 mL $CHCl_3$ to column. Using stirring device, vigorously agitate sample and top half of adsorbent layer to form slurry with solv. Withdraw stirring device, and rinse it and column with addnl $CHCl_3$ from wash bottle. Let solv. percolate thru column until level is few mm above diat. earth-sample layer.

Add ca 50 mL $CHCl_3$ to column, agitate sample and diat. earth with stirrer as above, and let solv. percolate thru column until upper level approaches sample layer. Repeat with two addnl 50 mL portions $CHCl_3$. When solv. ht has diminished to 2–3 mm, rinse column with three 10 mL portions $CHCl_3$, letting each portion enter diat. earth layer before adding next. Let column drain and rinse outlet tip with $CHCl_3$, collecting rinse in 250 mL vol. flask.

Transfer $CHCl_3$ eluate to evapg dish (125 mm diam.) marked at 40–50 mL. Evap. on steam bath to 40–50 mL. Remove dish and continue evapn at room temp. to 10–15 mL. Using $CHCl_3$, quant. transfer to vol. flask of such size to give DDVP concn of 0.5–1.0 g/100 mL when soln is dild to vol.

(**b**) *Emulsifiable concentrates.*—Weigh enough sample, to nearest 0.2 mg, to give ca 1 g DDVP/100 mL $CHCl_3$ when dild to vol. in 10, 25, or 50 mL vol. flask.

### D. Determination

Dil. $CHCl_3$ soln of DDVP to vol. with $CHCl_3$, mix thoroly, and fill calibrated liq. absorption cell with sample soln. Using same instrument settings as for calibration, scan sample soln over 10.7–9.9 μm.

Examine spectra for possible interference and use appropriate absorption min. as ref. point. (If solvs or other ingredients interfere at one of ref. points, use alternative ref. point.) For example, β-naphthol, often used as stabilizer in fly baits, exts with $CHCl_3$ and absorbs at ca 10.5 μm, requiring use of 10.0 μm ref. point.

Calc. $A$ of sample soln as in **964.04B**.

From calcd $A$, read g DDVP/L from calibration curve.

$$\% \text{ DDVP by wt} = [(\text{g DDVP/L}) \times \text{mL sample soln}]/(10 \times \text{g sample})$$

Ref.: JAOAC **47**, 268(1964).

CAS-62-73-7 (2,2-dichlorovinyl dimethyl phosphate)

## 966.07 DDVP in Pesticide Formulations

**Infrared Spectrophotometric Method**

**First Action 1966**

***Method II***

(Applicable to ca 0.5% (w/w) spray soln and ca 1.0% (w/w) cattle spray in hydrocarbon solvs)

### A. Apparatus and Reagent

(**a**) *Infrared spectrophotometer.*—Double beam instrument with specifications as in **964.04A(a)**.

(**b**) *2,2-Dichlorovinyl dimethyl phosphate.—See* **964.04A(c)**.

### B. Preparation of Compensating Solvent

Transfer ca 30 mL sample to 125 mL separator and ext (2–3 min per extn) with 4 ca 30 mL portions 0.5*N* NaOH. Dry DDVP-free hydrocarbon phase by passing it thru 2–3 g anhyd. $Na_2SO_4$. Reserve dried solv. for prepn of DDVP std soln and as compensating solv. in ref. cell.

### C. Determination

Prep. std DDVP soln in compensating solv. that approximates (on wt basis) DDVP content of sample. Calc. DDVP content of std soln to nearest 0.01% by wt.

After detg optimum instrument parameters for compensation technic, scan std soln over 9.9–10.7 μm (1010–935 $cm^{-1}$) region with ref. cell contg compensating solv. in ref. beam of spectrophtr. Scan sample against compensating solv. in same manner. Always use same cell in ref. beam.

### D. Calculations

From differential spectra, det. $A$ of DDVP at 10.2 μm (980 $cm^{-1}$) of std, $A'$, and sample, $A$, measured from baseline drawn between minima near 10.0 and 10.6 μm. Calc. DDVP as follows:

$$\% \text{ DDVP by wt} = \% \text{ DDVP in std} \times A/A'$$

Ref.: JAOAC **49**, 251(1966).

CAS-62-73-7 (2,2-dichlorovinyl dimethyl phosphate)

## Diazinon in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1982**

**Final Action 1988**

*See* **971.08**.

## 982.06 Diazinon in Microencapsulated Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1982**

### A. Principle

Sample is ground in tissue grinder and extd with $CH_3CN$, dibutyl phthalate is added as internal std, and diazinon is detd by GC with flame ionization detection.

### B. Apparatus and Reagents

(**a**) *Gas chromatograph.*—Equipped with flame ionization detector (Perkin-Elmer 900, or equiv.).

(**b**) *GC column.*—6 ft × 1/4 in. od, 2 mm id, glass, packed with 3% OV-17 on 80–100 mesh Supelcoport. Operating conditions: injection port 200°; column 190° (isothermal); detector 250°; He flow 35 mL/min; H flow optimum for instrument detector; chart speed 0.2 in./min; sample size: 1 μL.

(**c**) *Tissue grinder.*—40 mL capacity (Corning Glass Works, No. 441969 or 7726-L), or equiv.

(**d**) *Internal std soln.*—Accurately weigh ca 2.0 g dibutyl phthalate into 100 mL vol. flask. Dissolve in and dil. to vol. with $CH_3CN$.

(**e**) *Diazinon std soln.*—Accurately weigh ca 0.2 g diazinon ($W'$) into 50 mL vol. flask. Pipet in 10.0 mL internal std soln, dil. to vol. with $CH_3CN$, and mix well.

### C. Determination

Mix sample thoroly. With medicine dropper or disposable pipet, transfer ca 2 g sample to Al weighing dish and weigh accurately ($W$). Transfer to tissue grinder, add 30 mL $CH_3CN$, and grind 3 min. When sample is thoroly ground, quant. transfer to 100 mL vol. flask, wash grinder with $CH_3CN$, and add washings to vol. flask. Pipet in 20.0 mL internal std soln and dil. to vol. with $CH_3CN$. Using 10 $\mu$L syringe, make duplicate 1 $\mu$L injections of sample and std solns.

Measure peak hts of first peak, diazinon, and second peak, dibutyl phthalate, in sample and std soln and det. ratios of diazinon to dibutylphthalate peaks for each.

$$\text{Diazinon, wt\%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = peak ht ratios for sample and std solns, resp.; $W$ and $W'$ = g sample and diazinon std resp.; $P$ = % purity of diazinon std.

Ref.: JAOAC **65,** 115(1982).

CAS-333-41-5 (diazinon)

## 980.10 Disulfoton in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1980**
**Final Action 1981**

(*Caution:* See safety notes on pesticides.)

### A. Principle

Concn disulfoton in sample is detd by gas-liq. chromatgy, employing di-*n*-butyl phthalate as internal std and flame ionization detection. Response is compared to that of std of known purity.

### B. Apparatus and Reagents

(**a**) *Gas chromatograph.*—With on-column injection system and flame ionization detector. Conditions given are for use with Varian Model 3700. Other instruments may require changing operating parameters to obtain good peak response and proper resolution. Temps (°)—column 190, injection port 220, detector base 250; gas flow rates (mL/min)—carrier 30, $H_2$ 30, air 300; attenuation—$4 \times 10^{-10}$; sample size—3.0 $\mu$L; retention time (min)—disulfoton peak 5.5, internal std peak 9.25. Parameters ensure complete peak sepn with app. described. Adjust conditions to obtain peaks ca 60–80% full scale on chart at retention times given.

(**b**) *Column.*—1.8 m (6′) × 0.25″ (od) × 2 mm id glass column packed with 10% silicone SE-30 on 80/100 Chromosorb W-HP (available from Alltech Associates, 2051 Waukegan Rd, Deerfield, IL 60015). Condition new columns 16 hr at 275° before use.

(**c**) *Syringe.*—Precision syringe, 10 $\mu$L.

(**d**) *Di-N-butyl phthalate internal std soln.*—1.8%. Dil. appropriate amt internal std (Eastman Kodak, or equiv.) with acetone. Adjust concn, if needed, to obtain peaks nearly equiv. to disulfoton peak. Keep refrigerated in suitable container to avoid evapn. Internal std soln must contain no peaks eluting near disulfoton peak on chromatogram.

(**e**) *Disulfoton std solns.*—Approx. 3, 4, and 5 mg/mL. Weigh to nearest 0.1 mg ca 75, 100, and 125 mg disulfoton std of known purity (available as Di-Syston from Mobay Corp.) into pre-weighed 25 mL flasks. Pipet into each flask 5.0 mL internal std soln and dil. to vol. with acetone. Label these solns A, B, & C, resp. Soln B is working std. Use stds A and C for linearity check.

### C. Linearity Check

Perform when new working std is prepd, or when new column is installed, to check for weighing errors and instrument difficulties. Inject triplicate aliquots std solns A, B, and C and det. "response ratio" for each by dividing peak ht (or area) disulfoton peak by internal std peak. Obtain "response factor" by dividing av. response ratio by disulfoton content. Factors should agree within 2%.

### D. Preparation of Sample

(**a**) *Liquid samples and technical material.*—Accurately weigh amt sample contg ca 100 mg active ingredient into 25 mL vol. flask. Pipet in 5.0 mL internal std soln, dil. to vol. with acetone, and mix.

(**b**) *Granular formulations.*—Accurately weigh amt sample contg ca 100 mg active ingredient into 2 oz (60 mL) bottle. Pipet in 5.0 mL internal std soln and 20.0 mL acetone. Cover and shake 30 min. Let settle or centrf. before removing aliquot for injection.

### E. Determination

Inject 2 aliquots working std soln B. Response ratios should agree within 2%. If they do not, some difficulty is present. Correct before continuing.

If std response is satisfactory, make duplicate injections of each of the following:

Std B, Sample 1, Sample 2, Std B, Sample 3, Sample 4, Std B, etc.

For each injection

$$R = \text{response ratio} = \frac{\text{ht (area) disulfoton peak}}{\text{ht (area) internal std peak}}$$

$$\text{\% Disulfoton} = (R/R')\,(W'/W) \times P$$

where $R$ = av. response ratio for sample soln, $R'$ = av. response ratio for ref. std B, $W$ = mg sample, $W'$ = mg std, and $P$ = purity (%) of std.

For $R'$ use av. response ratios of duplicate std injections just before and after peaks. Average these ratios to det. $R'$.

Ref.: JAOAC **63,** 869(1980).

CAS-298-04-4 (disulfoton)

## 979.04 Ethion in Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1979**
**Final Action 1980**

(Applicable to dry and liquid formulations contg ethion as only active ingredient.)

### A. Apparatus

(**a**) *Liquid chromatograph.*—Waters Associates with Model 6000A pump, or equiv., with 254 nm UV detector (Waters Associates, Inc). Typical operating conditions: eluant flow rate 1 mL/min (ca 1100 psi), chart speed 0.25 in./min, detector sensitivity 0.2 *A* unit full scale, ambient temp, injection vol. 10 $\mu$L. Adjust operating conditions to elute ethion peak in 6 ± 2 min. Column condition and $H_2O$ content of MeOH eluant can change retention times. Ethion peak must be sepd completely from internal std peak which normally elutes in ca 7 min (Waters $C_{18}$ column).

(**b**) *Liquid chromatographic column.*—Either (*1*) Waters $\mu$Bondapak $C_{18}$, 300 × 3.9 mm id; or (*2*) DuPont ODS Permaphase, 0.5 m × 2.1 mm id.

### *B. Reagents*

(**a**) *Eluant*.—Either (*1*) *degassed MeOH-$H_2O$* (90 + 10), UV cutoff <230 nm, or (*2*) *degassed acetonitrile-$H_2O$* (40 + 60), UV cutoff <230 nm.

(**b**) *Light mineral oil*.—USP, viscosity 38.1 centistokes at 37.8°.

(**c**) *Internal std soln*.—(*1*) *For Waters column*.—Accurately weigh ca 0.24 g pentachloronitrobenzene (PCNB), ref. grade, with no interfering peaks on LC, into 200 mL vol. flask. Dil. to vol. with MeOH and mix. (*2*) *For DuPont column*.—Using $CH_3CN$ as solv., vary amt PCNB in internal std to give peak ht approx. same as ethion peak.

(**d**) *Ethion std solns*.—(*1*) *For Waters column*.—*Stock soln*.—Accurately weigh amt of std equiv. to 250 mg ethion, 95+% pure (available from Chemical and Biological Investigations, Environmental Protection Agency, Beltsville, MD 20705) into 25 mL vol. flask, dil. to vol. with MeOH, and mix. *Working soln*.—Pipet 10 mL stock soln into 50 mL vol flask, pipet 10 mL internal std soln, (**c**)(*1*), into flask, dil. to vol. with MeOH, and mix. Prep. std and samples daily. (*2*) *For DuPont column*.—Prep. as above, using $CH_3CN$ instead of MeOH. (*3*) *For oil formulations*.—Pipet 10 mL 1% stock soln (*1*) or (*2*) into 50 mL vol. flask contg ca same wt of light mineral oil as sample. Add 20 mL MeOH (or $CH_3CN$) and proceed as in **979.04C(c)** beginning with "Stopper and agitate . . ."

### *C. Preparation of Sample*

(**a**) *Dry powder*.—Accurately weigh sample contg ca 100 mg ethion into 250 mL g-s flask. Pipet in 40 mL MeOH (or $CH_3CN$) and 10 mL internal std soln. Shake 30 min on mech. shaker and centrf. to sep. phases.

(**b**) *Liquid concentrates*.—Prep. sample as in **979.04B(d)**.

(**c**) *Oil formulations*.—Accurately weigh sample contg ca 100 mg ethion into 50 mL vol. flask. Add 30 mL MeOH (or $CH_3CN$). Stopper and agitate vigorously 1 min, with side to side action, keeping mixt. in main body of flask. Pipet in 4 mL $H_2O$ and repeat vigorous mixing 1 min. Dil. to approx. vol. with MeOH (or $CH_3CN$). Cool to ambient temp and dil. to vol. Mix thoroly by inverting 10 times and swirling vigorously each time. Centrf. to sep. phases.

### *D. Determination*

Use high-pressure liq. syringe or sample injection loop to inject 10 μL portions of std until 2 peak ht ratios agree within ±1%. Alternately inject two 10 μL portions each of sample and std solns. Measure peak hts and calc. av. peak ht ratios for both std and sample. Adjust attenuation or amt injected for convenient size peaks (60–80% full scale). Measure peak hts from baseline between ethion and internal std peaks.

$$\%\ \text{Ethion} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. peak ht ratios for sample and std, resp.; $W'$ = mg ethion in working std soln (ca 100 mg); $W$ = mg sample in final diln; and $P$ = % purity of std.

Refs.: JAOAC **62,** 11(1979); **63,** 302(1980).

CAS-563-12-2 (ethion)

## 985.07 Fenitrothion Technical and Pesticide Formulations

### Gas Chromatographic Method

**First Action 1985**

### *A. Principle*

Samples of fenitrothion tech. and formulations are dissolved in $CHCl_3$ with fluoranthene added as internal std. Fenitrothion content is detd by gas chromatgy with flame ionization detection.

### *B. Apparatus and Reagents*

(**a**) *Gas chromatograph*.—Suitable for on-column injection and equipped with flame ionization detector.

(**b**) *Gas chromatographic column*.—2 mm id × 1.83 m glass column packed with 3.0% PPE-6R (polyphenylether, Alltech Associates, Inc., 2051 Waukegan Rd, Deerfield, IL 60015) on 100–120 mesh Chromosorb W-HP. Operating conditions: temps: injector, 200°; detector, 250°; column, 195°; N carrier gas flow ca 30 mL/min.

Approximate retention times for fenitrothion and internal std are 16 and 26 min, resp.

(**c**) *Internal std soln*.—Accurately weigh ca 1.5 g fluoranthene into 500 mL vol. flask. dil. to vol. with $CHCl_3$, and mix.

(**d**) *Fenitrothion std soln*.—Accurately weigh amt of std fenitrothion (Sumitomo Chemical Co., Ltd, Plant Protection Div. International, 15–5 Chome, Kitahama, Higashi-Ku, Osaka, Japan) contg ca 200 mg active ingredient into 50 mL screw-cap bottle. Add by pipet 25.0 mL internal std soln and mix to dissolve fenitrothion.

### *C. Preparation of Chromatographic Column*

Clean glass column by passing $H_2SO_4$ thru column, and rinse with $H_2O$. Draw ca 50 mL acetone thru column followed by 50 mL MeOH. Pass N thru column until it is dry. Treat column with 5% soln of dichlorodimethylsilane in toluene; rinse with toluene followed by MeOH. Pass N thru column to dry.

Attach 7.6 cm funnel to exit end of column. While tapping column with glass rod fitted with short length of heavy rubber tubing, add prepd packing in small quantity until exit end of column is filled to ca 0.5 cm from end of tube. Move funnel to entrance of column. Insert pledget of silane-treated glass wool in exit end of column, and attach a source of moderate vac. to exit end. Continue to add packing slowly with vigorous tapping until tube is filled to ca 0.5 cm from entrance end. Insert pledget of glass wool in entrance end; compress glass wool only enough to hold packing in place.

Condition column overnight at 230°. This step should be conducted with exit end of column disconnected from detector but with carrier gas flowing at recommended rate.

### *D. Preparation of Standard and Sample Solutions*

Accurately weigh samples of fenitrothion tech., emulsifiable conc., and $H_2O$-dispersible powder, each contg ca 200 mg active ingredient, into sep. 50 mL screw-cap bottles. To each bottle add by pipet 25.0 mL internal std soln and shake 30 s. Filter or centrf. $H_2O$-dispersible powder to remove particulates.

### *E. Determination*

Inject 2 μL portions of std soln until response ratios (area or peak ht) of fenitrothion to internal std agree ±2%. Make duplicate injections of std soln, followed by duplicate injections of sample solns (see Note 1). Recalibrate after not more than 4 injections of sample solns. (Note 1: To avoid interference from late-emerging impurity (retention time, ca 45 min), subsequent samples must be injected not earlier than 7 min after elution of internal std. Thus, total analysis time for each sample is ca 35 min.)

### *F. Calculation*

For each injection, response ratio ($R$) = area (or ht) of fenitrothion peak/area (or ht) of internal std peak.

$$\text{Fenitrothion, wt}\ \% = (R/R') \times (W'/W) \times P$$

where $R'$ and $R$ = av. response ratio for std and sample solns, resp.; $W'$ and $W$ = wt (mg) of fenitrothion std and sample, resp.; and $P$ = purity (%) of fenitrothion std.

Ref.: JAOAC **68,** 576(1985).

CAS-122-14-5 (fenitrothion).

## 989.02 Fenitrothion Technical and Pesticide Formulations

### Alternative Gas Chromatographic Method

### First Action 1989

(Applicable to fenitrothion tech. and its emulsifiable conc. and $H_2O$-dispersible powder formulations)

Method Performance:

Technical:

$s_r = 0.50$; $s_R = 0.51$; $RSD_r = 0.53\%$; $RSD_R = 0.54\%$

Wettable powder:

$s_r = 0.19$; $s_R = 0.38$; $RSD_r = 0.50\%$; $RSD_R = 1.00\%$

Emulsifiable conc.:

$s_r = 0.25$; $s_R = 0.81$; $RSD_r = 0.48\%$; $RSD_R = 1.56\%$

#### *A. Principle*

Samples of fenitrothion tech. and formulations are dissolved in $CHCl_3$ with dibutyl sebacate is added as internal std. Fenitrothion content is detd by gas chromatgy with flame ionization detection using peak area measurements. Method is alternative to **985.07** which uses PPE-6R column packing.

#### *B. Apparatus and Reagents*

(**a**) *Gas chromatograph.*—Suitable for on-column injection and equipped with flame ionization detector.

(**b**) *Chromatographic column.*—2 mm id × 1.83 m glass column packed with 7.5% OV-210 (Alltech Associates, Inc., 2051 Waukegan Rd, Deerfield, IL 60015) on 100–120 mesh Chromosorb W-HP. Operating conditions: temps—injector, 190°; detector, 250°; column, 165°. N carrier gas flow ca 40 mL/min.

Approx. retention times for fenitrothion and internal std are 16.9 and 19.5 min, resp.

(**c**) *Internal std soln.*—Accurately weigh ca 3.0 g dibutyl sebacate into 500 mL vol. flask, dil. to vol. with $CHCl_3$, and mix.

(**d**) *Fenitrothion std soln.*—Accurately weigh amt std fenitrothion (Sumitomo Chemical Co., Ltd, Osaka, Japan) contg ca 200 mg active ingredient into 50 mL screw-cap bottle. Add by pipet 25.0 mL internal std soln and mix to dissolve fenitrothion.

#### *C. Preparation of Sample Solutions*

(Acute oral $LD_{50}$ of fenitrothion tech. for rats is 250–500 mg/kg.) Accurately weigh samples of fenitrothion tech., emulsifiable conc., and $H_2O$-dispersible powder, each contg ca 200 mg active ingredient, into sep. 50 mL screw-cap bottles. To each bottle add by pipet 25.0 mL internal std soln and shake 30 s. Filter or centrf. $H_2O$-dispersible powder to remove particulates.

#### *D. Determination*

Inject 2 μL portions of std soln until response ratios (peak area) of fenitrothion to internal std agree ±2%. Verify that small peak just preceding that of fenitrothion [due to isomeric impurity *O,O*-dimethyl-*O*-(4-methyl-3-nitrophenyl) phosphorothionate] is effectively sepd from fenitrothion peak. Make duplicate injections of std soln followed by duplicate injections of sample solns. At end of each run raise column temp. to 230° at 20°/min and hold 5 min to allow rapid elution of late-eluting peaks before next detn. Recalibrate after no more than 4 injections of sample solns.

#### *E. Calculation*

For each injection, response ratio ($R$) = area of fenitrothion peak/area of internal std peak.

$$\text{Fenitrothion, wt \%} = (R/R') \times (W'/W) \times P$$

where $R'$ and $R$ = av. response ratio for std and sample solns, resp.; $W'$ and $W$ = wt (mg) of fenitrothion std and sample, resp.; and $P$ = purity (%) of fenitrothion std.

Ref.: JAOAC **71,** 991(1988).

CAS-122-14-5 (fenitrothion).

## 986.07 Fensulfothion in Pesticide Formulations

### Gas Chromatographic Method

### First Action 1986

(Method is suitable for tech. and liq. formulations of fensulfothion.)

#### *A. Principle*

Sample is dissolved in $CH_2Cl_2$ contg 4-chlorophenyl sulfoxide as internal std, and fensulfothion is detd by gas chromatgy.

#### *B. Apparatus*

(**a**) *Gas chromatograph.*—Equipped with flame ionization detector (FID). Temps—column 225°, injection port 250°, detector 250°; carrier gas 30–40 mL/min (either He or N); air and H flows as recommended for FID; sample size 2.0 μL; retention times (min)—internal std 4.0, fensulfothion 5.5. Adjust parameters to cause fensulfothion to elute in 5–6 min, but do not use column temp. >240°. If internal std and fensulfothion peaks are not completely sepd, repack column.

(**b**) *Column.*—0.9 m (3 ft) or 1 m × 2 mm (id) glass column packed with 5% OV-330 on 80–100 mesh Chromosorb WHP (Supelco). Condition newly packed columns 8–16 h at 240° before use.

#### *C. Reagents*

(**a**) *4-Chlorophenyl sulfoxide.*—Aldrich Chemical Co., Cat. No. 12,104–5, or equiv. that contains no impurities eluting at retention time of fensulfothion.

(**b**) *Internal std soln.*—Weigh 1.0 g 4-chlorophenyl sulfoxide, dissolve in 1 L $CH_2Cl_2$, and mix well. Keep tightly stoppered.

(**c**) *Fensulfothion reference std soln.*—Accurately weigh amt of ref. std (Mobay Corp.) contg ca 100 mg fensulfothion into ca 100 mL glass bottle. Add by pipet 50.0 mL internal std soln. Stopper and mix well.

#### *D. Preparation of Sample*

Accurately weigh sample contg ca 100 mg fensulfothion into glass bottle (ca 100 mL). Pipet in 50.0 mL internal std soln. Stopper and mix well.

#### *E. Determination*

Make repetitive 2 μL injections of fensulfothion ref. std soln until response is stable and ratios of fensulfothion peak area to internal std peak area for successive injections agree within 1% of their mean. Peak ht may be substituted for peak area.

Make duplicate 2 μL injections of each sample. Response ratios ($R$) for fensulfothion internal std for 2 sample injections must agree ±1% of their mean. If not, repeat detn, starting with std injections.

After every 4–6 sample injections and after last sample injection, make 2 injections of fensulfothion std soln. Av. std soln ratios preceding and following sample must be ±1.0% of mean; otherwise, repeat series of injections.

### F. Calculation

Calc. ratios for each injection. Average 2 sample ratios and 4 std ratios (std injections immediately before and after sample injections).

$$\text{Fensulfothion, \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. sample and std ratios (fensulfothion peak/internal std peak), resp.; $W$ and $W'$ = mg sample and std, resp.; and $P$ = % purity of fensulfothion std.

Ref.: JAOAC **69,** 488(1986).

CAS-115-90-2 (fensulfothion)

## 983.09 Fensulfothion in Pesticide Formulations
**Liquid Chromatographic Method**
**First Action 1983**
**Final Action 1984**

### A. Principle

Sample is dissolved in or extd with MeOH, benzophenone is added as internal std, and fensulfothion is detd by liq. chromatgy and UV detection at 230 nm.

### B. Apparatus

(**a**) *Liquid chromatograph.*—Able to generate >1000 psi and equipped with detector able to measure $A$ at 230 nm. Typical operating conditions: temp., ambient; flow rate, 0.8 mL/min; wavelength, 230 nm; chart speed, 2 mm/min; sample size, 10 μL. Conditions may be varied to accommodate instrument and column differences.

(**b**) *Column.*—Whatman Partisil PXS 10/25 ODS-2, stainless steel 25 cm × 4.6 mm id (Whatman, Inc.) or equiv.

(**c**) *Filter.*—10 μm Teflon, or similar type.

### C. Reagents

(**a**) *Methanol.*—Distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).

(**b**) *Phosphoric acid.*—85% (Fisher Scientific Co.).

(**c**) *Internal std soln.*—0.25 mg benzophenone/mL. Accurately weigh ca 250 mg benzophenone (Eastman Kodak Co.) into small flask. Transfer to 1 L vol. flask and dil. to vol. with same MeOH to be used in mobile phase. Concn may be varied so that when std soln (**d**) is injected, peak ht of benzophenone matches peak ht of fensulfothion within 20%.

(**d**) *Std soln.*—0.3 mg fensulfothion/mL, within optimum linearity range. Accurately weigh ca 150 mg fensulfothion (Mobay Corp.) into 125 mL flask. Pipet in 100 mL MeOH, shake to mix. Pipet 10 mL aliquot of soln into 125 mL flask with screw cap, add exactly 40 mL internal std soln, and shake to mix. Prep. fresh std daily. Keep ref. std in freezer.

(**e**) *Mobile phase.*—MeOH–$H_2O$ (80 + 20) buffered to 0.0025M with $H_3PO_4$. Mix 800 mL MeOH + 200 mL $H_2O$ + 156 μL $H_3PO_4$, and degas. If using column other than ODS-2, adjust MeOH–$H_2O$ ratio as necessary.

### D. Preparation of Sample

(**a**) *Spray concentrate.*—Accurately weigh sample contg ca 150 mg fensulfothion into 125 mL flask. Pipet in 100 mL MeOH and shake to mix. Pipet 10 mL aliquot into 125 mL flask with screw cap, add exactly 40 mL internal std soln, and shake to mix.

(**b**) *Granular formulations.*—Pour sample into 400 mL beaker and thoroly mix, turning granules over ≥10 strokes with wide spatula. Take weighed amt from beaker before sample is poured back into sample container. Accurately weigh sample contg ca 150 mg fensulfothion into 125 mL flask. Pipet in 100 mL MeOH and place on mech. shaker 15–30 min. Filter thru 10 μm Teflon or similar type filter. Place 10 mL aliquot of filtrate in 125 mL flask with screw cap, add exactly 40 mL internal std soln, and shake to mix.

### E. Determination

Adjust liq. chromatgc operating parameters to elute fensulfothion in 4–7 min. Maintain all parameters const thruout analysis. Benzophenone will elute 2–4 min after fensulfothion.

Adjust injection size and attenuation to give 60–80% on-scale peaks. Make repetitive injections of std until response is stable, and ratios of fensulfothion peak ht to benzophenone peak ht for successive injections vary ≤1%. Then make duplicate injections of sample followed by injection of std. Calc. av. ratio of fensulfothion peak ht to benzophenone peak ht for each set of duplicate injections and calc. % fensulfothion.

$$\text{Fensulfothion, \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. peak ht ratios for sample and std, resp.; $W$ = mg sample; $W'$ = mg fensulfothion anal. std; and $P$ = % purity of fensulfothion anal. std. Integrator area ratios may be substituted for peak ht ratios.

Ref.: JAOAC **66,** 801(1983).

CAS-115-90-2 (fensulfothion)

## 974.03 Formothion in Pesticide Formulations
**Gas Chromatographic Method**
**First Action 1974**
**Final Action 1978**
***CIPAC-AOAC Method***

### A. Reagents

(**a**) *Solvent I.*—Toluene contg 2% $Ac_2O$.

(**b**) *Solvent II.*—Hexane-acetone (2 + 1) plus 2% $Ac_2O$.

(**c**) *Internal std soln.*—Prep. soln contg ca 100 mg, accurately weighed, of ethion/mL solv. I. Ethion must be >95% pure and contain no impurities interfering at formothion retention time.

(**d**) *Reference std soln.*—Accurately weigh ca 500 mg Formothion Ref. Std (Sandoz Ltd, Agro Division, Development, CH4002 Basel, Switzerland) into 50 mL vol. flask, add 5.0 mL internal std soln, and dil. to vol. with solv. I.

### B. Apparatus

(**a**) *Gas chromatograph.*—(Varian Model 1520, or equiv.) With flame photometric detector (Tracor Instruments, Inc., FPD 100AT, or equiv.), automatic injector (Hewlett-Packard 7600 A, or equiv.), integrator, and effluent splitter at column end with ratio 1:100–1:1000 in favor of outlet. Use glass spiral column, 1.0 m × 3.6 mm id, packed with 3% OV 225 on 80–100 mesh Chromosorb W-HP. Operating conditions: temps (°)—oven 210, injector and detector 220; N carrier gas 60 mL/min; no. theoretical plates for ethion is ca 2000. Alternatively, flame ionization may be used. Conditions are same, except effluent splitter is not necessary.

(**b**) *Bottles.*—50 mL with Mininert valve, or equiv. inert system for closure (Pierce Chemical Co.).

### C. Determination

Accurately weigh well mixed sample contg ca 500 mg formothion into bottle, (**b**). Add 5.0 mL internal std soln, (**c**), and dil. to 50 mL with solv. I. Close tightly and shake. Transfer 6 μL soln to vial contg 1 mL solv. II. Seal vial with inert valve system. (For automatic injections with Hewlett-Packard sampler, dil. in Al foil-sealed vials and use Teflon rubber laminated disks as septa.) Keep tightly closed. Inject 1.0 μL dild mixt. into column, bypassing solv. around detector by using splitter to avoid contamination and deterioration. Det. appropriate time for splitting by test chromatogram. Compds may be identified by retention times relative to ethion as 1.00 (ca 4.4 min): formothion 0.50, dimethoate (by-product) 0.36.

Inject 1 μL aliquots of reference std soln, (**d**), dild as above, until ht or area ratio of formothion to ethion varies <2% for successive injections. Precede and follow each sample by reference std soln and make 3 sep. detns with all peak area ratios of reference std solns within ±2% of first accepted values.

$$\% \text{ Formothion} = W' \times H \times f \times P/W \times H'$$

where $W$ and $W'$ = mg sample and internal std, resp.; $H$ and $H'$ = peak hts or areas of formothion and internal std, resp.; $P$ = % formothion in reference compd;

$$f = \text{correction factor} = w \times h'/w' \times h$$

where $w$ and $w'$ = mg formothion ref. std and internal std, resp., and $h$ and $h'$ = peak hts or areas of formothion and internal std, resp.

Ref.: JAOAC **57,** 771(1974).

CAS-2540-82-1 (formothion)

## 983.10 Glyphosate (Technical) and Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1983**

**Final Action 1984**

### A. Principle

Samples are dissolved in phosphate buffer mobile phase and injected directly into ion exchange chromatgc system using fixed vol. loop. Peak area response as measured by UV detector is quantitated by external std technic.

### B. Apparatus and Reagents

(**a**) *Liquid chromatograph.*—Able to generate over 1000 psi and measure $A$ at 195 nm.

(**b**) *Loop injector.*—Rheodyne Model 7125 syringe loading, or equiv.

(**c**) *Strip chart recorder.*—Houston Instrument 10 mV full scale (Industrial Scientific, Inc., PO Box 60002, Houston, TX 77060), or equiv.

(**d**) *Electronic integrator.*—Capable of handling detector output.

(**e**) *Chromatographic column.*—25 cm × 4.6 mm id, $^1/_4$ in. od, strong anion exchange, e.g., Partisil 10 SAX (available from Whatman, Inc., 9 Bridewell Pl, Clifton, NJ 07014).

(**f**) *Methanol.*—LC grade (available from Burdick & Jackson Laboratories, Inc.).

(**g**) *Water.*—LC grade (available from Burdick & Jackson Laboratories, Inc.). Use thruout.

(**h**) *Potassium dihydrogen phosphate.*—Primary std grade (available from Fisher Scientific Co.).

(**i**) *Phosphoric acid.*—85%, reagent grade (available from Fisher Scientific Co.).

(**j**) *Glyphosate std.*—Monsanto Agricultural Products Co., PO Box 174, Luling, LA 70070.

(**k**) *Mobile phase.*—Dissolve 0.8437 g $KH_2PO_4$ in 960 mL $H_2O$. Add 40 mL MeOH and mix well. Using pH meter buffered at pH 2.0, adjust pH to 1.9 with 85% $H_3PO_4$. Filter and degas before use.

### C. Preparation of Standard

Accurately weigh ca 400 ± 10 mg glyphosate std (dried 2 h at 105°) into 100 mL vol. flask. Dil. to vol. with mobile phase and stir to dissolve (30 min may be required to dissolve std). Soln is stable ≥1 week.

### D. Preparation of Sample

Accurately weigh sample contg ca 400 mg glyphosate into 100 mL vol. flask contg ca 50 mL mobile phase. Dil to vol. with mobile phase and mix well.

### E. Determination

Adjust operating parameters so that glyphosate elutes at 2.5–4.0 min. Maintain all parameters consistent thruout std and sample analysis. Typical values are as follows: flow rate 2.3 mL/min; pressure ca 1200 psi; chart speed 0.5 cm/min; $A$ range 0.2 AUFS; column temp. ambient; injector vol. 50 μL.

Let mobile phase flow thru system until steady baseline is obtained; 1 h may be required for new column. When new columns are installed or instrument has not been used for 24 h, make at least 6 rapid injections of std soln; then inject std soln until peak areas for successive injections agree ±1%. Then inject sample soln until peak areas for successive injections agree ±1%. Let all components from samples elute (ca 10–12 min) before making next injection.

### F. Calculation

Average peak areas from 2 successive injections that agreed ±1% from both std and sample solns.

$$\% \text{ Glyphosate} = (R/R') \times (W'/W) \times P$$

where $R$ = av. peak area of sample; $R'$ = av. peak area of std; $W$ = mg sample; $W'$ = mg std; and $P$ = % purity of std. To convert % glyphosate to isopropylamine salt, multiply by 1.3496.

Ref.: JAOAC **66,** 1214(1983).

CAS-1071-83-6 (glyphosate)

## 987.01 Isofenphos Technical and in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1987**

***AOAC-CIPAC Method***

(Method is suitable for tech. isofenphos and formulations with isofenphos as only active ingredient.)

### A. Principle

Isofenphos is extd with MeOH contg diisobutyl phthalate as internal std, analyzed by gas chromatgy with flame ionization or thermal conductivity detection, and quantitated by comparing peak areas (or hts) of sample and internal std.

### B. Apparatus and Reagents

(**a**) *Gas chromatograph.*—Equipped with thermal conductivity (TC) detector or flame ionization detector or flame ionization detector (FID). *Operating conditions:* Temps—inlet 250°, column 190°, detector 250°; carrier gas 20–30 mL/min (He for TC detector, either He or N for FID); bridge current 180 mA or as recommended for TC detector; air and H flows as recommended for FID; chart speed 1.0 cm/min; range 1 (10 for FID); attenuation (× 2) (× 8 for FID). Retention times:

internal std ca 1.7 min, isofenphos ca 3.5 min. Let chromatograph stabilize (flat baseline) before beginning injections and allow ca 5 min run time for each injection.

(**b**) *Chromatographic column*.—0.5 m × 2 mm id stainless steel or glass column packed with 10% SP-2100 on 80–100 mesh Supelcoport (Supelco Inc., No. 1-2140) or equiv. support.

(**c**) *Diisobutyl phthalate*.—Kodak Laboratory Chemicals (Eastman Kodak) No. 6830 or equiv. that contains no impurities eluting at retention time of isofenphos.

(**d**) *Isofenphos reference std*.—Mobay Corp.

(**e**) *Internal std soln*.—Pipet 10 mL diisobutyl phthalate into 1 L vol. flask, dil. with MeOH, and mix well. If necessary, adjust concn so that peak hts of isofenphos and internal std are within 20% (± 10%).

(**f**) *Filters*.—0.45 μm porosity (Gelman Acrodisc-CR, Gelman Scientific, Inc.) or equiv.

### C. Preparation of Standard

Accurately weigh ca 250 mg ref. std isofenphos into glass bottle (ca 50 mL for TC detector or 150 mL for FID). Pipet in 15 mL internal std soln. For FID, add addnl 100 mL MeOH. Cap securely and mix well.

### D. Preparation of Sample

Accurately weigh sample contg ca 250 mg isofenphos into glass bottle (ca 50 mL for TC detector or 150 mL for FID). Pipet in 15 mL internal std soln. For FID, add addnl 100 mL MeOH. Cap securely and mix well to ext.

### E. Determination

Adjust operating parameters so that isofenphos elutes in 3.2–3.7 min. Adjust injection vol. and attenuation to give largest on-scale peaks. Make successive 2 μL injections of std soln until response is stable and response ratios ($R$) of isofenphos peak area (ht) to internal std peak area (ht) agree within ±1% of their mean.

Make duplicate 2 μL injections of each sample. Ratios of isofenphos to internal std peak area (ht) must agree within ±1% of their mean. If not, repeat detn, starting with std injections.

After every 4–6 sample injections, and/or after last sample injection, make 2 successive injections of ref. std soln to bracket samples. Av. std ratios preceding and following bracketed samples must lie within ±1% of mean; otherwise, repeat series of injections.

### F. Calculation

$$\text{Isofenphos, wt } \% = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and ref. std solns, resp.; $W'$ and $W$ = wt (mg) of isofenphos std and sample solns, resp.; and $P$ = purity of isofenphos std (%).

Ref.: JAOAC **70,** 55(1987).

CAS-25311-71-1 (isofenphos)

## 979.05 Malathion in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1979**
**Final Action 1980**

### A. Apparatus

(**a**) *Gas chromatograph*.—With glass column, on-column injection system, flame ionization detector, and electrometer with sensitivity of $\geq 10^{-11}$ amp driving 1 mv recorder. Drift should be <1%/hr. Totally solid state amplifier with FET input is recommended. Electronic digital integrator or computer calcd area measurements must be used. Integrator should have independent controls for selection of up and down slope sensitivities so that start and stop integration points can be selected. Automated sample injection system contributes significantly to precision. Hewlett-Packard Model 7600 is suitable when equipped as described. Equiv. instrumentation may be used but may require modification of operating conditions to obtain good peak shape, adequate resolution, and appropriate retention times.

Typical conditions for Hewlett-Packard Model 7600 (instrument may have to be adjusted to give complete resolution of well shaped peaks): Cycle timers (min): analysis and stop integrate, 16; range, $10^3$; temps (°): oven 180, injection port 200, flame detector 300; gas flow rotameters (mL/min): H 35, air 425, He carrier gas 30; integrator settings (adjusted so that deflections on slope meter do not exceed ±50% before injection): noise suppression max., slope sensitivity up and down 0.1, BL reset delay 0.15, area threshold 1000; retention times (min): malathion 10, internal std 6, min. time between malathion and internal std 3.5.

(**b**) *Column*.—Borosilicate glass tube 1.22 m (6′) × 4 mm id, 6 mm od, bent to fit chromatograph and packed with 5% SP-2401 or OV-210 on Supelcoport (100–120 mesh). Can be purchased as prepd packing from Supelco, Inc. (specify "Pesticide Grade"); Alltech Associates, 202 Campus Dr, Arlington Heights, IL 60006; and Applied Science. Use exclusively for malathion analysis.

(**c**) *Glass wool*.—Silane treated (No. 14502, Applied Science).

(**d**) *Syringes*.—10 μL, Series 700, Hamilton Co.

(**e**) *HI-EFF Fluidizer*.—Applied Science.

### B. Reagents

(**a**) *Internal std soln*.—1.2% *m*-Diphenoxybenzene in $CHCl_3$. Must not contain any impurities which elute at or near malathion peak. Bring soln to consistent temp. above ambient (e.g. 25°) before taking aliquots.

(**b**) *Malathion std solns*.—Accurately weigh ca 170, 200, and 230 mg malathion std (anal. grade, available from American Cyanamid Co.) into sep. preweighed 25 mL vol. flasks. Add by pipet 5 mL internal std soln and dil. to vol. with $CHCl_3$. Label A, B, and C. Soln B is working std soln for detn; solns A and C are used for linearity check and to guard against weighing error in prepn of working std soln. Solns are stable ca 4 weeks if kept tightly sealed in refrigerator. Warm to room temp. before use. Soln B can be prepd independently of solns A and C, if conditions of linearity check are met.

### C. Preparation and Conditioning of Column

Weigh 6.25 g of trifluoropropylsilicone (SP-2401 or OV-210) in 250 mL beaker and dissolve in 125 mL EtOAc. Stir to obtain vortex and add 25 g solid support (Gas-Chrom Q or Supelcoport, 100–120 mesh) with continued agitation. Filter slurry thru Whatman No. 1 paper, or equiv., on buchner, using gentle vac. to minimize evapn of solv. Continue filtration until drop rate is ca 1/sec. Transfer packing to HI-EFF Fluidizer, connect source of N thru pressure reducer to base, and place fluidizer on controlled temp. hot plate set for 75°. Continue gas flow until solv. vapors can no longer be detected by odor, taking care that packing is not blown out top of fluidizer.

To pack column, attach 75 mm funnel to exit end of prebent glass tube. Tap tube with pencil or small wooden rod, and add prepd packing in small amts until exit end is filled to ca 15 mm from end. Move funnel to entrance end of column. Insert pledget of silane-treated glass wool in exit end and attach source of moderate vac. to this end. Continue to add packing slowly with tapping until tube is filled to ca 20 mm from entrance end. Insert pledget of silane-treated glass wool in entrance end, compressing it only enough to hold it in place.

Condition column with He carrier gas flowing at 30 mL/min $\geq$15 hr (overnight) at 255° or ca 20° below max. temp. recommended for liq. phase. Exit end of column should not be connected to detector during this conditioning.

Connect exit end of column to detector, adjust controls to conditions given in **979.05A(a)**, and let instrument come to equilibrium. Inject 3 µL aliquots std soln C until $\geq$3 consecutive injections give response ratios agreeing within 2%.

### D. Linearity Check

Check gas chromatograph for linearity at least weekly, whenever new std solns are prepd, and whenever column, new or used, is newly installed in instrument.

Using digital integration for peak area measurements, det. appropriate attenuation setting and injection aliquot (2–4 µL) of std soln B to give area count of $\geq$100,000 counts (optimum electrometer output with acceptable noise level). Use conditions so detd for all samples and stds in series.

Inject triplicate aliquots of detd vol. of std solns A, B, and C into chromatograph, det. response ratio for each, and average ratios for each soln. Divide av. ratio for each soln by corresponding malathion content in mg. Ratio/mg should agree within 2%. Failure to meet this specification indicates either weighing error in prepn of a std soln or instrumental difficulties which must be corrected before proceeding with analysis of samples.

### E. Preparation of Sample

(Analyze samples at least in duplicate.)

(**a**) *Liquid formulations and technical materials.*—Accurately weigh sample contg ca 200 mg malathion into preweighed 25 mL vol. flask. Pipet in 5 mL internal std soln, dil. to vol. with $CHCl_3$, and mix well.

(**b**) *Solid formulations containing 10% or more of malathion.*—Accurately weigh sample contg ca 1.0 g malathion and transfer to 200–250 mL (8 oz) bottle. Pipet in 50 mL $CHCl_3$, stopper tightly, and shake on reciprocating shaker 30 min. Let settle ca 15 min; if not clear, centrf. Layer of solids will float at interface. Avoid entrainment of particles by exerting pos. pressure from bulb on pipet while it is carefully inserted into soln for removal of aliquot. Particles in final soln can clog syringe needle. Transfer 10 mL aliquot clear soln to 25 mL vol. flask, pipet in 5 mL internal std soln, dil. to vol. with $CHCl_3$, and mix well.

(**c**) *Solid formulations containing less than 10% malathion.*—Accurately weigh sample contg ca 400 mg malathion and transfer to 500 mL (16 oz) bottle. Add exactly 200 mL $CHCl_3$ and shake 30 min on reciprocal shaker. Let settle, observing precautions given in (**b**). Pipet 100 mL aliquot to 500 mL r-b flask and evap. to dryness. Pipet in 5 mL internal std soln and 20 mL $CHCl_3$, swirl to dissolve residue, and mix well.

### F. Determination

Inject duplicate aliquots of appropriate vol. of std soln B as detd in linearity check, **979.05D**. Response ratios should agree within 2%; if not, repeat with 2 more injections. Failure to meet specification with second pair of injections indicates instrumental difficulties which must be resolved before proceeding with analysis.

Inject duplicate aliquots of each sample soln of same vol. as std soln. Average response ratios for each sample. Precision considerations stated for std soln also apply to sample soln injection response.

Inject duplicate aliquots std soln B after every 2 sample solns. Average response ratios of stds immediately before and after sample solns. Use this av. to calc. malathion content of the 2 sample solns.

Each detn of av. response ratio for std soln B should yield value within 2% of previously detd value. Failure to meet this specification indicates instrumental drift which must either be corrected or compensated for by more frequent measurements of response of std soln B. In extreme cases, follow each sample injection with std injection but this would indicate an instability which should be corrected at once.

### G. Calculations

For each sample injection, calc. response ratio:

$$R = \text{area of malathion peak/area of internal std peak}$$

$$\%\ \text{Malathion} = (R/R') \times (W'/W) \times P \times D$$

where R' and R = av. response ratio for std soln B and sample soln, resp.; W' and W = g malathion std and sample, resp.; P = % purity of malathion std; and D = diln factor (1 for liqs; (50/10)(25/25) = 5 for solids $\geq$10% malathion; and (200/100)(25/25) = 2 for solids <10% malathion).

Ref.: JAOAC **62,** 292(1979).

CAS-121-75-5 (malathion)

## 978.06 Parathion in Pesticide Formulations

### Gas Chromatographic Method

#### First Action 1978

(Not applicable to dusts and powders)

### A. Standard Solutions

(**a**) *Dipentyl phthalate internal std soln.*—Dissolve 2.0 ± 0.1 g dipentyl phthalate (Eastman Kodak Co., No. P2473, or equiv.) in $CS_2$ and dil. to 500 mL with $CS_2$.

(**b**) *Parathion std soln.*—Accurately weigh ca 125 mg parathion (Monsanto Chemical Co., or equiv.) into 50 mL g-s erlenmeyer, pipet in 25 mL internal std soln, and mix thoroly.

### B. Preparation of Sample

Accurately weigh sample contg ca 125 mg parathion into 50 mL g-s erlenmeyer. Pipet in 25 mL internal std soln and mix thoroly.

### C. Gas Chromatograph

*See* **977.04C**. Column should have $\geq$1200 theoretical plates for parathion. Vary attenuation and injection vol. (1–2 µL) so that peak hts of parathion and dipentyl phthalate are 60–80% full scale on 1 mv recorder. Retention times for parathion and dipentyl phthalate are 6–8 and 8–10.5 min, resp.

### D. Determination

Proceed as in **977.04D**, except substitute parathion for Me parathion and dipentyl phthalate for *p,p'*-DDE.

### E. Calculations

Proceed as in **977.04E**, except substitute parathion for Me parathion and delete *F* from equation.

Refs.: JAOAC **61,** 495(1978); **62,** 337(1979).

CAS-56-38-2 (parathion)

## 978.07 Parathion in Pesticide Formulations

### Liquid Chromatographic Method

### First Action 1978

(Not applicable to dusts and powders)

#### A. Apparatus

(**a**) *Liquid chromatograph.—See* **977.05A(a)**, except use eluant flow rate of 1.5 mL/min (ca 800 psi).

(**b**) *Liquid chromatographic column.—See* **977.03A(b)**.

#### B. Reagents

(**a**) *Choroform.—See* **977.05B(a)**.

(**b**) *Eluant.*—Stir 500 mL $CHCl_3$ on mag. stirrer 3–4 min under moderate vac. (ca 350 mm Hg).

(**c**) *Internal std soln.*—Accurately weigh ca 110 mg benzophenone (EM Science, No. BX0410, or equiv.) into 250 mL vol. flask, and dissolve and dil. to vol. with $CHCl_3$.

(**d**) *Parathion std solns.*—(*1*) *Stock soln.*—1500 μg/mL. Accurately weigh ca 75 mg anal. grade parathion (Monsanto Chemical Co., or equiv.) into 50 mL vol. flask, and dissolve and dil. to vol. with $CHCl_3$. (*2*) *Working soln.*—(150 μg parathion + 44 μg benzophenone)/mL. Pipet 5 mL stock soln and 5 mL internal std soln into 50 mL vol. flask, and dil. to vol. with $CHCl_3$.

#### C. Preparation of Sample

Accurately weigh sample contg ca 75 mg parathion into 50 mL vol. flask, and dissolve and dil. to vol. with $CHCl_3$. Pipet 5 mL sample soln and 5 mL internal std soln into 50 mL vol. flask, and dil. to vol. with $CHCl_3$.

#### D. Determination

Proceed as in **977.05D**, except substitute parathion for Me parathion and benzophenone for acetophenone, and delete *F* from equation. Retention times for parathion and benzophenone are 4.0–5.5 and 7–9 min, resp.

Refs.: JAOAC **61**, 495(1978); **62**, 337(1979).

CAS-56-38-2 (parathion)

## 978.08★ Parathion in Pesticide Formulations

### Volumetric Method

### First Action 1978
### Surplus 1984

(Applicable to dusts and powders only)

*See* **6.472–6.478,** 14th ed.

## 978.09★ Parathion in Pesticide Formulations

### Colorimetric Method

### First Action 1978
### Surplus 1984

(Applicable to dusts and powders only)

*See* **6.479–6.483,** 14th ed.

## 977.04 Methyl Parathion in Pesticide Formulations

### Gas Chromatographic Method

### First Action 1977

#### A. Standard Solutions

(**a**) *p,p'-DDE internal std soln.*—Dissolve 5.0 ± 0.1 g 2,2-bis(*p*-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE, No. 12,389-7, Aldrich Chemical Co., Inc., or equiv.) in $CS_2$ and dil. to 1 L with $CS_2$.

(**b**) *Methyl parathion std soln.*—Accurately weigh ca 125 mg Me parathion (Monsanto Chemical Co., 800 N Lindbergh Blvd, St. Louis, MO 63167, or equiv.) into 50 mL g-s erlenmeyer, pipet in 25 mL internal std soln, and mix thoroly.

#### B. Preparation of Sample

(**a**) *Liquid.*—Accurately weigh into 50 mL g-s erlenmeyer sample contg ca 125 mg Me parathion. Pipet in 25 mL internal std soln and mix thoroly.

(**b**) *Wettable powder.*—Accurately weigh into 100–150 mL (4 oz) round bottle sample contg ca 625 mg Me parathion. Pipet in 50 mL $CHCl_3$-acetone (9 + 1), cap, and shake mech. 30 min. Let settle and pipet 10 mL supernate into 50 mL g-s erlenmeyer. Place erlenmeyer in 55° $H_2O$ bath and evap. solv. under stream of dry air or N. Pipet in 25 mL internal std soln and mix thoroly.

#### C. Gas Chromatograph

Use instrument equipped with flame ionization detector and 1.2 m × 4 (id) mm glass column packed with 1.5% SE-30 plus 1.5% OV-210 on 80–100 mesh Gas-Chrom Q.

Prep. column by accurately weighing ca 0.12 g SE-30 and ca 0.12 g OV-210 into 250 mL beaker. Add 50 mL $CHCl_3$-acetone (3+2), cover with watch glass, and heat on steam bath until stationary phases are dissolved. Speed dissoln of SE-30 by spreading material on walls of beaker with small spatula or stirring rod. Add enough 80–100 mesh Gas-Chrom Q to yield 1.5% of each phase on solid support. Heat on steam bath, stirring frequently until all solv. is removed. Air dry 2–3 hr. Pack in column and condition 24 hr at 245° with N or He at 30 mL/min. Column should have ≥1200 theoretical plates for *p,p'*-DDE.

Typical operating conditions: temps (°)—inlet 210, column 180 ± 10, detector 250; N or He carrier gas, 55–75 mL/min; air and H as specified by manufacturer; attenuation and injection vol. (1–2 μL) varied so that peak hts of Me parathion and *p,p'*-DDE are 60–80% full scale on 1 mv recorder. Retention times for Me parathion and *p,p'*-DDE are 3.5–5.5 and 6–8 min, resp.

#### D. Determination

Inject aliquots of std soln until peak ht ratio of Me parathion:*p,p'*-DDE varies ≤1% for successive injections. Then make duplicate injections of sample followed by duplicate injections of std. Peak ht ratios of stds must be within ±1% of first accepted std values or repeat series of injections. Repeat for addnl samples.

#### E. Calculations

Calc. peak ht ratios for both duplicate std injections preceding and following samples. Average the 4 values ($R'$). Calc. and average peak ht ratios of the 2 samples ($R$).

$$\% \text{ Me parathion} = (R/R') \times (W'/W) \times F \times P$$

where $W$ and $W'$ = mg sample and std, resp.; $F$ = 1 for liq. and 5 for wettable powder samples; and $P$ = % purity of std.

Ref.: JAOAC **60**, 720(1977).

CAS-298-00-0 (methyl parathion)

## 977.05 Methyl Parathion in Pesticide Formulations

### Liquid Chromatographic Method
### First Action 1977

#### *A. Apparatus*

(**a**) *Liquid chromatograph*.—Waters Model ALC 202/GPC 204 (Waters Associates, Inc.), or equiv., with 254 nm UV detector and 10 mv recorder. Typical operating conditions: eluant flow rate, 1.2 mL/m in (ca 700 psi); detector sensitivity, 0.16 *A* unit full scale; temp., ambient; valve injection vol., 10 μL.

(**b**) *Liquid chromatographic column*.—See **977.03A(b)**.

(**c**) *Chromatographic tubes*.—Glass, 900 × 25 (id) mm, with coarse porosity frit in bottom (Lurex Scientific, No. 131–1044, or equiv.).

#### *B. Reagents*

(**a**) *Chloroform*.—Alcohol-free with <0.01% $H_2O$ (Burdick & Jackson Laboratories, Inc., distd in glass, or equiv.).

(**b**) *Silicic acid-water*.—75% (w/v). Add 25 mL $H_2O$ to 75 g silicic acid (Mallinckrodt Chemical Works, Code 2847, or equiv.), and shake until lumps disappear.

(**c**) *Water-saturated chloroform*.—Shake 700 mL $CHCl_3$ with 150 mL $H_2O$ 2–3 min, and pass thru 900 × 25 mm glass tube packed with 100 g silicic acid-$H_2O$.

(**d**) *Eluant*.—Blend 200 mL $H_2O$-satd $CHCl_3$ with 300 mL $CHCl_3$ on mag. stirrer 2–3 min under moderate vac. (ca 350 mm Hg).

(**e**) *Internal std soln*.—Accurately weigh ca 115 mg acetophenone (Aldrich Chemical Co. Inc., or equiv.) into 250 mL vol. flask, and dissolve and dil. to vol. with $CHCl_3$.

(**f**) *Methyl parathion std solns*.—(*1*) *Stock soln*.— 700 μg/mL. Accurately weigh ca 70 mg anal. grade Me parathion (Monsanto Chemical Co., or equiv.) into 100 mL vol. flask, and dissolve and dil. to vol. with $CHCl_3$. (*2*) *Working soln*.—(70 μg Me parathion + 46 μg acetophenone)/mL. Pipet 5 mL stock soln and 5 mL internal std soln into 50 mL vol. flask, and dil. to vol. with $CHCl_3$.

#### *C. Preparation of Sample*

Accurately weigh ca 95 mg tech. Me parathion into 100 mL vol. flask, or accurately weigh emulsifiable sample contg ca 35 mg Me parathion into 50 mL vol. flask, and dil. to vol. with $CHCl_3$. Pipet 5 mL sample soln and 5 mL internal std soln into 50 mL vol. flask, and dil. to vol. with $CHCl_3$.

#### *D. Determination*

Pump sufficient eluant thru column to equilibrate system. Inject 10 μL working std soln onto column thru sampling valve, and adjust operating conditions to give peak hts 60–80% full scale and retention times of 3.5–5.0 and 5.5–8.0 min for Me parathion and acetophenone, resp. Repeat injections until ratio of Me parathion to acetophenone peak hts is within ±1% of previous injection. Without changing conditions, alternately inject 10 μL aliquots of working std soln and duplicate 10 μL aliquots of sample soln until peak ht ratios for sample soln vary ≤1% for successive injections. Average last 2 peak ht ratios for sample and for std, resp., and calc. % Me parathion.

$$\% \text{ Me parathion} = (R/R') \times (W'/W) \times (P/F)$$

where $R$ and $R'$ = av. peak ht ratios of Me parathion to acetophenone for sample and std, resp.; $W$ and $W'$ = mg sample and std, resp.; $P$ = % purity of std; and $F$ = 1 for tech. and 2 for emulsifiable samples.

Ref.: JAOAC **60**, 724(1977).

CAS-298-00-0 (methyl parathion)

## 980.11 Methyl Parathion or Ethyl Parathion in Microencapsulated Pesticide Formulations

### Gas Chromatographic Method
### First Action 1980
### Final Action 1982

(*Caution:* See safety notes on pesticides and acetonitrile.)

#### *A. Principle*

Me or Et parathion is released from microcapsules by grinding, and compd is extd into $CH_3CN$. Internal std is added and concn of parathion compd is detd by gas chromatgy.

#### *B. Apparatus*

(**a**) *Gas chromatograph*.—With flame ionization detector, such as Perkin-Elmer 900, or equiv., and strip chart recorder, with full scale reading for 1 mv and 1 sec full scale response. Glass column, 1.8 m (6′) × 0.25″ (od), 2 mm id, packed with 3% OV-17 on 80–100 mesh Supelcoport (Supelco, Inc.). Typical operating conditions: temps (°): column 200 (Me) or 190 (Et) (isothermal), injection port 225–250 (Me) or 225 (Et), detector 250; flow rates (mL/min): He 30, H and air optimize for max. sensitivity; chart speed 0.2″ (5 mm)/min.

(**b**) *Syringe*.—10 μL. Hamilton 702N, or equiv.

(**c**) *Grinder*.—(*1*) *Mixer mill*.—With tool steel vial and ball bearing pestles. Spex Mixer Mill No. 8000-115, or equiv. (Spex Industries, 3380 Park Ave, Edison, NJ 08820); or (*2*) *Tissue grinder*.—Corning Glass Works No. 7726-L, large, 40 mL.

(**d**) *Weighing dishes*.—Light Al, 60 × 15 (depth) mm; Fisher Scientific Co., or equiv.

#### *C. Reagents*

(**a**) *Ethyl and methyl parathion*.—Anal. grade.

(**b**) *Internal stds*.—(*1*) *For Me parathion*.—Bis(2-methoxyethyl) phthalate, available from Pfaltz and Bauer, 375 Fairfield Ave, Stamford, CT 06902, Cat. No. D34510. (*2*) *For Et parathion*.—Dibutyl phthalate (Fisher Scientific Co.).

(**c**) *Std solns*.—Accurately weigh ca 0.3 g Et or Me parathion and ca 0.1 g (to 0.1 mg) of appropriate internal std, (**b**) (*1*) or (*2*), into Al dish, and transfer mixt. to 50 mL vol. flask by washing carefully with $CH_3CN$. Dil. to vol. with $CH_3CN$. Shake several min. to ensure homogeneity. Prep. in duplicate.

#### *D. Determination of Correction Factor*

Inject 1 μL std soln until peaks are reproducible. Calc. correction factor

$$C = (H'/H) \times (W/W') \times (P/P')$$

where $H$ and $H'$ = peak ht × attenuation of Me or Et parathion and of internal std, resp.; $W$ and $W'$ = g Me or Et parathion and of internal std in std soln, resp.; and $P$ and $P'$ = purity of Me or Et parathion and internal std, resp.

Run the duplicate std solns daily and average the 2 results to obtain correction factor for that day. Duplicates ordinarily differ by ≤0.01.

#### *E. Preparation of Sample*

Prep. duplicate sample solns as follows: Thoroly shake sample container to ensure no sediment remains on bottom and remove ca 1 g with medicine dropper (use sep. dropper for each sample) while stirring. Weigh sample to nearest 0.1 mg in weighed Al dish and record wt.

Transfer weighed sample to grinder, add ca 30 mL $CH_3CN$, and grind ca 4 min. Accurately weigh ca 0.2 g appropriate internal std in weighed Al dish and quant. transfer to 100 mL vol. flask with $CH_3CN$. Quant. transfer ground sample to same vol. flask with $CH_3CN$, dil. to vol. with $CH_3CN$, and mix thoroly. Let any sediment settle before withdrawing samples.

#### F. Determination

Inject 1–2 μL sample soln, using 10 μL syringe. Identify peaks on basis of retention times: solv. elutes almost immediately; Me parathion 2 min and bis(2-methoxyethyl) phthalate 3 min; Et parathion 4 min and dibutyl phthalate 3 min.

$$\text{Wt \% parathion} = (H/H') \times (W_I/W_S) \times C \times 100$$

where $W_I$ and $W_S$ = g internal std and sample weighed, resp.; and other symbols are defined in **980.11D**.

Ref.: JAOAC **63,** 999(1980).

CAS-298-00-0 (methyl parathion)
CAS-56-38-2 (parathion)

## 964.05 Phorate in Pesticide Formulations

### Infrared Spectroscopic Method

**First Action 1964**
**Final Action 1966**

(Applicable to analysis of 5 and 10% granules. Presence of other pesticides and extractable org. materials such as dispersing agents, emulsifiers, and solvs requires testing for interference.)

#### A. Apparatus

(**a**) *Infrared spectrophotometer.*—Capable of measurement in 7.9–8.6 μm range; with 0.5 mm cell.

(**b**) *Chromatographic tube.*—15 × 450 mm with stopcock or Ultramax valve (Fischer & Porter Co., Lab Crest Scientific Div., Cat. No. 274–019 or 274–100).

#### B. Reagents

(**a**) *Phorate reference std.*—Purified (obtainable from American Cyanamid Co.).

(**b**) *Phorate std soln.*—Accurately weigh by difference from Smith or Lunge pipet 1.0–1.1 g Phorate Ref. Std into 250 mL beaker contg 45 mL $CH_3CN$.

(**c**) *Cyclohexane.*—Practical grade.

(**d**) *Acetonitrile.*—Practical grade, bp 82–84°.

#### C. Preparation of Sample Solution

(*Caution*: See safety notes on distillation, toxic solvents, and acetonitrile.)

Accurately weigh 20 ± 0.01 g sample of 5% granular material (10 ± 0.01 g for 10%). Place small glass wool plug in bottom of chromatgc tube, transfer sample to tube, and gently tap sides with spatula or rod to settle contents. Place 250 mL beaker under column. Add 50 mL $CH_3CN$ to column and let percolate thru at rate of 40–50 drops/min until flow stops. Place beakers contg std (from **964.05B**(**b**)) and sample solns in shallow $H_2O$ bath at 30–35° and evap. under gentle stream of air until odor of $CH_3CN$ is no longer detectable. (Sample solns on evapn will change from clear to cloudy and then to residue of 2 layers.) Treat residue with four 5 mL portions and one 4 mL portion cyclohexane, quant. transferring cyclohexane layers to 25 mL vol. flask. (Keep cyclohexane-immiscible layer in beaker during each extn.) Dil. to vol. with cyclohexane.

#### D. Determination

Using hypodermic syringe, fill 0.5 mm cell with prepd std soln, and obtain IR spectrum from 7.9 to 8.6 μm. (With single beam instrument, adjust to give 75% *T* at 8.2 μm with cell contg std soln in position.) Using same instrument settings, treat prepd sample solns similarly.

Draw baseline from inflection points 8.10 to 8.48 μm. Draw perpendicular from 0 radiation line thru absorption peak, and measure distance from 0 to baseline ($Y$) and from 0 to absorption peak ($X$) in same units. Calc. $A = \log(Y/X)$ for sample ($A$) and std ($A'$).

$$\text{\% Phorate} = (A/A') \times (\text{wt std/wt sample}) \times \text{\% purity of std}$$

Ref.: JAOAC **47,** 245(1964).

CAS-298-02-2 (phorate)

## 980.12 Sulprofos in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1980**
**Final Action 1981**

(*Caution:* See safety notes on pesticides.)

#### A. Standard Solutions

(**a**) *Tetracosane internal std soln.*—Dissolve 2.5 g tetracosane, $CH_3(CH_2)_{22}CH_3$ (Aldrich Chemical Co., No. T875–2) in toluene and dil. to 1 L.

(**b**) *Sulprofos std soln.*—Accurately weigh ca 90 mg anal. grade sulprofos (Mobay Corp.) into 50 mL g-s erlenmeyer. Pipet 25 mL tetracosane soln into flask, and swirl to dissolve.

#### B. Apparatus

(**a**) *Gas chromatograph.*—With flame ionization detector, recorder, integrator, and provisions for on-column injection. GC conditions: temps (°)—inlet 200, column oven 185 ± 5, detector 250; N carrier gas flow (50–75 mL/min) to give retention time of ca 8 min for sulprofos; injection vol. 1.5–2.5 μL; recorder attenuation to give ca 70% full scale deflection for peaks on 1 mV recorder; integrator adjusted to give optimum slope sensitivity, baseline signal, and area response for peaks.

(**b**) *Column.*—1.2 m (4′) × 4 mm (id) Pyrex column packed with 1.5% SE-30/1.5% OV-210 on Gas-Chrom Q. For prepn of column packing, *see* **977.04C**.

#### C. Preparation of Sample

Accurately weigh tech. sulprofos or sulprofos emulsifiable conc. contg ca 90 mg pure material into 50 mL g-s erlenmeyer. Pipet 25 mL tetracosane soln into flask, and swirl to dissolve.

#### D. Determination

Inject aliquots of std soln until response ratios of sulprofos to tetracosane vary ≤1% on successive injections. Then make duplicate injections of sample followed by std injection. Calc. av. ratio of sulprofos to tetracosane area for each set of duplicate injections, and calc. % sulprofos.

$$\text{\% Sulprofos} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. integrator area ratios for sample and std, resp.; $W$ = mg tech. material or emulsifiable conc. sample; $W'$ = mg sulprofos anal. std in std soln; and $P$ = purity (%) of sulprofos anal. std.

Ref.: JAOAC **63,** 120(1980).

CAS-35400-43-2 (sulprofos)

## 982.07 Temephos in Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1982**

***CIPAC-AOAC Method***

(Method is suitable for tech. temephos and formulations with temephos as only active ingredient.)

### *A. Principle*

Sample is dissolved in ethyl acetate, *p*-nitrophenyl *p*-nitrobenzoate is added as internal std, and, after diln with *n*-hexane, sample is injected into liq. chromatgc column. LC response ratio of insecticide to internal std is compared with response ratio of std to give content in sample.

### *B. Apparatus and Reagents*

(**a**) *Liquid chromatograph.*—Able to generate >2000 psi and measure *A* at 254 nm.

(**b**) *Chromatographic column.*—Stainless steel, 300 × 3.9 mm id packed with 10 μm silica gel (μ-Porasil, Waters Associates, Inc., is suitable).

(**c**) *Ethyl acetate.*—Burdick & Jackson Laboratories, Inc. Dry over molecular sieve, 5Å, 8–12 mesh beads (W.R. Grace & Co., Davison Chemical Div., 10 E. Baltimore St, PO Box 2117, Baltimore, MD 21203-2117). Filter thru 0.45 μm Millipore filter (Millipore Corp., Bedford, MA 01730).

(**d**) *n-Hexane.*—Non-spectro, distd in glass (Burdick & Jackson Laboratories, Inc.). Dry over molecular sieve, 5Å, 8–12 mesh, and filter thru 0.45 μm Millipore filter.

(**e**) *p-Nitrophenyl p-nitrobenzoate internal std.*—1.5 g/250 mL ethyl acetate. React *p*-nitrophenyl *p*-nitrobenzoate with excess *p*-nitrophenyl Na salt (Eastman Kodak Co.) in $CH_3CN$. Alternatively, prep. 1.1% (w/v) dimethyl 4-nitrophthalate in ethyl acetate.

(**f**) *Reference std soln.*—Accurately weigh ca 50, 60, and 70 mg temephos, anal. reagent (American Cyanamid Co.) into sep. 50 mL vol. flasks. Add by pipet 5 mL internal std soln and 25 mL dry ethyl acetate to each flask. Shake flasks to ensure dissolution of std, and dil. to vol. with *n*-hexane. Designate solns as A, B, and C. Use soln B as working std soln for liq. chromatgy; use solns A and C to check linearity of liq. chromatograph (see *Linearity Check*) and to guard against weighing error in prepn of std soln. Supply of soln B can be replenished from time to time without prepg new supplies of solns A and C, provided linearity requirement described under *Linearity Check* can be met.

(**g**) *LC mobile phase.*—Add 100 mL dry ethyl acetate to 1 L vol. flask and dil. to vol. with dry *n*-hexane.

(**h**) *LC operating conditions.*—Column temp. ambient; flow rate 1.0 mL/min (ca 450 psi); retention times: internal std ca 9.6 min, temephos ca 11.5 min. Pump 50 mL anhyd. MeOH thru column followed by 100 mL dry ethyl acetate. Pump LC mobile phase thru column until system is equilibrated (flat baseline). Inject 5 μL aliquots of std soln B until const. response is obtained. If necessary, adjust instrument or injection vol. (usually 3–6 μL) to give 50–60% FSD for internal std peak. Use same injection vol. and instrument settings for all samples and stds.

### *C. Linearity Check*

Inject triplicate aliquots of appropriate vol. (as detd above) of std solns A, B, and C into liq. chromatograph, det. response ratio for each injection, and av. resulting ratios for each soln. Divide av. response ratio for each soln by corresponding content (in mg) and compare resulting response factors. These factors should agree within 2%.

Liq. chromatograph should be checked for linearity at least once a week, and same check should be carried out whenever new std solns are prepd and whenever column, new or used, is installed in instrument.

### *D. Sample Preparation*

(**a**) *Technical and emulsifiable concentrates.*—Accurately weigh amt sample contg ca 60 mg temephos directly into tared 50 mL vol. flask. For temephos tech., warm and thoroly mix before sampling. Add by pipet exactly 5 mL internal std soln and 25 mL dry ethyl acetate. Shake flask to ensure dissolution and dil. to vol. with *n*-hexane.

(**b**) *Water-dispersible powders and sand granules.*—Accurately weigh amt sample contg ca 60 mg temephos directly into 2 oz bottles fitted with plastic screw cap. Add by pipet 5 mL internal std soln and 25 mL dry ethyl acetate and shake 1 min. Add 20 mL *n*-hexane, mix thoroly, and let particles settle. Filter portion of soln and hold for LC analysis. (In some cases, centrifugation may be sufficient to remove particles before LC analysis.)

### *E. Analysis of Sample Solutions*

Inject duplicate aliquots of std soln B. Calc. response ratios by dividing area (or ht) of temephos peak by that of internal std peak. Response ratios should agree within 2%. Average duplicate response ratios obtained with std solns.

Inject duplicate aliquots of each sample soln. Average duplicate response ratios for each sample soln. Note: After first injection of any sample, let instrument run ≥30 min after emergence of temephos peak to det. late-eluting peaks due to impurities. Subsequent injections should be timed so that late-eluting peaks from sample injections do not interfere with internal std or temephos peaks of subsequent samples.

Inject duplicate aliquots of std soln B. Average response ratios of stds immediately before and after sample solns, which should agree within 2%. Use this av. to calc. temephos content of sample solns.

### *F. Calculations*

For each injection, response ratio ($R$) = (area temephos peak/area internal std peak).

$$\text{Temephos, wt\%} = (R/R') \times (W'/W) \times P$$

where $R'$ and $R$ = av. response ratio for std soln B and sample soln, resp.; $W'$ and $W$ = wt (mg) of temephos std taken (for std soln B) and sample, resp.; and $P$ = purity of temephos std (%).

Ref.: JAOAC **65,** 580(1982).

CAS-3383-96-8 (temephos)

## 949.06 TEPP in Pesticide Formulations

**Titrimetric Method**

**Final Action**

(*Caution:* See safety notes on pesticides.)

### *A. Reagents*

(**a**) *Indicator.*—0.1% aq. soln Me red or chlorophenol red.

(**b**) *Amberlite IR-4B(OH) (free base form) resin.*—Anal. grade. Amberlite IR-45, Dowex 3, or equiv., are satisfactory.

### B. Preparation of Resin Column

Screen resin to remove particles <30 mesh. Slurry 30 g screened resin with $H_2O$, and pour into 100 mL buret contg small plug of glass wool at bottom. Wash resin column with 150 mL 3% NaOH soln at flow rate of ca 5 mL/min and then rinse with $H_2O$ until effluent is acid to phthln, adjusting stopcock of buret so flow rate is ca 25 mL/min. Wash with aq. acetone (1 + 3) to displace $H_2O$. Column is now ready for use.

*Notes:* Because channeling may result if column runs dry, keep liq. level ca 2.5 cm above resin bed at all times. Because resin tends to pack in column as it adsorbs acidic material, expand resin bed after each detn before adding new sample by back-washing with acetone (1 + 3) as follows: Connect large funnel to tip of buret with rubber hose, and add the dil. acetone from funnel until liq. level reaches top of buret; let resin settle, and then let soln flow from buret until surface is 2.5 cm above resin bed. Column is now ready to receive next sample.

After 8–10 samples have passed thru column, regenerate resin by repeating original treatment with 3% NaOH soln, $H_2O$, and acetone (1 + 3). Washing with dil. acetone must be continued until effluent is colorless.

### C. Determination

(**a**) *In purified or technical grades of tetraethylpyrophosphate not mixed with solvent, emulsifying agent, etc.*—From 5–10 mL weighing buret, weigh by difference, to nearest mg, 2.5 g sample (1.0 g if tetraethylpyrophosphate content is >50%) into 50 mL acetone (1 + 3) in 125 mL separator. Mix by swirling, and let soln stand 15 min at 25 ± 2°. Let soln flow thru resin column by gravity at ca 25 mL/min, and catch effluent in 250 mL vol. flask. Wash separator and column with three 50 mL portions acetone (1 + 3), collecting washings in same flask. Dil. combined effluent to vol. with $H_2O$, mix, and transfer 100 mL aliquot to 250 mL beaker. Add 50 mL 0.1*N* NaOH to beaker, stir well, let stand 30 min at room temp., and back-titr. with 0.1*N* HCl, using pH meter (or indicator, **949.06A(a)**, if pH meter is not available). Calc. % tetraethylpyrophosphate = net mL 0.1*N* NaOH × 3.67/wt sample taken.

(**b**) *In formulations of tetraethylpyrophosphate containing organic solvent and emulsifying agent.*—Proceed as in (**a**), except filter acetone soln thru 25 mm cotton plug in cylindrical funnel (25 mm diam., 75 mm long) before adding it to column if oil seps from soln. Pass acetone washings successively thru separator, cylindrical funnel, and resin column as in (**a**). (Cotton plug absorbs oil.)

Ref.: Anal. Chem. **21,** 808(1949).

CAS-107-49-3 (tetraethylpyrophosphate)

## CARBAMATE, SUBSTITUTED UREA, AND TRIAZINE PESTICIDES

## 974.04 Aldicarb in Pesticide Formulations

**Infrared Spectrophotometric Method**

**First Action 1974**
**Final Action 1976**

(*Caution:* See safety notes on pesticides.)

### A. Apparatus and Reagents

(**a**) *Infrared spectrophotometer.*—Perkin-Elmer Model 337, or equiv. Adjust conditions as required by specific instrument.

(**b**) *Soxhlet extractor.*—With 125 mL flask and 25 × 80 mm cellulose thimble.

(**c**) *Aldicarb std soln.*—0.18 g/100 mL. Accurately weigh (to 0.1 mg) 0.18 ± 0.01 g anal. grade aldicarb (available from Rhône-Poulenc Ag Co., 2 T.W. Alexander Dr, PO Box 12104, Research Triangle Park, NC 27709) into 100 mL g-s vol. flask, add ca 80 mL $CH_2Cl_2$, mix to dissolve, and dil. to vol. with $CH_2Cl_2$.

### B. Determination

Transfer accurately weighed sample contg 0.18 ± 0.01 g aldicarb to extn thimble, cover with wad of surgical grade cotton, and place thimble in extractor. Add 2–3 Alundum boiling stones and ca 80 mL $CH_2Cl_2$ to flask, and ext at rate to provide 5 extns within 60 min. Let cool to room temp., transfer quant. to 100 mL g-s vol. flask with $CH_2Cl_2$, and dil. to vol.

Using matched 0.5 mm NaCl cells, scan sample and std solns from 5.2 to 6.0 μm (1900 to 1600 $cm^{-1}$) against $CH_2Cl_2$. Calc. $A$ of sample and $A'$ of std at 5.75 μm (1740 $cm^{-1}$), using corresponding $A$ at 5.4 μm (1850 $cm^{-1}$) as $I_0$. ($A$ and $A'$ should both be ca 0.45.)

### C. Determination of Binder Correction

Pipet 50 mL sample soln into 100 mL beaker and place in room temp. $H_2O$ bath in hood. Evap. to dryness, using gentle stream of clean, dry air. Add 25 mL MeOH, stir well, and filter thru 30 mL coarse fritted glass gooch. Rinse beaker and gooch with 25 mL MeOH, applying vac. until all liq. is in filter flask. Place gooch and contents in original beaker, place 20 mL $CH_2Cl_2$ in gooch, and swirl to dissolve binder, letting solv. drip into beaker. Repeat with addnl 20 mL $CH_2Cl_2$. Quant. transfer solv. to 50 mL g-s vol. flask and dil. to vol. with $CH_2Cl_2$. Scan soln as in detn and subtract $A$ of binder soln (should be <0.005) from that of sample (=$\Delta A$).

% Aldicarb by wt = ($\Delta A$/g sample) × (g std/$A'$) × $P$, where $P$ is % purity of ref. std.

Ref.: JAOAC **57,** 642(1974).

CAS-116-06-3 (aldicarb)

## 985.02 Aminocarb Technical and Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1985**
**Final Action 1987**

***CIPAC-AOAC Method***

(Method is suitable for tech. aminocarb and formulations with aminocarb as only active ingredient.)

### A. Principle

Aminocarb is detd by liq. chromatgy, using *n*-butrophenone as internal std.

### B. Apparatus

(**a**) *Liquid chromatograph.*—Able to generate >17.5 MPa (>2500 psi) and measure $A$ at 246 nm.

(**b**) *Chromatographic column.*—250 × 4.6 mm id packed with ≤10 μm C18 bonded silica gel (Partisil-10 ODS-3, Whatman Chemical Separations, Inc.; MicroPak MCH-10, Varian Instrument Group; Ultrapack-ODS, Beckman Instruments, Inc., 2350 Camino Ramon, PO Box 5101, San Ramon, CA 94583-0701; Zorbax Sil, Du Pont Co.; or equiv. is suitable). Operating conditions: column temp. ambient; mobile phase flow rate 1.5 mL/min (ca 2000 psi); chart speed 0.5 cm/min; injection vol. 10 μL; $A$ range 0.320 AUFS; retention times: aminocarb ca 2.65 min, internal std ca 3.80 min. Pump LC mobile phase thru column until system is equilibrated (flat baseline). Allow ca 6 min after each injection.

(c) *Filters*.—0.45 μm porosity (Gelman Acrodisc-CR, Gelman Scientific, Inc., or equiv.).

### C. Reagents

(a) *n-Butrophenone internal std soln*.—3 g/100 mL tetrahydrofuran.
(b) *Tetrahydrofuran*.—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).
(c) *Buffer soln*.—Dissolve 1.36 g $KH_2PO_4$ and 2.68 g $Na_2HPO_4.7H_2O$ in 1 L $H_2O$.
(d) *Water*.—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).
(e) *Mobile phase*.—Tetrahydrofuran-buffer soln (60 + 40).
(f) *Aminocarb reference std soln*.—Accurately weigh ca 250 mg ref. std (Mobay Corp.) into 100 mL vol. flask. Pipet 5.0 mL internal std soln into flask, dil. to vol. with tetrahydrofuran, and mix well. Pipet 5.0 mL of this soln into 100 mL vol. flask, dil. to vol. with mobile phase, and mix well. Filter portion of soln and hold for LC analysis.

### D. Preparation of Sample

Accurately weigh amt sample contg ca 250 mg aminocarb into 100 mL vol. flask. Pipet 5.0 mL internal std soln into flask, dil. to vol. with tetrahydrofuran, and shake 1 min. Pipet 5.0 mL of this soln into 100 mL vol. flask, dil. to vol. with mobile phase, and mix well. Filter portion of soln and hold for LC analysis.

### E. Determination

Adjust operating parameters to cause aminocarb to elute in 2.6–3.1 min. Adjust injection size and attenuation to give largest possible on-scale peaks. Using same injection vol. for samples and stds, make repetitive injections of std soln and calc. response ratios by dividing peak ht of aminocarb by that of internal std peak. (Note: Peak area measurements are unacceptable.) Response ratios must agree within ±1%. Average duplicate response ratios obtained with std solns.

Inject duplicate aliquots of each sample soln. Average duplicate response ratios for each sample soln. Response ratios must agree within ±1%. If not, repeat detn, starting with std injections.

Re-inject std soln twice. Average response ratios of stds immediately preceding and following sample injections. These must agree within ±1%. If not, repeat detn.

### F. Calculation

$$\text{Aminocarb, wt\%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std solns, resp.; $W'$ and $W$ = wt (mg) of aminocarb std and sample solns, resp.; and $P$ = purity of aminocarb std (%)

Ref.: JAOAC **68,** 567(1985).

CAS-2032-59-9 (aminocarb)

## 988.04 Anilazine in Pesticide Formulations

**Liquid Chromatographic Method**
**First Action 1988**
***AOAC-CIPAC Method***

(Method is suitable for formulations with anilazine as only active ingredient.)

### A. Principle

Anilazine is detd by liq. chromatgy using octanophenone as internal std. Adequate resolution is controlled by monitoring sepn of bis-compound (major impurity of anilazine) from anilazine and internal std peaks.

### B. Apparatus

(a) *Liquid chromatograph*.—Able to generate >7 MPa (>1000 psi) and measure $A$ at 250 nm. Operating conditions: column temp. ambient; mobile phase flow rate ca 1.7 mL/min (ca 800 psi); chart speed 0.5 cm/min; injection vol. 20 μL; $A$ range 0.32 AUFS. Retention times: anilazine ca 2.5 min, bis-compound ca 4.0 min, octanophenone ca 6.6 min. Pump LC mobile phase thru column until system is equilibrated (flat baseline). Allow 1 min after elution of internal std before next injection.
(b) *Chromatographic column*.—250 × 4.6 mm id packed with ≤10 μm $C_{18}$ bonded silica gel capable of resolving bis-compound from anilazine and internal std peaks (DuPont ODS, or equiv.)
(c) *Chart recorder*.—Min. 250 mm span, 10 mV range, 30 cm/h speed.
(d) *Bath*.—Ultrasonic.
(e) *Filters*.—0.45 μm porosity (Gelman Acrodisc-CR, Gelman Scientific, Inc., or equiv.).

### C. Reagents

(a) *Acetonitrile*.—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).
(b) *Octanophenone internal std soln*.—Dil. 4 mL octanophenone (Aldrich Chemical Co., Inc., or equiv.) to 250 mL with $CH_3CN$.
(c) *Water*.—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).
(d) *LC mobile phase*.—$CH_3CN$-$H_2O$ (80 + 20).
(e) *Anilazine std soln*.—Accurately weigh ca 230 mg anilazine ref. std (Mobay Corp.) into 100 mL vol. flask. Pipet 10 mL internal std soln into flask, dil. to vol. with $CH_3CN$, and mix well. Pipet 5 mL of this soln into 100 mL vol. flask, dil. to vol. with $CH_3CN$, and mix well. Filter portion of final soln for LC analysis.

### D. Preparation of Sample

(a) *Formulations excluding flowable*.—Accurately weigh sample contg ca 230 mg anilazine into 100 mL vol. flask. Pipet 10 mL internal std soln into flask, dil. to vol. with $CH_3CN$, and sonicate 1 min. Mix well. Pipet 5 mL of this soln into 100 mL vol. flask, dil. to vol. with $CH_3CN$, and mix well. Filter portion of final soln for LC analysis.
(b) *Flowable*.—Accurately weigh sample contg ca 230 mg anilazine into 100 mL vol. flask. Add 5 mL LC grade or distd in glass $H_2O$ and swirl until sample is thoroughly dispersed. Pipet 10 mL internal std soln into flask, dil. to vol. with $CH_3CN$, and sonicate 1 min. Mix well. Pipet 5 mL of this soln into 100 mL vol. flask, dil. to vol. with $CH_3CN$, and mix well. Filter portion of final soln for LC analysis.

### E. Determination

Inject anilazine std soln and adjust operating parameters so that anilazine elutes in 2.5–3.0 min. Adjust injection vol. and attenuation to give largest possible on-scale peaks. Bis-compound must be resolved from anilazine and octanophenone peaks. If not, change or repack column.

Using same injection vol. for all sample and std injections, make repetitive injections of ref. std soln and calc. response ratios by dividing anilazine peak ht by internal std peak ht. Response ratios must agree within ±1%. Average duplicate response ratios obtained with std solns.

Inject duplicate aliquots of each sample soln. Average duplicate response ratios for each sample soln. Response ratios must agree within ±1%. If not, repeat detn, starting with std injections.

Re-inject ref. std soln twice. Average response ratios of stds immediately preceding and following sample injection. These must agree within ±1%. If not, repeat detn.

### F. Calculation

$$\text{Anilazine, wt\%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std solns, resp.; $W'$ and $W$ = wt (mg) of anilazine in std and sample solns, resp.; and $P$ = % purity of anilazine std.

Ref.: JAOAC **71,** 23(1988).

CAS-101-05-3 (anilazine)

## 986.09 Bendiocarb in Technical and Wettable Powder Pesticide Formulations

### Liquid Chromatographic Method

### First Action 1986

(Applicable to tech. bendiocarb and its 20 and 80% wettable powder formulations)

### A. Principle

Bendiocarb is extd from sample with fixed vol. of $CH_3CN$ contg 0.1 % v/v propiophenone internal std. Soln is filtered and chromatographed on reverse phase column with $CH_3CN$-$H_2O$ (40 + 60) mobile phase. Compd is quantitated by comparison of response ratio for bendiocarb/propiophenone in sample and std.

### B. Apparatus and Reagents

(**a**) *Liquid chromatograph.*—Const vol. pump, UV detector, injector. Injection system may be manual or automatic. Response may be measured by peak ht or peak area. Column: 250 × 4.6 mm id, 6.4 mm od, type 316 stainless steel, slurry-packed with Partisil 10 ODS 2 (Whatman Ltd). Mobile phase: Mix 800 mL $CH_3CN$ with $H_2O$ and dil. to 2 L with $H_2O$. Degas by applying reduced pressure until solv. just boils. Maintain this pressure 10 min.

Set UV detector to 254 nm. At 2 mL/min, pump 50 mL $CH_3CN$ thru column, followed by 50 mL $CH_3CN$-$H_2O$ (75 + 25). Change to mobile phase, pump 50 mL to waste, then connect system to recycle mobile phase.

(**b**) *Internal std soln.*—0.1% v/v propiophenone in $CH_3CN$.

(**c**) *Bendiocarb reference std soln.*—Cambridge Animal and Public Health Ltd, Hauxton, Cambridge CB2 5HU, UK. Weigh 0.49–0.51 g bendiocarb into 100 mL g-s conical flask. Add, by pipet, 25.0 mL internal std soln. Inject 5 μL into system. Adjust flow rate to give bendiocarb peak at 3–5 min and internal std peak at 1.5 times elution time of bendiocarb.

### C. Preparation of Sample

Accurately weigh amt of sample contg ca 0.50 g bendiocarb into 100 mL g-s conical flask. Use 0.49–0.51 g for tech. material, 0.61–0.64 g for 80% formulation, and 2.50–2.55 g for 20% formulation.

Using same pipet as for std, add 25.0 mL internal std soln. Swirl to dissolve. Filter soln, which contains suspended solids, thru suitable filter and use filtrate for liq. chromatgy.

### D. Determination

It is necessary to establish that LC system has achieved stability and that it remains stable. After any period of idling, whether pump is running or not, make at least 3 injections of std soln. For each injection, measure bendiocarb and internal std peaks and calc. response ratio, bendiocarb/internal std. Rerun stds until response ratio achieves acceptable repeatability, then inject sample soln in duplicate. Follow duplicate sample injections with std. Average sample responses. Use as std response ratio the mean of those which occur on either side of sample injections.

### E. Calculation

$$\text{Bendiocarb, \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std, resp.; $W$ and $W'$ = wt (g) of sample and std, resp.; and $P$ = % purity of std.

Ref.: JAOAC **69,** 908(1986).

CAS-22781-23-3 (bendiocarb)

## 984.09 Benomyl in Pesticide Formulations

### Liquid Chromatographic Method

### First Action 1984

(Method dets benomyl equiv. of benomyl and methyl-2-benzimidazole carbamate present.)

### A. Principle

Benomyl is extd from inerts with $CH_3CN$ contg 3% (v/v) *n*-butylisocyanate (BIC). Equilibrium of benomyl spontaneous decomposition is driven toward benomyl and no significant degradation occurs. Ext is filtered, chromatographed on reverse phase ($C_{18}$) column, using $CH_3CN$-2% HOAc mobile phase, and quantitated by comparing peak hts of sample exts and std from UV detector set at 280 or 290 nm. *Caution:* BIC is a severe lachrymator!

### B. Reagents

(**a**) *Extractant.*—3% (v/v) *n*-butyl isocyanate (Aldrich Chemical Co.) in $CH_3CN$ (LC grade).

(**b**) *Mobile phase.*—$CH_3CN$ (LC grade)-2% HOAc (80 + 20), or as adjusted to give $k' > 2$ for analyte when delivered at 1.0 mL/min, and retention time for benomyl of 4–6 min.

(**c**) *Reference std.*—Benomyl (E.I. DuPont de Nemours & Co., Inc.).

### C. Apparatus

(**a**) *Liquid chromatograph.*—Able to generate >1000 psi and measure UV absorbance at 290 or 280 nm. Also must be capable of reproducibly injecting 10 μL.

(**b**) *Chromatographic column.*—10 μm reverse phase $C_{18}$ column which produces ≥1000 theoretical plates for benomyl (defined as $5.5\,(t/w)^2$, where $t$ = retention time and $w$ = width at half ht).

(**c**) *Filtration.*—13 mm glass fiber disc (Gelman Sciences) inserted into 5 mL disposable syringe.

### D. Procedure

Accurately weigh stds and samples to contain ca 25 mg benomyl, add 50.0 mL extractant, and shake 30 min. Filter thru glass fiber pad and inject 10 μL aliquot. Bracket each 2 sample injections with std injections and av. std response for calcn of sample concn.

### E. Calculation

$$\%\ \text{Benomyl} = (R/R') \times (W'/W) \times \%\ P$$

where $R$ and $R'$ = peak ht of sample and std, resp.; $W$ and $W'$ = wt of sample and std; and $P$ = % purity of std.

Ref.: JAOAC **67**, 303(1984).

CAS-17804-35-2 (benomyl)

## 976.04 Carbaryl in Pesticide Formulations

### Infrared Spectrophotometric Method
### First Action 1976
### Final Action 1979

(*Caution:* See safety notes on pesticides, pipets, toxic solvents, and chloroform.)

#### A. Apparatus

(**a**) *Centrifuge.*—Clinical model, 8 place, or equiv.

(**b**) *Hypodermic syringe.*—1 mL, glass barrel with rubber-tipped plastic plunger (1 mL B-D Glaspak Tuberculin disposable syringe supplied by Becton, Dickinson, and Co., Stanley St, Rutherford, NJ 07070, is suitable). Disposable syringe may be used repeatedly. Wash with $H_2O$ and acetone or MeOH, air-dry, and lubricate rubber plunger tip with silicone stopcock grease.

(**c**) *Infrared spectrophotometer.*—Perkin-Elmer Corp., Model 337, or equiv. Operator must adapt conditions to instrument.

(**d**) *Rotator.*—Tube type, Scientific Equipment Products (SEPCO), or equiv.

(**e**) *Shaking machine.*—Wrist-action shaker (Burrell Corp., or equiv.).

(**f**) *Tubes.*—Culture tubes, borosilicate glass, 16 × 150 mm with screw caps and Teflon liners (Corning Glass Works No. 9826, or equiv.).

#### B. Reagents

(**a**) *Methanol-chloroform soln.*—10% (v/v) MeOH in $CHCl_3$.

(**b**) *Carbaryl std solns.*—(*1*) *8 mg/mL.*—Transfer 0.12 ± 0.01 g carbaryl (anal. grade, available from Rhône-Poulenc Ag Co., 2 T.W. Alexander Dr, PO Box 12014, Research Triangle Park, NC 27709), weighed to nearest 0.1 mg, to culture tube. Pipet 15 mL MeOH-$CHCl_3$ soln into tube, cap securely, and rotate or shake mech. 30 min. (*2*) *2.5 mg/mL.*— Transfer 0.25±0.01 g carbaryl, weighed to nearest 0.1 mg, to 250 mL g-s erlenmeyer. Pipet 100 mL $CHCl_3$ into flask, stopper, and swirl to dissolve.

#### C. Preparation of Sample

(**a**) *Carbaryl dust and powder formulations.*— Transfer weighed sample (≤2.4 g) contg 0.12 ± 0.01 g carbaryl to culture tube. Pipet 15 mL MeOH-$CHCl_3$ soln into tube and cap securely. Rotate or shake mech. 30 min and centrf. 10 min.

(**b**) *Liquid suspensions.*—Following steps must be performed in order described, as any deviation can cause erroneous results due to faulty sample transfer and incomplete extn: Place ca 20 g $Na_2SO_4$ in 250 mL g-s erlenmeyer. Pipet 100 mL $CHCl_3$ into flask. Vigorously shake sample bottle. Draw appropriate vol. sample into hypodermic syringe without needle. Use ca 0.5 mL sample for carbaryl 4 lb/gal. and ca 1.0 mL for carbaryl 2 lb/gal. Wipe outside of syringe with paper towel and weigh syringe and contents to nearest 0.1 mg. Add sample to erlenmeyer by slowly depressing syringe plunger. Do not let syringe or sample touch sides of flask. Sample must drop into $CHCl_3$. Reweigh syringe and calc. sample wt by difference. Stopper flask and shake vigorously 30 min on mech. shaker.

#### D. Determination

(**a**) *Carbaryl dust and powder formulations.*—Using matched 0.2 mm NaCl cells, scan sample soln against MeOH-$CHCl_3$ soln from 5.2 to 6.0 μm (1900–1600 $cm^{-1}$). Repeat scan with std soln. Measure $A$ of carbaryl peak at 5.75 μm (1740 $cm^{-1}$), using $A$ at 5.40 μm (1850 $cm^{-1}$) as 0 point. $A$ = ca 0.4 for both std and sample.

$$\% \text{ Carbaryl by wt} = (A \times B' \times P)/(A' \times B)$$

where $A$ and $A'$ = absorbance of sample and std, resp., at 5.75 μm; $B$ and $B'$ = mg sample and mg std/mL, resp.; and $P$ = % purity of carbaryl std.

(**b**) *Liquid suspensions.*—Proceed as in (**a**), except use matched 0.5 mm NaCl cells and scan sample soln against $CHCl_3$.

Refs.: JAOAC **50,** 566(1967); **56,** 576(1973); **59,** 753, 1196(1976).

CAS-63-25-2 (carbaryl)

## 986.10 Carbofuran in Pesticide Formulations

### Liquid Chromatographic Method
### First Action 1986

#### A. Principle

Carbofuran is extd from sample with MeOH contg acetophenone as internal std. Soln is centrfgd and chromatographed on reverse phase column with MeOH-$H_2O$ (50 + 50) mobile phase. Compd is quantitated by comparison of response ratio for carbofuran/acetophenone in sample and std.

#### B. Apparatus and Reagents

(**a**) *Liquid chromatograph.*—High pressure pump, capable of 5000 psi up to desired flow rate; sensitivity of 0.2 AUFS; UV detector with 8–12 μL flow-thru cells, operating at 280 nm; guard column (optional), Brownlee RP-18, No. 140–200 and guard cartridge No. ODS-GU (Rainin Instrument Co., Mack Rd, Woburn, MA 01801-4628), ambient temp.; column, 150–300 × 3–5 mm id C18 (typically 250 × 4.1 mm), ca 40°; injector, Waters Associates Model U6K, Rheodyne Model 7120 or 7125, or Model 728, Alcott Chromatographies, Inc. (One Micromeritics Dr, Norcross, GA 30093) or equiv.; recorder, 10 in. 10 mV full scale at 0.5 cm/min.

(**b**) *Mobile phase.*—Use distd or distd, deionized $H_2O$ and distd in glass MeOH. Use prefiltered solvs or filter thru sub-micron filters, 0.5 μm (Millipore Corp. or Gelman Scientific Inc.). Measure $H_2O$ and MeOH (50 + 50) and combine; do not add one to the other to det vol. Alternatively, use 2-pump liq. chromatgc system with sep. metering for each solv. Flow rate 1 mL/min.

(**c**) *Internal std soln.*—0.5 mg acetophenone (≥98%, no. A1070-1, Aldrich Chemical Co. Inc.; Pfaltz and Bauer, 375 Fairfield Ave, Stamford, CT 06902; or equiv.)/mL MeOH, distd in glass.

(**d**) *Carbofuran reference std soln.*—0.5 mg carbofuran (FMC Corp., Agricultural Chemical Group, Group Quality Assurance, 100 Niagara St, Middleport, NY 14105)/mL MeOH. Also contg 0.5 mg/mL of 7-hydroxycarbofuran (FMC Corp.).

#### C. Preparation of Sample

Use balance capable of 0.01 mg resolution, or increase all wts and vols by factor of 10. Accurately weigh amt of sample contg ca 8–10 mg carbofuran into 100 mL g-s conical flask. Add 20 mL internal std soln. Swirl to dissolve. Ext ca 30 min on reciprocating shaker or 2 min on vortex mixer. Centrf. and use supernate for liq. chromatgy.

#### D. Determination

Optimize column as follows: Set column temp. to 40°. Det. retention time of carbofuran (preferably 12–15 min). Alter mobile phase if necessary. Det. retention time and resolution of acetophenone relative to carbofuran. If acetophenone is not resolved at baseline from carbofuran, alter mobile phase by reducing MeOH. This may result in retention time >15 min for carbofuran on some columns.

Det. retention time of 7-hydroxycarbofuran. If it interferes with carbofuran, further optimization is required: Increase or decrease column temp., in 5° increments, and alter mobile phase composition to maintain the resolution and retention times of carbofuran and acetophenone const. Note retention time and resolution of 7-hydroxyfuran peak. When it is well resolved from carbofuran, impurity interferences are minimized and sample analysis can begin.

Inject 10 μL sample. Det. response ratio $R$ for peak ht (or area) of carbofuran/peak ht (or area) of internal std.

$$\text{Carbofuran, wt \%} = (R/R') \times (W'/W) \times P$$

where $R'$ and $R$ = av. response ratio for std and sample solns, resp.; $W'$ and $W$ = wt (mg) of carbofuran std and sample, resp.; and $P$ = % purity of carbofuran std.

On some high resolution columns, flow of 2 mL/min can be used to reduce analysis time. If signs of decomposition are noted, 3–5 drops of $H_3PO_4$/L may be added to mobile phase.

Ref.: JAOAC **69,** 915(1986).

CAS-1563-66-2 (carbofuran)

## 977.06 Chlorotoluron, Chloroxuron, or Metoxuron in Pesticide Formulations

**Thin Layer Chromatographic Method**

**First Action 1977**
**Final Action**

***CIPAC-AOAC Method***

### A. Principle

Pesticide is extd from formulations with $CH_2Cl_2$, free amines are removed with acid, and ext is hydrolyzed by alkali to $Me_2NH$ which is distd and titrd. Related byproducts, 3-(3-chloro-4-methylphenyl)-1-methylurea (I), 3-(4-methylphenyl)-1,1-dimethylurea (II) (from chlorotoluron), 3-[4-(4-chlorophenoxy)phenyl]-1-methylurea (III) and 3-(4-chlorophenyl)-1,1-dimethylurea (IV) (from chloroxuron), and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (V), 3-(3-chloro-4-hydroxyphenyl)-1,1-dimethylurea (VI), and 3-(4-methoxyphenyl)-1,1-dimethylurea (VII) (from metoxuron), which may interfere, are detd by semiquant. TLC. Limit of detection for TLC is 0.1% for each byproduct. On same TLC plate for chloroxuron, free amine 4-(4-chlorophenoxy)aniline (VIII) is detd by sep. detection technic. Other byproducts, 1,3-bis(3-chloro-4-methylphenyl) urea (IX), 1,3-bis[4-(chlorophenoxy)-phenyl]urea (X), and 1,3-bis(3-chloro-4-methoxyphenyl) urea (XI), do not interfere with chlorotoluron, chloroxuron, and metoxuron detns, resp.

### B. Preparation of Sample

(**a**) *Technical formulation*.—Accurately weigh ca 3 g sample (4 g for chloroxuron) and transfer, using 100 mL $CH_2Cl_2$, into 250 mL separator, dissolve, and add 50 mL 1*N* HCl.

(**b**) *Wettable powder*.—Accurately weigh ca 3.5–4.0 g sample (for 80%) or 6.0–6.5 g (for 50%) into 200 mL beaker. Add 100 mL $CH_2Cl_2$ and stir mag. 5 min. Filter thru fritted glass crucible contg paper and 0.5 g layer of Celite, and rinse beaker and crucible with portions of $CH_2Cl_2$ to total vol. of ca 200 mL. Use only slight vac. to prevent crystn of pesticide on walls of crucible. Transfer quant. to 500 mL separator, and add 50 mL 1*N* HCl.

### C. Determination

Vigorously shake mixt. 1 min and drain lower org. layer into second separator. Add 25 mL (50 mL for chloroxuron) 1*N* HCl, shake 30 sec, and drain lower layer into 500 mL r-b flask. Wash the 2 acid layers successively with same 100 mL portion $CH_2Cl_2$ (with two 50 mL portions for chloroxuron) and drain lower layer into the 500 mL r-b flask. Discard acid.

Vac.-evap. $CH_2Cl_2$ in rotary evaporator to dryness at max. of 40°. Remove all solv. to prevent interference in subsequent titrn. Add 100 mL propylene glycol, 40 g KOH, and some boiling stones to residue. Immediately connect flask securely to distn app. (Fig. **977.06**) whose joints are lubricated with thin film of silicone grease. Place end of condenser delivery tube (≥10 mm id) in 400 mL beaker below level of absorbing soln of 0.2 g $H_3BO_3$ and 1 mL mixed indicator soln (40 mg methylene blue and 60 mg Me red dissolved in 100 mL alcohol) in 150 mL $H_2O$. (To enhance end point, use 150 mL MeOH (2+1).)

Gently warm flask until all particles dissolve; then boil 10 min or until propylene glycol distils into condenser. Titr. distd $Me_2NH$ continuously with stdzd 1*N* HCl, **936.15**. Complete distn by carefully adding $H_2O$ dropwise from dropping funnel at rate of 1 drop/sec. Continue titrn until end point persists 2 min ($V$ mL). Perform blank detn ($B$ mL) with each series.

$$\text{\% Pesticide} = [(V - B) \times N \times F/\text{g sample}] - \text{\% byproducts (from 977.06D)}$$

where $F$ = 21.27 for chlorotoluron, 29.07 for chloroxuron, or 22.87 for metoxuron, and $N$ = normality of stdzd HCl.

### D. Determination of Byproducts

(**a**) *For chlorotoluron*.—Dissolve 100 mg each of byproducts I and II (**977.06A**) (available from Ciba-Geigy Muenchwilen Ltd, Analytical Development Agro, CH-4333 Muenchwilen, Switzerland) together in tetrahydrofuran and dil. to 50 mL in vol. flask. Dil. aliquots of 1, 2, 3, 4, and 5 mL to 20

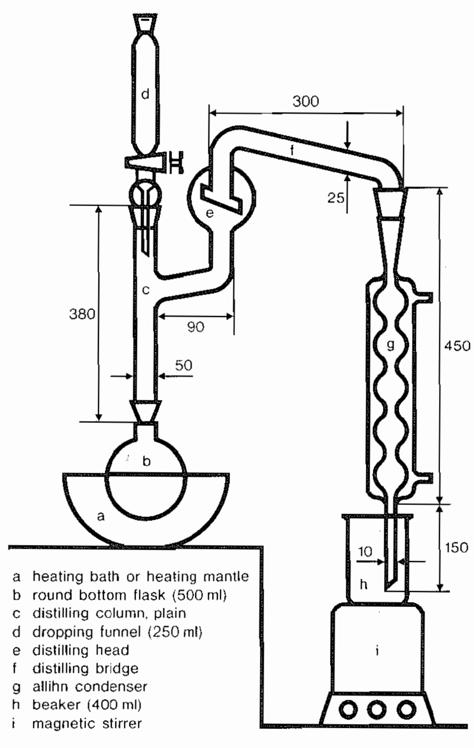


**FIG. 977.06—Distillation apparatus (all dimensions in mm)**

mL with tetrahydrofuran, equiv. to 0.2, 0.4, 0.6, 0.8, and 1.0%, resp., of each byproduct.

Dissolve 1.0 g sample in tetrahydrofuran, and dil. to 20 mL with same solv.

Spot 5 µL each of sample and std solns on 20 × 20 cm glass plates precoated with 0.25 mm layer of silica gel 60 F-254 (No. 5715, E. Merck, Frankfurterstrasse 250, Postfach 4119, D6100 Darmstadt, West Germany, or equiv.), and develop by ascending technic in tank, presatd 30 min with developing solv. $CHCl_3$-EtOAc (4 + 1), without filter paper linings, for ca 70 min (13 cm migration). Expose plate to 254 nm UV light and compare spots of samples with those of stds to est. concn of byproducts. Approx $R_f$ values: chlorotoluron, 0.50; byproduct I, 0.25; byproduct II, 0.35; and byproduct VI (does not interfere), 0.82.

(**b**) *For chloroxuron.*—Dissolve 100 mg each of byproducts III, IV, and VIII (available from Ciba-Geigy Muenchwilen Ltd) together in acetone and dil. to 100 mL in vol. flask. Dil. aliquots of 1, 3, 5, 8, and 10 mL to 50 mL with acetone, equiv. to 0.1, 0.3, 0.5, 0.8, and 1% resp., of each byproduct.

Dissolve 1.0 g sample in acetone, and dil. to 50 mL with same solv. Proceed as in (**a**), but use $CHCl_3$-dioxane (9 + 2) as developing solv. for ca 80 min (14 cm). Approx $R_f$ values: chloroxuron 0.75; byproduct III, 0.40; byproduct IV, 0.65; and byproduct X (does not interfere), 0.90.

Det. byproduct VIII on same TLC plate. Place beaker contg ca 2 g $NaNO_2$ in empty developing tank and pour ca 3 mL HCl over salt. After 2 min, insert plate into tank 3 min, remove, and dry 5 min at room temp. with hair dryer. Spray with 1% soln of *N*-(1-naphthyl)ethylenediamine.2HCl in 0.1*N* HCl and compare violet sample spots with those of stds ($R_f$, 0.85).

(**c**) *For metoxuron.*—Proceed as in (**a**), except use 100 mg each of byproducts V, VI, and VII (available from Sandoz Ltd, AgroDivision, Development, CH-4002 Basle, Switzerland). Approx. $R_f$ values: metoxuron, 0.25; byproduct III, 0.34; byproduct IV, 0.08; byproduct V, 0.13; and byproduct VII (does not interfere), 0.46.

### *E. Identification*

(**a**) *Technical chloroxuron.*—Record IR spectrum of 1% $CH_2Cl_2$ soln of sample and compare with spectrum of 1% $CH_2Cl_2$ soln of authentic ref. std.

(**b**) *50% Wettable powder.*—Stir ca 2 g sample and 2 g silica gel (70–230 mesh) with 100 mL $CH_2Cl_2$ 5 min and percolate thru fluted filter. Record IR spectrum of filtrate in NaCl cell (0.5 mm path length) from 3000 to 650 $cm^{-1}$, using blank solv. as ref. Identity is established if sample spectrum corresponds qual. to that of std.

Refs.: JAOAC **59,** 716(1976); **61,** 1499(1978); **62,** 334(1979).

CAS-11111-56-1 (chlorotoluron)
CAS-1982-47-4 (chloroxuron)
CAS-59587-03-0 (metoxuron)

## 965.15 Dithiocarbamates in Pesticide Formulations

**Carbon Disulfide Evolution Method**

**First Action 1965**
**Final Action 1966**

(Applicable only to concs or formulations contg ferbam, maneb, nabam, zineb, or ziram and free from interfering substances)

(*Caution:* See safety notes on pesticides.)

### *A. Principle*

Dithiocarbamates decompose on heating in acid medium. Evolved $CS_2$ is passed thru $Pb(OAc)_2$ soln traps to remove $H_2S$ and $SO_2$ formed from sample impurities. Washed $CS_2$ is reacted with methanolic KOH, and xanthate formed is titrd with I soln.

### *B. Apparatus*

*Carbon disulfide evolution apparatus.*—See Fig. **965.15**. Available from Lurex Scientific, No. JE-1000–0000.

### *C. Reagent*

*Methanolic potassium hydroxide.*—2*N*. Dissolve 112 g KOH pellets in 500 mL anhyd. MeOH, filter thru cotton, and add addnl 500 mL anhyd. MeOH.

### *D. Determination*

Add 20 mL 10% $Pb(OAc)_2$ soln to each $Pb(OAc)_2$ trap and pipet 50 mL 2*N* MeOH-KOH soln into MeOH-KOH absorber (Fig. **965.15**). (Absorber must be dry at time of addn and kept at 25 ± 1°.) Add 50 mL $H_2SO_4$ (1 + 4) to reaction flask and heat acid to boiling. Adjust aspiration rate to ≤1 bubble/sec thru MeOH-KOH soln, using stopper in reaction flask.

Weigh ≤5 g sample (contg 0.1–0.3 g dithiocarbamates) into small filter paper cone and fold cone to prevent sample loss. Remove stopper from reaction flask, insert wrapped sample, and immediately stopper flask. Adjust air flow if necessary and maintain steady, moderate boil. Do not let acid soln enter air inlet tube. Some dust formulations react vigorously and require special care to prevent ejection of hot acid. As reaction proceeds, adjust system so that rates of boiling and aspiration are almost in equilibrium, producing only very slow rate of bubbling thru MeOH-KOH soln. Continue boiling 1.5 hr. Disconnect MeOH-KOH absorber and rinse contents into 500 mL erlenmeyer, using ca 250 mL $H_2O$. (To remove absorber con-

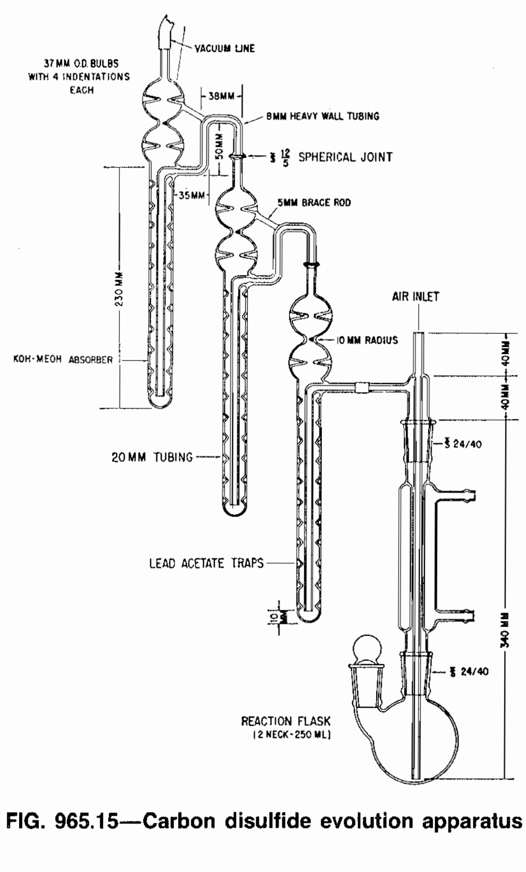


**FIG. 965.15—Carbon disulfide evolution apparatus**

tents, apply slight air pressure to top of absorber and force soln thru side arm. Rinse with 4 ca 25 mL portions $H_2O$, forcing out rinse $H_2O$ in same manner with air pressure.)

Add 3 drops phthln, and titr. with 30% HOAc until red just disappears. Immediately titr. with 0.1*N* I; near end point, add 5 mL starch indicator soln, **922.03A(f)**, and titr. to faint but definite color change.

Det. blank (usually 0.1–0.2 mL 0.1*N* I) by dilg 50 mL MeOH-KOH soln with 250 mL $H_2O$, neutzg with 30% HOAc, and titrg as above.

Calc. % dithiocarbamate = (Sample titrn − blank) × (I normality) × (equiv. wt dithiocarbamate)/ (g sample × 10)

Equiv. wts ($^1/_2$ MW) of zineb, maneb, ziram, nabam, and ($^1/_3$ MW) ferbam are 137.87, 132.65, 152.91,128.18, and 138.82, resp.

Ref.: JAOAC **48,** 562(1965); **52,** 385(1969).

CAS-301-05-3 (ferbam)
CAS-301-03-1 (maneb)
CAS-142-59-6 (nabam)
CAS-142-14-3 (zineb)
CAS-137-30-4 (ziram)

## 977.07 Fluometuron in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1977**
**Final Action 1978**

### *A. Standard Solutions*

(**a**) *Diethyl phthalate internal std soln.*—Weigh 1.5 ± 0.1 g tech. diethyl phthalate, dissolve in ca 100 mL alcohol-free $CHCl_3$, dil. to 250.0 mL with $CHCl_3$, and mix well. Std should be >98% pure and contain no impurities eluting at retention time of fluometuron.

(**b**) *Fluometuron std soln.*—Accurately weigh ca 125 mg tech. fluometuron of known purity (available from Ciba-Geigy Corp., PO Box 11422, Greensboro, NC 27409) into 2 oz round bottle with Teflon-lined or Poly-Seal screw cap. Pipet in 25 mL diethyl phthalate internal std soln and shake to dissolve. Pipet in 3 mL trifluoroacetic anhydride and shake mech. 15 min; then place bottle in 55° $H_2O$ bath 30 min. Let cool to room temp.

### *B. Preparation of Sample*

Accurately weigh sample contg ca 125 mg fluometuron into 2 oz round bottle with Teflon-lined or Poly-Seal screw cap. Pipet in 25 mL diethyl phthalate internal std soln and shake well. Pipet in 3 mL trifluoroacetic anhydride and shake mech. 15 min; then place bottle in 55° $H_2O$ bath 30 min. Let cool to room temp. Let insol. materials settle or centrf. portion of ext to obtain clear soln.

### *C. Gas Chromatography*

Use instrument equipped with flame ionization detector and 1.83 m × 2 (id) mm glass column packed with 2% OV-3 (Applied Science) on 80–100 mesh Gas-Chrom Q. Condition 24 hr at 240° with N or He at ca 40 mL/min. Column should have ≥1500 theoretical plates. Use on-column injection to prevent decomposition of derivative.

Typical operating conditions: temps (°)—inlet 150, column 115 ± 10, detector 250; N or He carrier gas, 20–22 mL/min; air and H as specified by manufacturer; attenuation varied so that peak hts of pesticide and internal std are 60–80% full scale. Retention times for fluometuron derivative and diethyl phthalate are 3–5 and 8–10 min, resp.

### *D. Determination*

Proceed as in **971.08C** and **D**, except inject 1 μL aliquots.

### *E. Calculations*

*See* **971.08E**.

Refs.: JAOAC **60,** 716(1977); **62,** 334(1979).

CAS-2164-17-2 (fluometuron)

## 984.10 Methiocarb Technical and Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1984**
**Final Action 1987**

***CIPAC-AOAC Method***

(Method is suitable for tech. methiocarb and formulations with methiocarb as only active ingredient.)

### *A. Principle*

Methiocarb is detd by liq. chromatography using acetophenone as internal std.

### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Able to generate >1500 psi and measure *A* at 266 nm.

(**b**) *Chromatographic column.*—250 × 4.6 mm id packed with ≤10 μm C18 bonded silica gel. (Partisil-10 ODS-3, Whatman Inc., or equiv., is suitable.) *LC operating conditions.*—Column temp. ambient; mobile phase $CH_3CN$-$H_2O$ (60 + 40); flow rate 2.5 mL/min (ca 1500 psi); chart speed 0.5 cm/min; injection vol. 10 μL; *A* range 0.160 AUFS; retention times: internal std ca 2.50 min, methiocarb ca 3.70 min. Pump LC mobile phase thru column until system is equilibrated (flat baseline). Allow 6 min after each injection and then pump $CH_3CN$ 6 min to remove impurities. Pump LC mobile phase ca 8 min, allowing system to re-equilibrate before next injection.

(**c**) *Filters.*—0.45 μm porosity (Gelman Acrodisc-CR, or equiv.).

### *C. Reagents*

(**a**) *Water.*—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc.).

(**b**) *Acetonitrile.*—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc.).

(**c**) *Acetophenone internal std soln.*—10 g/200 mL $CH_3CN$.

(**d**) *Tetrahydrofuran.*—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc.).

(**e**) *Methiocarb ref. std soln.*—Accurately weigh ca 500 mg ref. std (Mobay Corp.) into 100 mL vol. flask. Pipet 10 mL tetrahydrofuran into flask, and swirl to dissolve. Pipet 10.0 mL internal std soln into flask, dil. to vol. with $CH_3CN$, and mix well. Pipet 10.0 mL this soln into 100 mL vol. flask, dil. to vol. with $CH_3CN$, and mix well.

### *D. Preparation of Sample*

Accurately weigh amt sample contg ca 500 mg methiocarb into 100 mL vol. flask. Pipet 10 mL tetrahydrofuran into flask and swirl. Pipet 10.0 mL internal std soln into flask, dil. to vol. with $CH_3CN$, and shake 1 min. Pipet 10.0 mL this soln into 100 mL vol. flask, dil. to vol. with $CH_3CN$, and mix well. Filter portion of soln and hold for LC analysis.

***E. Determination***

Adjust LC operating parameters to cause methiocarb to elute in 3.5–4.5 min. Adjust injection size and attenuation to give >60% FSD peaks for std soln. Make repetitive injections of std soln and calc. response ratios by dividing peak area (or ht) of methiocarb by that of internal std. Response ratios must agree within ±1%. Average duplicate response ratios obtained with std soln.

Inject duplicate aliquots of each sample soln. Average duplicate response ratios for each sample soln. Response ratios must agree within ±1%. If not, repeat detn, starting with std injections.

Re-inject std soln twice. Average response ratios of stds immediately preceding and following sample injection. These must agree within ±1%. If not, repeat detn.

***F. Calculation***

$$\text{Methiocarb, wt \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std solns, resp.; $W'$ and $W$ = wt (mg) of methiocarb std and sample, resp.; and $P$ = purity of methiocarb std (%).

Ref.: JAOAC **67**, 492(1984).

CAS-3566-00-5 (methiocarb)

## 984.11 Metribuzin in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1984**
**Final Action 1986**

(Method is suitable for tech. metribuzin and formulations.)

***A. Principle***

Sample is extd with $CH_2Cl_2$ contg di-*n*-butyl phthalate as internal std and metribuzin is detd by gas chromatgy.

***B. Apparatus and Reagents***

**(a)** *Gas chromatograph.*—Equipped with flame ionization detector (FID).

**(b)** *Chromatographic column.*—1–2 m × 2 mm id glass column packed with 3% OV-225 on 60–80 or 80–100 mesh Gas-Chrom Q, or equiv.

**(c)** *Di-n-butyl phthalate.*—Eastman No. 1403 or equiv. that contains no impurities eluting at retention time of metribuzin.

**(d)** *Reference std metribuzin.*—Mobay Corp.

***C. Preparation of Standards***

**(a)** *Internal std soln.*—Weigh 1.6 g di-*n*-butyl phthalate, dil. to1 L with $CH_2Cl_2$, and mix well.

**(b)** *Metribuzin std soln.*—Accurately weigh amt ref. std contg ca 200 mg ref. std metribuzin into ca 250 mL glass bottle. Add by pipet 100.0 mL internal std soln. Mix well.

***D. Preparation of Sample***

Shake liq. flowable formulations vigorously ≥1 min before sampling. Accurately weigh sample contg ca 200 mg metribuzin into glass bottle (ca 250 mL). Pipet in 100.0 mL internal std soln. Stopper and mech. shake or ultrasonify 1–5 min. Let insol. materials settle and use supernate for injection.

***E. Determination***

Adjust operating parameters to cause metribuzin to elute in 3–5 min. Maintain all parameters const thruout analysis. Typical values are as follows: temps (°)—inlet 250, column 210, detector 250; carrier gas 20–40 mL/min (either He or N); air and H flows as recommended for FID. Measure peak areas by electronic integration, or alternatively, peak hts. Retention times (min)—*N*-methyl isomer (impurity in tech. metribuzin) ca 1.5–2, di-*n*-butyl phthalate ca 2–3, metribuzin ca 3–5. If internal std and *N*-methyl isomer are not resolved on1 m column, substitute longer column, but do not exceed 2 m.

Make repetitive 2 µL injections of metribuzin std soln until response is stable and ratios of metribuzin peak area (or ht) to internal std peak area (or ht) for successive injections agree with ±1% of their mean.

Make duplicate 2 µL injections of each sample. Metribuzin/internal std ratios for 2 sample injections must agree within ±1% of their mean. If not, repeat detn, starting with std injections. After every 4–6 sample injections and after last sample injection, make 2 injections of calibration soln. Av. metribuzin std soln ratios preceding and following samples must lie ±1.0% of the mean; otherwise, repeat series of injections.

***E. Calculations***

Calc. ratios for each injection. Average 2 sample ratios and 4 std ratios (std injections immediately before and after sample injections).

$$\text{\% Metribuzin} = (R/R') \times (W'/W) \times P$$

where $R$ = av. sample ratio (metribuzin peak/internal std peak); $R'$ = av. std ratio (metribuzin peak/internal std peak); $W'$ = mg std; $W$ = mg sample; $P$ = % purity of metribuzin std.

Ref.: JAOAC **67**, 840(1984).

CAS-21087-64-9 (metribuzin)

## 982.08 Pirimicarb in Pesticide Formulations

### Gas Chromatographic Method

**First Action 1982**

(*Caution:* See safety notes on pipets, toxic solvents, pesticides, and chloroform.)

***A. Principle***

Pirimicarb is detd by gas chromatgy, using nonadecane as internal std and flame ionization detection. Peak areas are compared with that of std of known purity.

***B. Apparatus***

**(a)** *Gas chromatograph.*—With heated, glass-lined, injection port and flame ionization detector. Conditions given are for Hewlett-Packard Model 5710A. Other instruments may require changing operating parameters to obtain good resolution and response. Temps (°)—column 210, injection port 240, detector 250; gas flow rates (mL/min)—N carrier gas 40, H 60, air 240; attenuation 32 × 10; sample size 1.0 µL; retention times (min)—pirimicarb 6.8, internal std 8.9. Adjust parameters to assure complete sepn of peaks, and peak hts ca 60–80% full scale on chart at quoted retention times.

**(b)** *Column.*—1.8 m (6 ft) × 0.25 in. (od) × 2 mm (id) glass column packed with 10% silicone SE-30 on 100–120 mesh Chromosorb W(HP) (Applied Science). Silanize with 30 µL Silyl 8 (Pierce Chemical Co. and heat to 300° for 16 h before use.

***C. Reagents***

**(a)** *Nonadecane internal std soln.*—Accurately weigh ca 1 g nonadecane (Aldrich Chemical Co., Cat. No. N2890-6) and dissolve in 100 mL $CHCl_3$. Store in tightly capped bottle to avoid evapn. Check internal std soln for interfering components by injecting 1 µL into chromatograph.

(**b**) *Pirimicarb std soln.*—Accurately weigh ca 150 mg pirimicarb std of known purity (ICI Americas Inc.) into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake to dissolve pirimicarb. Store tightly capped to avoid evapn.

### D. Determination

(**a**) *Powder and technical material samples.*—Accurately weigh amt sample contg ca 150 mg pirimicarb into vial. Pipet 10.0 mL internal std soln into vial, cap, and shake to dissolve pirimicarb. Keep tightly capped to avoid evapn. Allow insoluble inerts to settle before use.

(**b**) *Granular formulations.*—Grind sample in mortar and pestle or mech. mill. Accurately weigh amt sample contg ca 150 mg pirimicarb into vial. Add 5.0 mL MeOH and mix to release pirimicarb. Add 10.0 mL internal std soln, cap, and shake to dissolve pirimicarb. Store tightly stoppered to avoid evapn. Allow insoluble inerts to settle before use.

Inject 2 or more aliquots of std soln to set integration parameters and stabilize instrument. Monitor response factor until results agree within 2%. Inject 4 aliquots of std soln and 2 aliquots of sample soln in succession. Calc. response factor, $R$, for each:

$$R = \text{area pirimicarb peak/area internal std peak}$$

$$\text{Pirimicarb, \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response factor for sample and std solns, resp.; $W$ and $W'$ = mg sample and std, resp.; and $P$ = purity (%) of std.

Ref.: JAOAC **64,** 1315(1981).

CAS-23103-98-2 (pirimicarb)

## 984.12 Propoxur Technical and Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1984**
**Final Action 1987**

***CIPAC-AOAC Method***

(Method is suitable for tech. propoxur and formulations with propoxur as only active ingredient.)

### A. Principle

Propoxur is detd by liq. chromatography using butyrophenone as internal std.

### B. Apparatus

(**a**) *Liquid chromatograph.*—Able to generate >1500 psi and measure $A$ at 280 nm.

(**b**) *Chromatographic column.*—250 × 4.6 mm id packed with ≤10 μm C18 bonded silica gel. (Partisil-10 ODS-3, Whatman Chemical Separations, Inc., or equiv., is suitable.) *LC operating conditions.*—Column temp. ambient; mobile phase $CH_3CN$-$H_2O$ (60+40); flow rate 1.5 mL/min (ca 1000 psi); chart speed 0.5 cm/min; injection vol. 20 μL; $A$ range 0.160 AUFS; retention times: propoxur ca 3.75 min, internal std ca 6.50 min. Pump LC mobile phase thru column until system is equilibrated (flat baseline). Allow 8 min between injections.

(**c**) *Rotary evaporator.*—Equipped with 40–50° $H_2O$ bath.

(**d**) *Soxhlet extractor.*—30 mm id with 25 × 80 mm cellulose extn thimbles.

(**e**) *Filters.*—0.45 μm porosity (Gelman Scientific, Inc., Acrodisc-CR, or equiv.).

### C. Reagents

(**a**) *Water.*—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc.).

(**b**) *Acetonitrile.*—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc.).

(**c**) *n-Butyrophenone internal std soln.*—6 g/200 mL $CH_3CN$.

(**d**) *Propoxur ref. std soln.*—Accurately weigh ca 300 mg ref. std (Mobay Corp.) into 50 mL vol. flask. Pipet 10.0 mL internal std into flask, dil. to vol. with $CH_3CN$, and mix well. Pipet 10.0 mL this soln into 100 mL vol. flask, dil. to vol. with $CH_3CN$, and mix well.

### D. Preparation of Sample

(**a**) *Technical and formulations excluding bait.*—Accurately weigh amt sample contg ca 300 mg propoxur into 50 mL vol. flask. Pipet 10.0 mL internal std into flask, dil. to vol. with $CH_3CN$, and shake 1 min. Pipet 10.0 mL this soln into 100 mL vol. flask, dil. to vol. with $CH_3CN$, and mix well. Filter portion of soln and hold for LC analysis.

(**b**) *Bait.*—Accurately weigh ca 13.5 g sample into extn thimble. Cover bait with glass wool. Ext 1 h in Soxhlet extractor contg 75 mL $CH_3CN$. Strip ext to oil on rotary evaporator (or steam bath). Using 30–35 mL $CH_3CN$, transfer all soluble material to 50 mL vol. flask. Pipet 10.0 mL internal std into flask, dil. to vol. with $CH_3CN$, and mix well. Pipet 10.0 mL this soln into 100 mL vol. flask, dil. to vol. with $CH_3CN$, and mix well. Filter portion of soln and hold for LC analysis.

### E. Determination

Adjust LC operating parameters to cause propoxur to elute in 3.5–4.5 min. Adjust injection size and attenuation to give >60% FSD peaks. Make repetitive injections of std soln and calc. response ratios by dividing peak area (or ht) of propoxur by that of internal std peak. Response ratios must agree within ±1%. Average duplicate response ratios obtained with std soln.

Inject duplicate aliquots of each sample soln. Average duplicate response ratios for each sample soln. Response ratios must agree within ±1%. If not, repeat detn, starting with std injections.

Re-inject std soln twice. Average response ratios of stds immediately preceding and following sample injection. These must agree within ±1%. If not, repeat detn.

### F. Calculation

$$\text{Propoxur, wt \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std solns, resp.; $W'$ and $W$ = wt (mg) of propoxur std and sample, resp.; and $P$ = purity of propoxur std (%).

Ref.: JAOAC **67,** 497(1984).

CAS-114-26-1 (propoxur)

## 981.04 Terbuthylazine in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1981**
**Final Action 1983**

***CIPAC-AOAC Method***

(*Caution:* See safety notes on pipets and pesticides.)

### A. Principle

Terbuthylazine is detd by gas chromatgy using di-*n*-pentyl phthalate as internal std. Identity is confirmed simultaneously by comparing retention times with std.

### B. Standard Solutions

(**a**) *Internal std soln.*—Weigh 4.0 ± 0.2 g di-*n*-pentyl phthalate, dil. to 1 L with $CH_2Cl_2$, and mix well. Std should be ≥98% pure and contain no impurities eluting at retention time of terbuthylazine.

(**b**) *Terbuthylazine calibration soln.*—Accurately weigh, in duplicate, ca 250 mg terbuthylazine ref. std of known purity (available from PAC-Switzerland, Swiss Federal Research Station, CH-8820 Waedenswil, Switzerland) into 100 mL g-s flasks. Pipet 50.0 mL internal std soln, (**a**), into each flask, stopper, and dissolve terbuthylazine by swirling.

### C. Preparation of Sample

Accurately weigh sample contg ca 250 mg terbuthylazine into 100 mL g-s flask. Pipet in 50.0 mL internal std soln, (**a**), stopper, and shake 3 min. Let insol. materials settle and use supernate for injection.

### D. Gas Chromatography

Use instrument equipped with flame ionization detector and 1.8 m × 2 mm id glass column packed with 3% Carbowax K 20M (Applied Science) on 80–100 mesh Gas-Chrom Q. Condition 24 h at 230° with N or He at ca 30 mL/min. Column must give baseline sepn between peaks of terbuthylazine and internal std; otherwise, prep. new column.

Operating conditions: temps (°)—inlet 250, column 210, detector 270; N or He carrier gas 35 mL/min; air and H as recommended for detector. For monitoring sepn, record chromatograms using suitable attenuation. Measure peak areas by electronic integration. Retention times (min)—terbuthylazine ca 6, di-*n*-pentyl phthalate ca 8.

### E. Calibration

Alternately inject 2 μL aliquots of the 2 calibration solns until calibration factors $F = W_c/R_c$ of 2 successive chromatograms vary ≤1%, where $W_c$ = mg terbuthylazine std for calibration solns $C_1$ and $C_2$, resp.; $R_c$ = peak area ratios of terbuthylazine/internal std. For the next steps, use only calibration soln $C_1$.

### F. Determination

Inject 2 μL calibration soln. Then make duplicate injections of sample followed by one injection of calibration soln. Individual calibration factors must lie within ±1%, otherwise repeat series of injections. Repeat for addnl samples.

### G. Calculation

Average calibration factors ($F$) preceding and following samples. Calc. and average peak area ratios of terbuthylazine/internal std of the 2 sample injections.

$$\% \text{ Terbuthylazine} = R_s \times F_{av.} \times P/W_s$$

where $R_s$ = av. peak area ratio of the 2 sample injections; $F_{av.}$ = av. calibration factor; $P$ = % purity of terbuthylazine std; and $W_s$ = mg sample.

### H. Identity Test

Identity of terbuthylazine is confirmed if differences of retention times between terbuthylazine and internal std of sample and calibration solns do not deviate >0.2 min.

Ref.: JAOAC **64,** 825(1981).

CAS-5915-41-3 (terbuthylazine)

## 974.05 Thiocarbamates in Herbicide Formulations

### Gas Chromatographic Method

**First Action 1974**
**Final Action 1988**

(Applicable to liq. and granular formulations of EPTC, butylate, molinate, cycloate, vernolate, and pebulate.)

(*Caution:* See safety notes on on pesticides.)

### A. Apparatus

(**a**) *Gas chromatograph.*—With flame ionization detector. Operating conditions: temps (°)—injection port 225, column 130 (EPTC and butylate), 170 (molinate), 140 (cycloate, vernolate), 150 (pebulate), detector 250; gas flows (mL/min)—N carrier 30–35, H 25–30, air 200–300 (or as specified by manufacturer).

(**b**) *Recorder.*—1 mv full scale sensitivity and 1 sec response.

(**c**) *Columns.*—6′ (1.8 m) × 0.25″ od, Pyrex, Al, or stainless steel, packed with 3% OV-17 on 60–80 mesh Gas-Chrom Q, or equiv. (for molinate), and 3% SE-30 or OV-1 on 60–80 mesh Gas-Chrom Q, or equiv. (for other 5 compds). Condition columns 12 hr at 250° under N flow of 30 mL/min.

### B. Preparation of Standards

(**a**) *Internal std solns.*—Accurately weigh ca 400 mg each ref. grade thiocarbamate (EPTC, cycloate, butylate, or pebulate; Stauffer Chemical Co., 1200 S 47th St, Richmond, CA 94804; or ICI Americas, Inc.) and transfer to sep. 100 mL vol. flasks. Dil. to vol. with $CS_2$-$CHCl_3$-MeOH (80 + 15 + 5), and mix thoroly.

(**b**) *Std solns.*—Accurately weigh ca 100 mg each ref. grade thiocarbamate into sep. 2 oz (50 mL) polyethylene-lined screw-cap, conical bottles. Add 25 mL internal std soln indicated below, and mix thoroly.

| Std soln | Approx. retention time, min | Internal std soln added | Approx. retention time, min |
|---|---|---|---|
| EPTC | 2.0 | Butylate | 2.4 |
| Molinate | 4.3 | Cycloate | 4.8 |
| Cycloate | 5.4 | Pebulate | 2.6 |
| Butylate | 2.4 | EPTC | 2.0 |
| Pebulate | 4.0 | Cycloate | 8.0 |
| Vernolate | 3.5 | Cycloate | 5.5 |

### C. Preparation of Sample

Accurately weigh sample contg ca 100 mg thiocarbamate into 2 oz (50 mL) polyethylene-lined screw-cap, conical bottle. Add 25 mL appropriate internal std soln, (**a**), as indicated in (**b**), and shake thoroly. Vigorously shake granular formulations 30 min on wrist-action shaker.

### D. Determination

Inject 2 μL clear supernate or soln into chromatograph preadjusted to appropriate conditions. Make triplicate injections of sample and appropriate std soln in random order. Det. peak areas, preferably with digital integrator.

Adjust sensitivity of gas chromatograph so that larger component or internal std peak is ca ¾ full scale.

### E. Calculations

$$\text{Wt \% compd} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. ratios of compd peak area to internal std peak area for sample and std solns, resp.; $W$ = g sample; $W'$ = g compd std in std soln; $P$ = % purity of compd.

Ref.: JAOAC **57,** 53(1974).

CAS-759-94-4 (EPTC)
CAS-1137-23-2 (cycloate)
CAS-2008-41-5 (butylate)
CAS-2212-67-1 (molinate)
CAS-1114-71-2 (pebulate)
CAS-1929-77-7 (vernolate)

## 966.08 Thiram in Pesticide Formulations

**Distillation Method**

**First Action 1966**
**Final Action 1977**

***CIPAC-AOAC Method***

(*Caution:* See safety notes on pesticides.)

### *A. Principle*

Thiram is decomposed by boiling with HOAc and $Zn(OAc)_2$ to $Me_2NH$, $CS_2$, and carbonyl sulfide. The gaseous mixt. is carried by air stream thru $CdSO_4$ scrubber to remove $H_2S$, and then into absorption system contg MeOH-KOH soln. Mixed xanthate-monothiocarbamate soln is neutzd and titrd with std aq. I.

Method is not specific for thiram. Sep. characterization test, **972.29G**, must be made.

### *B. Apparatus*

*Assembly and operating conditions.*—Assemble app. as shown in Fig. **966.08** with 30 mL $CdSO_4$ soln in first absorber, 25 mL KOH soln in second absorber, and 5 mL in each bubbler. Turn on condenser $H_2O$ and maintain $H_2O$ bath surrounding $CdSO_4$ scrubber at 70–80° thruout test. Keep main KOH absorber at <25° by immersion in beaker of cold $H_2O$. Absorber must be dry or rinsed with MeOH before adding KOH soln. Air bleed must reach nearly to bottom of digestion flask. Make all joints gas-tight, using small amts $H_3PO_4$, petrolatum, or silicone grease.

Check app. for absorber leaks and efficiency periodically, using pure Na diethyldithiocarbamate. Recoveries should be 99–101%. Check purity of Na diethyldithiocarbamate by dissolving ca 0.5 g, accurately weighed, in 100 mL $H_2O$ and titrg directly with 0.1*N* I, using ca 2% starch soln as indicator. 1 mL 0.1*N* I = 0.02253 g Na diethyldithiocarbamate. % Na diethyldithiocarbamate = 2.253 × mL 0.1*N* I/g sample.

### *C. Reagents*

(**a**) *Acid mixture.*—Dissolve 2.5 g ZnO in 100 mL HOAc (1+1).

(**b**) *Cadmium sulfate soln.*—Dissolve 18.5 g $3CdSO_4.8H_2O$ in 100 mL $H_2O$.

(**c**) *Potassium hydroxide soln.*—2*N* in MeOH and contg <1 ppm Cu or Fe.

(**d**) *Iodine std soln.*—0.1*N*. Stdze as in **939.13B**.

### *D. Determination*

Accurately weigh and transfer sample contg ca 0.3 g thiram to digestion flask, using small amt $H_2O$, if necessary. Assemble air bleed and dropping funnel, Fig. **966.08**, and add 20 mL acid mixt. thru funnel. Connect app. to controlled aspiration (vac. or compressed air) so that ca 3 bubbles/sec pass thru absorbers. After sample is evenly dispersed, heat and reflux 30 min at moderate rate. Turn off cooling $H_2O$ and flush condenser and first absorber with steam from flask ≤1 min. Remove burner and disconnect train. Wash contents of KOH absorber and bubblers into 600 mL beaker with 300–400 mL $H_2O$, add 1–2 drops phthln, just neutze with HOAc (1+9) from buret, and add 3 drops excess. With continual stirring, titr. immediately (preferably within 1 min, as decomposition of mixed xanthate/monothiocarbamate soln is extremely rapid under acidic conditions) with 0.1*N* I (*t* mL), using ca 2% starch soln as indicator. Det. blank in same manner, omitting sample (*b* mL). 1 mL 0.1*N* I = 0.01202 g thiram.

$$\% \text{ Thiram} = 1.202\ (t - b)/\text{g sample}$$

Refs.: J. Sci. Food Agric. **15,** 509(1964). JAOAC **49,** 40(1966); **51,** 447(1968).

CAS-137-26-8 (thiram)

## 971.08 Triazines in Pesticide Formulations

**Gas Chromatographic Method**

**First Action 1971**
**Final Action 1976**

***AOAC-CIPAC Method***

(*See* Table **971.08** for applicability to specific compds.)

### *A. Standard Solutions*

(*Caution:* See safety notes on pipets and pesticides.)

(**a**) *Dieldrin internal std soln.*—Std should be ≥90% pure and contain no impurities eluting at retention time for pesticide being detd. (*1*) *For propazine.*—Weigh 14.0 ± 0.1 g tech. dieldrin, dissolve in ca 300 mL $CHCl_3$, and dil. to 1 L with $CHCl_3$. (*2*) *For other compounds.*—Weigh 2.00 ± 0.02 g tech. dieldrin, dissolve in ca 200 mL $CHCl_3$, and dil. to 250 mL with $CHCl_3$.

(**b**) *Aldrin internal std soln.*—(For Diazinon®.) Weigh 4.0 ± 0.1 g tech. aldrin into 600 mL beaker. Slurry with 400 mL acetone to dissolve, filter thru paper into 1 L vol. flask, washing with several 100 mL portions acetone, and dil. to vol. Std should be ≥90% pure and contain no impurities eluting at retention time of Diazinon.

(**c**) *Dibenzyl succinate internal std soln.*—(For chlorobenzilate and chloropropylate.) Weigh 5.0 ± 0.1 g dibenzyl succinate, dissolve in ca 300 mL acetone, and dil. to 1 L with

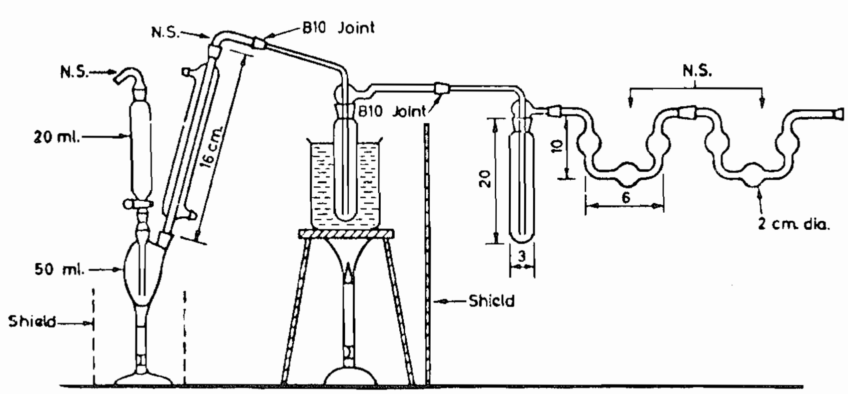


**FIG. 966.08—Absorption system for thiram. Dimensions in cm; N.S. = nonstandard; B10 = $\mathbb{T}$ 10/30**

acetone. Std should be >98% pure and contain no impurities eluting at retention time for pesticide being detd.

(**d**) *Pesticide std solns*.—Accurately weigh 250 mg (125 mg for Diazinon and 150 mg for simazine) of ref. std of pesticide being detd (available from Ciba-Geigy Corp., PO Box 11422, Greensboro, NC 27409) into 4 oz (125 mL) round bottle with Al-lined screw cap. Pipet in 50 mL internal std soln (*see* Table **971.08**) and shake mech. 30 min.

(**e**) *Dioctyl phthalate internal std soln*.—(For simazine.) Weigh 3.0 ± 0.1 g tech. dioctyl phthalate, dissolve in ca 200 mL DMF, and dil. to 1 L with DMF. (*Caution:* See safety notes on dimethylformamide.) Std should be >98% pure and contain no impurities eluting at retention time of simazine.

### *B. Preparation of Sample*

Accurately weigh amt sample specified in Table **971.08** into 4 oz (125 mL) round bottle with Al-lined screw cap. Pipet in same vol. internal std used for prepn of std soln, (**d**), and shake mech. 30 min. Let insol. materials settle or centrf. portion of ext to obtain clear soln.

### *C. Gas Chromatography*

Use instrument equipped with flame ionization detector and 4 mm id glass column (length specified in Table **971.08**) packed with 3% Carbowax 20M (Applied Science) on 80–100 mesh Gas-Chrom Q. (For Diazinon, use 10% silicone DC-200 viscosity 12500.) Condition 24 hr at 240° with N or He at ca 40 mL/min. Column should have ≥2000 (≥1500 for chlorobenzilate, chloropropylate, propazine, and simazine) theoretical plates (*see* **973.12B**(a)).

Operate at following conditions: temps—as specified in Table **971.08**; N or He carrier gas, 80–100 mL/min; air and H, 80–100 mL/min; attenuation varied so that peak hts of pesticide and internal std are 60–80% full scale. Retention times are specified in Table **971.08**. (Ametryn and dieldrin peaks must be resolved. Prep. new column if variation of flow rate or temp. does not resolve peaks. Resolution may be improved by increasing column temp.)

### *D. Determination*

Inject 3 μL aliquots std soln until peak ht ratio of pesticide:internal std varies ≤1% for successive injections. Then make duplicate injections of sample followed by duplicate injections of std. Peak ht ratios of stds must be within ±1% of first accepted std values or repeat series of injections. Repeat for addnl samples.

### *E. Calculations*

Calc. peak ht ratios for both duplicate std injections preceding and following samples. Average the 4 values ($R'$). Calc. and average peak ht ratios of the 2 samples ($R$).

$$\% \text{ Pesticide} = (R/R') \times (W'/W) \times P$$

where $W$ and $W'$ = mg sample and std, resp.; and $P$ = % purity of std.

Refs.: JAOAC **54,** 450, 452(1971); **56,** 586(1973); **58,** 513, 516(1975); **59,** 758(1976).

## MISCELLANEOUS PESTICIDES

## 967.06 Amitrole in Pesticide Formulations

**Titrimetric Method**

**First Action 1967**
**Final Action 1973**

(*Caution:* See safety notes on pipets and pesticides.)

### *A. Preparation of Sample Solution*

(**a**) *50% Dry powder formulation*.—Transfer 10.00 g sample to 100 mL g-s vol. flask, using powder funnel. Add 50

**Table 971.08 Chemical and Gas Chromatographic Parameters for Triazines and Other Pesticides**

| Chemical Name | Common or Trade Name | CA Registry No. | Internal Std Soln 971.08A | Wt Sample | Length Column (m) | Temperature (°) Inlet | Temperature (°) Column | Temperature (°) Detector | Retention Times (min) Pesticide | Retention Times (min) Internal Std |
|---|---|---|---|---|---|---|---|---|---|---|
| 2-(Ethylamino)-4-(isopropylamino)-6-(methylthio)-*s*-triazine | Ametryn | 834-12-8 | (**a**)(*2*) | 300 mg 80% wettable powder | 1.8 | 240 | 215±15 | 240 | 8–12 | 9–15 |
| 2-Chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine | Atrazine | 1912-24-9 | (**a**)(*2*) | 300 mg 80% wettable powder | 1.8 | 240 | 200±10 | 240 | 5–7 | 9–15 |
| Ethyl-4,4′-dichlorobenzilate | Chlorobenzilate | 510-15-6 | (**c**) | 500 mg liq. formulation | 1.2 | 260 | 230±10 | 260 | 5–8 | 8–10 |
| Isopropyl-4,4′-dichlorobenzilate | Chloropropylate | 5836-10-2 | (**c**) | 1 g liq. formulation | 1.2 | 260 | 230±10 | 260 | 4–6 | 8–10 |
| *O,O*-Diethyl-*O*-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate | Diazinon | 333-41-5 | (**b**) | Sample contg 110 mg | 1.8 | 240 | 190±10 | 240 | 5–6 | 10–12 |
| 2,4-Bis(isopropylamino)-6-methoxy-*s*-triazine | Prometon | 1610-18-0 | (**a**)(*2*) | 1 g liq. formulation | 1.8 | 240 | 200±20 | 240 | 3–5 | 9–15 |
| 2,4-Bis(isopropylamino)-6-(methylthio)-*s*-triazine | Prometryn | 7287-19-6 | (**a**)(*2*) | 300 mg 80% wettable powder | 1.8 | 240 | 200±10 | 240 | 6–8 | 9–15 |
| 2-Chloro-4,6-bis(isopropylamino)-*s*-triazine | Propazine | 139-40-2 | (**a**)(*1*) | 300 mg 80% wettable powder | 1.2 | 250 | 210±10 | 240 | 3–5 | 7–9 |
| 2-Chloro-4,6-bis(ethylamino)-*s*-triazine | Simazine | 122-34-9 | (**e**) | 190 mg 80% wettable powder | 1.8 | 250 | 210±5 | 250 | 6–8 | 10–14 |
| 2-(*tert*-Butylamino)-4-chloro-6-(ethylamino)-*s*-triazine | Terbuthylazine | 5915-41-3 | | | | | | | | |
| 2-(*tert*-Butylamino)-4-(ethylamino)-6-(methylthio)-*s*-triazine | Terbutryn | 886-50-0 | (**a**)(*2*) | 300 mg 80% wettable powder | 1.8 | 240 | 200±20 | 240 | 8–10 | 9–15 |

mL DMF. Shake 2–3 min to dissolve amitrole. (Undissolved amitrole is powder and can be differentiated visually from inerts which are usually crystals.) Let settle and carefully decant supernate into 100 mL vol. flask. Repeat extn of residue with three 15 mL portions DMF, letting settle each time before decanting into vol. flask. Dil. combined exts to vol. with DMF and shake well. Filter 40–50 mL thru fritted glass filter of medium porosity. Pipet 25 mL into 400 mL beaker contg 50 mL $H_2O$.

**(b)** *90% Dry powder formulation.*—Dissolve 1.0000 g sample in 100 mL $H_2O$ in 400 mL beaker.

**(c)** *Aqueous amitrole.*—Pipet 5 mL sample into 400 mL beaker contg 50 mL $H_2O$.

### *B. Determination*

Adjust sample soln or dild aliquot to pH 1.8 with 0.5*N* HCl. Stir mech. and titr. with 0.5 mL increments 0.5*N* NaOH to pH 3.5–4.0 using pH meter. Add 0.5*N* NaOH rapidly to pH 6.5 and then dropwise to pH 7.5 (second inflection point). Plot pH against mL 0.5*N* NaOH and det. first inflection point (occurs at pH 2.5–2.9).

$$\% \text{ Amitrole by wt} = (B - C) \times 0.5 \times 8.408/F$$

where $C$ = mL 0.5*N* NaOH required to titr. to first inflection point; $B$ = mL 0.5*N* NaOH required to titr. to pH 7.5; and $F$ = 2.5 for 50% dry powder formulation, (**a**), g sample for 90% dry powder formulation, (**b**), and 5.0 × sp gr sample for aq. amitrole, (**c**).

$$\text{lb Amitrole in aq. amitrole/U.S. gal.} = \% \text{ amitrole} \times \text{sp gr} \times 8.32/100$$

Ref.: JAOAC **50,** 568(1967).

CAS-61-82-5 (amitrole)

## 983.11 Brodifacoum (Technical) and Pesticide Formulations

### Liquid Chromatographic Method

**First Action 1983**
**Final Action 1989**

(Not applicable to wax bait formulations)

### *A. Principle*

Weighed sample of tech. brodifacoum, brodifacoum conc., or bait ext is dissolved in triphenylbenzene internal std soln and detd by reverse phase liq. chromatgy and UV detection.

### *B. Apparatus*

**(a)** *Liquid chromatograph.*—Flow rate 1 mL/min; loop injection 10 μL; mobile solv. MeOH–$H_2O$–HOAc (94.2 + 5.0 + 0.8), filtered and degassed; UV detection at 254 nm with range to give peak hts ca 60–80% full scale. Retention times (min)—brodifacoum 6.2, internal std 11.7.

**(b)** *Column.*—25 cm × 4.6 mm Zorbax ODS 5 μm reverse phase column (E.I. DuPont de Nemours & Co., Instrument Products Div.).

**(c)** *Centrifuge.*—Equipped with 15 mL capped tubes.

**(d)** *Macerator.*—With 400 mL stainless steel cup/impeller assembly, such as Sorvall Omnimixer (DuPont Instruments Inc.).

**(e)** *Rotary evaporator.*—Fitted with vac. and cold $H_2O$ supplies.

**(f)** *Filter.*—Spread 10 g Celite 545 on 9 cm No. 5 filter paper wetted with MeOH in buchner. Press filter and prewash with 30 mL MeOH.

### *C. Reagents*

**(a)** *1,3,5-Triphenylbenzene internal std soln.*—Accurately weigh ca 100 mg pure 1,3,5-triphenylbenzene into 500 mL vol. flask, dissolve in 200 mL $CH_2Cl_2$, and dil. to vol. with MeOH.

**(b)** *Brodifacoum std soln.*—Accurately weigh ca 100 mg brodifacoum std of known purity (ICI Americas Inc.) into 100 mL vol. flask. Dissolve in 40 mL $CH_2Cl_2$. Dil. to vol. with MeOH. Transfer 10.0 mL each of brodifacoum std soln and internal std soln to 50 mL vol. flask and dil. to vol. with dilg soln.

**(c)** *Diluting soln.*—$CH_2Cl_2$–MeOH (2 + 3).

**(d)** *Extracting soln.*—$CH_2Cl_2$–formic acid (50 + 1).

Store reagents in tightly capped dark bottles to avoid evapn and decomposition. Check internal std soln for interfering components by injecting 10 μL into liq. chromatograph.

### *D. Determination*

**(a)** *Technical material.*—Accurately weigh ca 100 mg sample into 100 mL vol. flask. Dissolve in 40 mL $CH_2Cl_2$. Dil. to vol. with MeOH. Transfer 10.0 mL each of sample soln and internal std soln to 50 mL vol. flask. Dil. to vol. with dilg soln.

**(b)** *Powder concentrate.*—Accurately weigh amt sample contg ca 5 mg brodifacoum into 250 mL capped conical flask. Add 100 mL extg soln and shake 1 min. Filter thru Celite, using two 30 mL washes of extg soln. Evap. filtrate at 60° under vac. Dissolve residue in 20.0 mL dilg soln and 5.0 mL internal std soln.

**(c)** *Liquid concentrate.*—Accurately weigh amt sample contg ca 5 mg brodifacoum into 25 mL vol. flask. Add 5.0 mL internal std soln, and dil. to vol. with dilg soln.

**(d)** *Pelleted bait.*—Grind amt sample contg ca 2 mg brodifacoum in anal. mill. Transfer to tared macerator cup and accurately weigh. Add 250 mL extg soln and homogenize 10 min. Filter thru Celite using three 50 mL washings of extg soln. Rotary-evap. filtrate at 60° under vac. Dissolve residue in 8.0 mL dilg soln and 2 mL internal std soln. Centrf. sample to remove remaining solids.

Inject 2 or more aliquots of std soln into liq. chromatograph to set integration parameters and stabilize instrument. Monitor response factor until results agree within 2%. Inject 4 aliquots of std soln and 2 aliquots of sample soln in succession. Calc. response factor, $R$, for each:

$$R = \text{area brodifacoum peak/area internal std peak}$$

Peak hts can be used in place of peak areas for tech. material and bait formulations, but not for liq. formulations.

$$\text{Brodifacoum, } \% = (R/R') \times (W'/W) \times P \times F$$

where $R$ and $R'$ = av. response factor for sample and std solns, resp.; $W$ and $W'$ = mg sample and std, resp.; $P$ = purity (%) of std; $F$ = scaling factor = 1 for technical material, $^1/_{20}$ for powder and liq. concs, $^1/_{50}$ for pelleted bait.

Ref.: JAOAC **66,** 993(1983).

CAS-56073-10-0 (brodifacoum)

## 988.02 Cyhexatin Technical and in Pesticide Formulations

**Liquid Chromatographic Method**

**First Action 1988**

***CIPAC-AOAC Method***

### A. Principle

Sample is extd with *n*-decylbenzene internal std soln contg HOAc, MeOH, and $H_2O$. Cyhexatin is detd by liq. chromatgy using peak ht for quantitation.

### B. Apparatus

(**a**) *Liquid chromatograph.*—With peak ht integrator or recorder, 10 μL sample loop, and detector at 214 nm. Operating conditions: column ambient; flow rate 2.0 mL/min; *A* range 1.0 AUFS; injection vol. 10 μL; retention times, cyhexatin ca 7 min and internal std ca 10 min. Adjust parameters to give peak ht for cyhexatin ca 75% full scale.

(**b**) *Chromatographic column.*—ODS bonded silica, 10 μm particle size, stainless steel, 25 cm × 4.6 mm id (E. Merck, available from Curtin Matheson Scientific, Inc., or VWR Scientific), or equiv.

### C. Reagents

(**a**) *Methanol.*—LC grade,

(**b**) *Acetic acid.*—Glacial.

(**c**) *HCl soln.*—1M.

(**d**) *Sodium chloride.*—Analytical reagent grade.

(**e**) *Mobile phase.*—MeOH-$H_2O$-HCl-NaCl (93 + 7 + 0.0001M + 0.005M). In 1 L g-s flask, combine 1 mL 1M HCl, 69 mL $H_2O$, and 0.29 g NaCl. Add 930 mL MeOH, mix, and degas.

(**f**) *n-Decylbenzene.*—Eastman Laboratory Chemicals No. 9195 (Eastman Kodak Co.), or equiv.

(**g**) *Cyhexatin reference std.*—Available from Dow Chemical Co.

### D. Preparation of Standards

(**a**) *n-Decylbenzene internal std soln.*—Weigh 1.0 g *n*-decylbenzene into 1 L vol. flask. Add 49 mL $H_2O$ and 1 mL HOAc. Dil. to vol. with MeOH and sonicate until dissolved.

(**b**) *Cyhexatin std soln.*—Accurately weigh ca 110 mg pure cyhexatin ref. std into 150 mL g-s flask. Add by pipet 100 mL internal std soln, shake well, sonicate for 10 min, and cool to ambient temp.

### E. Preparation of Sample

(**a**) *Technical material.*—Accurately weigh ca 120 mg sample into 150 mL g-s flask. Add by pipet 100 mL internal std soln, shake well, and sonicate for 10 min. Cool to ambient temp. and centrifuge at 2000 rpm.

(**b**) *Wettable powder.*—Accurately weigh sample contg 110 mg cyhexatin into 150 mL g-s flask. Proceed as for technical material, beginning, "Add by pipet. . ."

(**c**) *Suspension concentrate.*—Accurately weigh sample contg ca 120 mg cyhexatin into 150 mL g-s flask. Add 10 mL $H_2O$ and swirl until completely homogeneous. Proceed as for technical material, beginning. "Add by pipet. . ."

### F. Determination

Inject 10 μL std solns until response ratio (cyhexatin peak ht/internal std peak ht) varies <2%. Make 2 sample injections followed by 1 std injection. Average peak ht ratios of stds immediately preceding and following sample injections, and average peak ht ratios of the 2 samples. Calc. cyhexatin as follows:

$$\text{Cyhexatin, \%} = (R/R') \times (W'/W) \times P$$

where *R* and *R'* = av. peak ht ratios for sample and std, resp.; *W'* = g cyhexatin in std soln; *W* = g sample extd for analysis; and *P* = % purity of std.

Ref.: JAOAC **71,** 26(1988).

CAS-13121-70-5 (cyhexatin)

## 969.08 Diquat in Pesticide Formulations

**Spectrophotometric Method**

**First Action 1969**
**Final Action 1972**

***AOAC-CIPAC Method***

### A. Reagents

(**a**) *Acetate buffer soln.*—pH 4.05. Dissolve 10.88 g $NaOAc.3H_2O$ in $H_2O$, add 19 mL HOAc, dil. to 2 L with $H_2O$, and mix.

(**b**) *Diquat std solns.*—(*1*) *Stock soln.*—0.2 mg diquat/mL. Prep. stock soln by dissolving 0.1968 g pure diquat dibromide monohydrate ($C_{12}H_{12}N_2Br_2.H_2O$, MW 362.1; 50.87% cation; ICI Americas, Inc.) in buffer soln, dil. to 500 mL with buffer soln, and mix. (*2*) *Working soln.*—0.02 mg diquat/mL. Dil. 10.0 mL stock soln to 100 mL with buffer soln. Prep. dild stds fresh as required.

### B. Determination

Using buret, transfer 10.0, 20.0, and 30.0 mL std diquat soln, contg 0.2, 0.4, and 0.6 mg diquat, resp., to three 100 mL vol. flasks, dil. each soln to vol. with buffer soln, and mix. Measure *A* of stds at 310 nm in 1 cm silica cell, with buffer soln as ref., and draw std curve relating *A* to mg diquat.

Accurately weigh portion (*w* g) of well mixed sample contg ca 0.5 g diquat, transfer to 250 mL vol. flask, dil. to vol. with buffer soln, and mix (*Soln 1*). Transfer 10.0 mL *Soln 1* to 200 mL vol. flask, dil. to vol. with buffer soln, and mix (*Soln 2*). Transfer 5.0 mL *Soln 2* to 100 mL vol. flask, dil. to vol. with buffer soln, and mix (*Soln 3*).

Measure *A* of *Soln 3* at 310 nm in 1 cm silica cell, with buffer soln as ref. Read diquat content of *Soln 3* (*y* mg) directly from std curve or calc. diquat content by interpolation.

$$\text{\% Diquat, w/w} = 100\ y/w$$

Refs.: Analyst **92,** 375(1967). JAOAC **51,** 1304, 1306(1968).

CAS-2764-72-9 (diquat)

## 970.07 Dodine in Pesticide Formulations

**Titrimetric Method**

**First Action 1970**
**Final Action 1971**

(*Caution:* See safety notes on acetic acid, perchloric acid, and pesticides.)

### A. Reagents

(**a**) *Perchloric acid.*—0.05*N*. Dissolve 4.2 mL 72% $HClO_4$ in HOAc and dil. to 1 L with HOAc. Stdze as follows: Accurately weigh 0.200 g $KHC_8H_4O_4$ into 250 mL erlenmeyer. Dissolve in 20 mL HOAc by gently heating flask on hot plate. Add 80 mL $Ac_2O$ and 8 drops metanil yellow indicator, (**b**). Place erlenmeyer contg bar on mag. stirrer and titr. with $HClO_4$

to first definite red (magenta). Titr. reagent blank and correct sample titer.

$$\text{Normality} = 0.200/(0.20422 \times \text{net mL } HClO_4)$$

(**b**) *Metanil yellow*.—0.20%. Dissolve 0.200 g metanil yellow powder in 100 mL MeOH.

(**c**) *Potassium acid phthalate*.—NIST SRM $KHC_8H_4O_4$.

### B. Determination

Accurately weigh sample contg ca 0.600 g dodine into 250 mL erlenmeyer. Add 10 mL HOAc followed by 90 mL $Ac_2O$. Mix by swirling 5 min. Filter slurry with vac. thru large, medium porosity fritted glass buchner into 250 mL vac. flask. Wash erlenmeyer and residue in funnel with two 10 mL portions HOAc-$Ac_2O$ (10 + 90). Place vac. flask contg bar on mag. stirrer, add 8 drops metanil yellow indicator, and titr. with stdzd ca 0.05$N$ $HClO_4$ to first definite red (magenta). Titr. reagent blank and correct sample titer.

% Dodecylguanidine acetate
$$= (\text{net mL } HClO_4 \times \text{normality} \times 28.75)/\text{g sample}$$

Ref.: JAOAC **52,** 1292(1969).

CAS-2439-10-3 (dodine)

## 898.01 Formaldehyde in Pesticide Formulations

### Hydrogen Peroxide Method

### Final Action

(Applicable only to solns)

### A. Reagents

(**a**) *Sulfuric acid std soln*.—1$N$. Prep. and stdze as in **890.01.**

(**b**) *Sodium hydroxide std soln*.—1$N$. Stdze against (**a**), using litmus or bromothymol blue indicator. 1 mL = 30.03 mg HCHO.

(**c**) *Hydrogen peroxide soln*.—Com., contg ca 3% $H_2O_2$. If acid, neutze with NaOH, (**b**), using litmus or bromothymol blue indicator.

(**d**) *Litmus indicator*.—Soln. of purified litmus of such concn that 3 drops gives distinct blue color to 50 mL $H_2O$.

(**e**) *Bromothymol blue indicator*.—Dissolve 1 g bromothymol blue in 500 mL alcohol, 50% by vol.

### B. Determination

Pipet 50 mL 1$N$ NaOH soln into 500 mL erlenmeyer and add 50 mL $H_2O_2$, (**c**). Add weighed amt sample (ca 3 g) from weighing pipet, letting point of pipet reach nearly to liq. in flask. Place funnel in neck of flask and heat on steam bath 5 min, shaking occasionally. Remove from bath, wash funnel with $H_2O$, cool to room temp., and titr. excess NaOH with std acid, using bromothymol blue or litmus. (Cool flask before titrn to obtain sharp end point with litmus.) From mL 1$N$ NaOH used and wt sample, calc. % HCHO according to following equation

$$NaOH + HCHO + H_2O_2 = HCOONa + 2H_2O$$

If HCHO soln contains appreciable free acid, titr. sep. portion and calc. acidity as % HCOOH. Correct for this acidity in calcg % HCHO.

Refs.: Ber. **31,** 2979(1898). J. Am. Chem. Soc. **27,** 1183(1905). USDA Bur. Chem. Bull. **99,** p. 30; **132,** p. 49; **137,** p. 47.

CAS-50-00-0 (formaldehyde)

## 897.01 Formaldehyde in Pesticide Formulations

### Cyanide Method

### Final Action

(Applicable only to dil. solns)

Treat 15 mL 0.1$N$ $AgNO_3$, **941.18A–C**, with 6 drops $HNO_3$ (1+1) in 50 mL vol. flask, add 10 mL *KCN soln* (3.1 g in 500 mL $H_2O$), dil. to vol., shake well, filter thru dry filter, and titr. 25 mL filtrate with 0.1$N$ $NH_4SCN$, **942.26**, as in **915.01B**. Acidify another 15 mL portion 0.1 $N$ $AgNO_3$ with 6 drops $HNO_3$ (1 + 1) and treat with 10 mL of the KCN soln to which has been added measured amt of sample (wt calcd from sp gr) contg $\leq$25 mg HCHO. Dil. to 50 mL, filter, and titr. 25 mL aliquot with the 0.1$N$ $NH_4SCN$ as before. Difference between mL $NH_4SCN$ used in these 2 titrns × 2 = mL 0.1$N$ $NH_4SCN$ corresponding to KCN used by the HCHO. Calc. % HCHO present. 1 mL 0.1$N$ $NH_4SCN$ = 3.003 mg HCHO.

Refs.: Z. Anal. Chem. **36,** 18(1897). USDA Bur. Chem. Bull. **132,** p. 49.

## 931.03 Formaldehyde in Seed Disinfectants

### Final Action

(Applicable to detn of HCHO absorbed in inert carrier, e.g., bentonite, talc, charcoal, sawdust)

Weigh ca 5 g sample contg 0.3–0.5 g HCHO in weighing bottle and transfer to 800 mL Kjeldahl flask. Add 25 mL $H_2O$ and 12 mL $H_2SO_4$ (1 + 4). Steam distil rapidly, passing vapors thru condenser with delivery end dipping into 25 mL $H_2O$ in 500 mL vol. flask. Collect ca 450 mL distillate, keeping vol. in distg flask nearly const with aid of small flame. After distn, wash delivery tube, and dil. distillate to vol. with $H_2O$.

Into each of two 200 mL vol. flasks measure 20 mL 0.1$N$ $AgNO_3$. To each flask add 12 drops $HNO_3$ (1 + 1) and 30 mL $H_2O$. To one flask add slowly, with const shaking, 30 mL *KCN soln* (3.1 g in 1 L $H_2O$). Dil. to vol., shake well, and filter thru dry filter. To 100 mL filtrate add 3 mL $HNO_3$ and 5 mL ferric indicator, **929.04A(e)**, and titr. with 0.1$N$ KSCN.

Pipet 25 mL HCHO distillate into small beaker contg 30 mL of the KCN soln, mix well, and add slowly, with const shaking, to second flask contg the acidified $AgNO_3$ soln. Dil. to vol. with $H_2O$, filter, acidify 100 mL filtrate with 3 mL $HNO_3$, and titr. with the KSCN soln, using $FeNH_4(SO_4)_2$ indicator.

Difference between mL KSCN soln used in these 2 titrns × 2 = mL 0.1$N$ KSCN equiv. to HCHO. Calc. % HCHO present. 1 mL 0.1$N$ KSCN = 3.003 mg HCHO.

Refs.: Ind. Eng. Chem. Anal. Ed. **3,** 357(1931). JAOAC **25,** 80, 668(1942).

CAS-50-00-0 (formaldehyde)

## 986.08 Oxythioquinox in Pesticide Formulations

### Liquid Chromatographic Method

### First Action 1986

### Final Action 1989

### *AOAC-CIPAC Method*

(Method is suitable for tech. oxythioquinox and formulations with oxythioquinox as only active ingredient.)

### A. Principle

Sample with 1-phenyl-1-pentanone internal std is extd with $CH_3CN$, and oxythioquinox is detd by reverse phase liq. chromatgy.

### B. Apparatus and Reagents

(a) *Liquid chromatograph*.—Able to generate >10 MPa (>1430 psi) and measure $A$ at 280 nm. Operating conditions: column temp. ambient; flow rate 2 mL/min (ca 5 MPa); chart speed 0.5 cm/min; injection vol. 10 μL; $A$ range 0.320 AUFS; retention times: 1-phenyl-1-pentanone ca 3.1 min, oxythioquinox ca 5.4 min. Pump LC mobile phase thru column until system is equilibrated (flat baseline). Allow each injection ca 7 min run time, then pump $CH_3CN$ ca 4 min to remove impurities. Pump LC mobile phase ca 4 min, allowing system to re-equilibrate before next injection.

(b) *Chromatographic column*.—250 × 4.6 mm id packed with ≤10 μm C18 bonded silica gel.

(c) *Acetonitrile*.—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).

(d) *Chloroform*.—Spectrophtric grade or equiv.

(e) *Filters*.—0.45 μm porosity (Gelman Acrodisc-CR, or equiv.).

(f) *1-Phenyl-1-pentanone (n-valerophenone) internal std soln*.—1 g/100 mL $CHCl_3$.

(g) *Reference std oxythioquinox*.—Mobay Corp.

(h) *Water*.—LC grade or distd in glass (Burdick & Jackson Laboratories, Inc., or equiv.).

(i) *LC mobile phase*.—$CH_3CN$-$H_2O$ (80 + 20).

### C. Preparation of Standard

Accurately weigh ca 100 mg ref. std into 100 mL vol. flask. Pipet 10 mL internal std soln into flask and swirl to mix. Add ca 50 mL $CH_3CN$, sonicate 4 min, dil. to vol. with $CH_3CN$, and mix well. Filter portion of soln for LC analysis.

### D. Preparation of Sample

Accurately weigh amt of sample contg ca 100 mg oxythioquinox into 100 mL vol. flask. Pipet 10 mL internal std soln into flask, and swirl to mix. Add ca 50 mL $CH_3CN$, sonicate 4 min, dil. to vol. with $CH_3CN$, and mix well. Filter portion of soln for LC analysis.

### E. Determination

Adjust operating parameters to elute oxythioquinox in 5.0–5.9 min. Adjust injection size and attenuation to give largest possible on-scale peaks. Make repetitive injections of ref. std soln and calc. response ratios ($R$) = oxythioquinox peak area (or ht)/internal std peak area (or ht). Response ratios must agree ±1%. Average duplicate response ratios obtained with ref. std soln.

Inject duplicate aliquots of each sample soln. Average response ratios for each sample soln. Response ratios must agree ±1%. If not, repeat detn, starting with std injections.

Re-inject ref. std soln twice. Average response ratios of stds immediately preceding and following sample injections. These must agree ±1%. If not, repeat detn.

### F. Calculations

$$\text{Oxythioquinox, wt\%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std solns, resp.; $W'$ $W$ = wt (mg) of oxythioquinox std and sample, resp.; $P$ = % purity of std oxythioquinox.

Ref.: JAOAC **69,** 490(1986).

CAS-2439-01-2 (oxythioquinox)

## 969.09 Paraquat in Pesticide Formulations

**Spectrophotometric Method**

**First Action 1969**
**Final Action 1971**

### A. Reagents

(a) *Sodium dithionite*.—1% soln in 0.1$N$ NaOH. (Sodium dithionite, $Na_2S_2O_4.2H_2O$, is also called sodium hydrosulfite and sodium hyposulfite.) Do *not* keep soln >3 hr; solid is unstable in presence of moisture. Store solid in small air-tight bottles in vac. desiccator.

(b) *Paraquat std soln*.—0.25 mg paraquat/mL. Dry anal. std (ICI Americas, Inc.) to const wt at 100–120° before weighing (paraquat salts are hygroscopic). Dissolve 0.1728 g paraquat dichloride (72.40% cation) in $H_2O$, dil. to 500 mL with $H_2O$, and mix. Prep. soln fresh as required.

(c) *Extracting soln*.—Dissolve 11 g $Na_2SO_4.10H_2O$ in 500 mL $H_2O$, add 500 mL alcohol, and mix.

### B. Preparation of Standard Curve

Pipet 50 mL std soln into 250 mL vol. flask, dil. to vol. with $H_2O$, and mix. Pipet 5, 10, 15, and 20 mL aliquots of this dild std soln into sep. 100 mL vol. flasks. (When dild to vol. these solns contain 2.5, 5.0, 7.5, and 10.0 μg paraquat/mL, resp.) Proceed as in **969.09D**. Plot $A$ against μg paraquat/mL at final diln.

### C. Preparation of Sample

(*Caution:* Open aerosol can behind safety shield.)

(a) *Formulations not containing oil base*.—Accurately weigh portion well mixed sample contg ca 0.25 g paraquat. Transfer to 500 mL vol. flask, dil. to vol. with $H_2O$, and mix well (*Soln 1*). Pipet 10 mL *Soln 1* into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix well (*Soln 2*). Pipet 10 mL *Soln 2* into 100 mL vol. flask and proceed as in **969.09D**.

(b) *Aerosol formulations containing oil base*.— Weigh aerosol can to nearest 0.1 g ($C$). Clamp can with bottom up and puncture smallest possible hole with punch and hammer. After hiss of escaping propellent is no longer heard, cut bottom 7/8 open with hand can opener. Push nearly detached lid into can. Immerse can 15 min in 50–70° $H_2O$ bath or in hot tap $H_2O$ running into 1 L beaker.

Add 50 mL extg soln, (c), and 50 mL *pentane* to 250 mL separator. Remove can from $H_2O$ bath, dry well (especially inside cap and around valve), and weigh ($D$). Place pipet with capacity to deliver ca 20 mg paraquat in can, and weigh both ($E$). Withdraw liq., transfer contents to separator, replace pipet in can, and weigh ($F$). (Disregard material left in and on pipet.) Empty can, rinse completely with acetone, air dry, and weigh ($G$).

Stopper separator and shake 30 sec, venting frequently. Let layers sep, and drain lower layer into 200 mL vol. flask. Add 25 mL extg soln to separator, repeat extn, and drain lower layer into same vol. flask. Dil. to vol. with extg soln and mix well. Pipet 5 mL into 100 mL vol. flask and proceed as in **969.09D**.

### D. Determination

(Complete analysis of one soln before adding dithionite to next soln.)

Add 10 mL Na dithionite soln to one 100 mL vol. flask and dil. to vol. with $H_2O$. Mix by inverting end-over-end 3 times at such speed that air bubble travels from one end to other; do *not* shake flask vigorously, as this tends to cause fading of color due to oxidn. Immediately measure $A$ of soln at 600 nm, using reagent blank (no paraquat) to set the 100% $T$ or for ref. side for dual beam instruments. Similarly, treat each flask in turn, completing color measurement without delay before adding dithionite to next soln.

$$\% \text{ Paraquat} = (\mu g/mL \text{ from std curve}) \times 5/g \text{ sample}$$

% Paraquat (in aerosol formulations)

$$= [(\mu g/mL \text{ from std curve}) \times (D - G) \times 0.4]/[(C - G) \times (E - F)]$$

Refs.: Analyst **92,** 375(1967). JAOAC **51,** 1304, 1306(1968); **55,** 857(1972).

CAS-4685-14-7 (paraquat)

## 960.14 Quaternary Ammonium Compounds

**First Action 1960**
**Final Action 1961**

### *A. Potentiometric Titration Method*

Transfer sample contg 30–35 mg Cl to 600 mL beaker, dil. to 200 mL with $H_2O$, and add 5 mL $HNO_3$ (1 + 1). Add just enough acetone to dissolve ppt that forms and titr. with 0.1$N$ $AgNO_3$, using app. for potentiometric titrn. Calc. % Cl (1 mL 0.1$N$ $AgNO_3$ = 3.545 mg Cl) and equiv. % quaternary $NH_4$ salt.

### *Adsorption Indicator Method*

### *B. Reagents*

(**a**) *Bromothymol blue indicator.*—Dissolve 1 g indicator in 500 mL 50% alcohol.

(**b**) *Dichlorofluorescein soln.*—0.1%. Dissolve 100 mg indicator in 100 mL 70% alcohol.

### *C. Determination*

Transfer sample contg 30–140 mg Cl (usually ca 1 g quaternary $NH_4$ salt) into 300 mL erlenmeyer, dil. to 75 mL with $H_2O$, and add 25 mL isopropanol. Neutze if necessary with HOAc (1 + 9), using 1 drop bromothymol blue (pH 4–6). Add 10 drops dichlorofluorescein, and titr. with 0.1$N$ $AgNO_3$, avoiding direct sunlight. Ppt becomes red at end point and may flocculate just before end point. Calc. % Cl and equiv. % quaternary $NH_4$ salt.

Ref.: JAOAC **43,** 352(1960).

## 951.02 Organic Thiocyanate in Livestock or Fly Sprays

**Kjeldahl Method**
**Final Action**

(*Caution:* See safety notes on pesticides.)

### *A. Reagents*

(**a**) *Strong potassium polysulfide soln.*—Dissolve 180 g KOH in 120 mL $H_2O$. Sat. 100 mL of this soln with $H_2S$ (ca 42 g) (*Caution:* See safety notes on hydrogen sulfide.) while cooling. Add remaining 100 mL KOH soln and 80 g S. Shake until dissolved.

(**b**) *Mixed sulfide soln.*—To 100 mL (**a**) add 50 g $Na_2S.9H_2O$, 30 g KOH, and 200 mL $H_2O$.

(**c**) *Sodium bisulfite.*—$Na_2S_2O_5$ or $NaHSO_3$.

(**d**) *Copper sulfate soln.*—20% aq. soln $CuSO_4.5H_2O$.

(**e**) *Wash soln.*—To 300 mL $H_2O$ add 1 mL $H_2SO_4$ (1+4), 1 g (**c**), 10 mL (**d**), and 12 g $Na_2SO_4$, and pass $SO_2$ into soln 10 min.

### *B. Preparation of Sample*

Weigh sample preferably contg ca 0.03 g thiocyanate N into 250 mL g-s erlenmeyer. (If SCN content is very low, do not unduly increase amt sample without correspondingly increasing amt mixed sulfide soln used; 20–25 g fly spray is usually enough.) Add 35 mL mixed sulfide soln and shake vigorously at room temp. 10 min, during which time reaction is nearly completed. Heat to 70° on steam bath, carefully releasing pressure resulting from heating, shake 15 min at 70°, and cool.

*Removal of petroleum oil.*—Transfer mixt. to separator with ca 200 mL $H_2O$. Add 50 mL pet ether, shake, and drain aq. layer into 600 mL beaker. Wash pet ether layer with two 10 mL portions $H_2O$, adding washings to main soln. (If emulsions form during washing, break by acidifying with $H_2SO_4$ (1 + 4).) Drain aq. layer and wash pet ether layer with $H_2O$ as above. Discard pet ether layer.

### *C. Determination of Thiocyanate Nitrogen*

Dil. combined aq. soln to ca 300 mL and neutze with $H_2SO_4$ (1 + 4), using litmus paper as outside indicator. Add 2 mL $H_2SO_4$ (1 + 4), quickly bring mixt. to bp, and boil 8 min to remove $H_2S$. Cool. If fatty acids or oils are present, transfer to separator, ext with pet ether, and return aq. phase to original beaker. Filter thru small buchner and transfer filtrate to beaker. Neutze to litmus paper with 10% KOH soln and add 1 mL $H_2SO_4$ (1 + 4). Add 1 g Na bisulfite and stir until dissolved. Add excess (ca 15 mL) $CuSO_4$ soln and pass $SO_2$ into soln 10 min.

Let pptd CuSCN settle 2 hr, and filter with suction thru 56 mm buchner coated with layer of asbestos (*Caution:* See safety notes on asbestos.), upon which is placed No. 42 Whatman paper, or equiv., second layer of asbestos, layer of diatomite, and finally third layer of asbestos. If filtrate is not clear, centrf. soln at 2000 rpm 10–15 min, and pour thru filter again. Wash filter and ppt once or twice with wash soln, continue suction until filter pad is dry, and transfer to 800 mL Kjeldahl flask. (Filter pad may be folded in filter paper together with bits of moist filter paper used to wipe out buchner, and whole placed in Kjeldahl flask.) Add few glass beads, 35 mL $H_2SO_4$, 10 g $K_2SO_4$, and ca 0.7 g HgO or 0.65 g Hg. (*Caution:* See safety notes on sulfuric acid and mercury.) Digest until colorless; then 15 min more. Det. N as in **955.04C**, second par. Perform blank analysis on paper, filter pad, and reagents.

Ref.: JAOAC **34,** 677(1951).

## 985.08 Triadimefon Technical and Pesticide Formulations

**Liquid Chromatographic Method**
**First Action 1985**
**Final Action 1987**
***CIPAC-AOAC Method***

(Method is suitable for tech. triadimefon and formulations with triadimefon as only active ingredient.)

### *A. Principle*

Triadimefon is detd by liq. chromatgy, using 4-chlorophenyl sulfoxide as internal std.

### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Able to generate >7 MPa (>1000 psi) and measure $A$ at 475 nm.

(**b**) *Chromatographic column.*—250 × 4.6 mm id packed with ≤10 μm silica gel capable of resolving 4-chlorophenol from triadimefon and internal std peaks (Du Pont Zorbax-Sil or equiv.). *LC operating conditions.*—Column temp. ambient; mobile phase flow rate 1.5 mL/min (ca 500 psi); chart speed 0.5 cm/min; injection vol. 20 μL; *A* range 0.320 AUFS; retention times: 4-chlorophenyl sulfoxide ca 4.0 min, triadimefon ca 5.9 min. Pump LC mobile phase until system is equilibrated 15 min (flat baseline). Allow 6.5 min between injections.

(**c**) *Mechanical shaker.*

(**d**) *Filters.*—0.45 μm porosity (Gelman Acrodisc-CR, Gelman Scientific, Inc., or equiv.).

### C. Reagents

(**a**) *Butyl chloride.*—LC grade or distd in glass (Burdick and Jackson Laboratories, Inc.).

(**b**) *Ethanol.*—Anhyd., 200 proof.

(**c**) *Mobile phase.*—Butyl chloride–EtOH (100 + 1). Pipet 10 mL anhyd. EtOH into 1 L butyl chloride, mix, and degas.

(**d**) *4-Chlorophenol stock soln.*—Weigh ca 20 mg 4-chlorophenol into 100 mL vol. flask and dil. to vol. with mobile phase.

(**e**) *4-Chlorophenyl sulfoxide internal std soln.*—About 275 mg/250 mL mobile phase; sonicate to dissolve.

(**f**) *Triadimefon reference std soln.*—Accurately weigh ca 200 mg ref. std (Mobay Corp.) into 100 mL vol. flask. Pipet 10 mL cholorophenol stock soln into flask. Pipet 20 mL internal std soln into flask, dil. to vol. with mobile phase, and mix well. Filter portion of soln for LC analysis.

### D. Preparation of Sample

Accurately weigh amt sample contg ca 200 mg triadimefon into 100 mL vol. flask. Pipet 20.0 mL internal std into flask. Add ca 50 mL mobile phase and shake 20 min. Dil. to vol. with mobile phase and mix. Filter portion of soln for LC analysis.

### E. Determination

Inject triadimefon std soln and adjust operating parameters to cause triadimefon to elute in 5.5–6.0 min. Adjust injection size and attentuation to give largest possible on-scale peaks. Chlorophenol in std injection must be resolved from triadimefon and internal std peaks (Fig. **985.08**). If not, change silica columns.

Using same injection vol. for all sample and std injections, make repetive injections of std and calc. response ratios by dividing peak area (or ht) of triadimefon by that of internal std peak. Response ratios must agree within ±1%. Average duplicate response ratios obtained with std solns.

Inject duplicate aliquots of each sample soln. Average duplicate response ratios for each sample soln. Response ratios must agree within ±1%. If not, repeat detn, starting with std injections.

Reinject std soln twice. Average response ratios of stds immediately preceding and following sample injection. These must agree within ±1%. If not, repeat detn.

### F. Calculation

$$\text{Triadimefon, wt \%} = (R/R') \times (W'/W) \times P$$

where $R$ and $R'$ = av. response ratios for sample and std solns, resp.; $W'$ and $W$ = wt (mg) of triadimefon std and sample solns, resp.; and $P$ purity of triadimefon std (%).

Ref.: JAOAC **68,** 586(1985).

CAS-43121-43-3 (triadimefon)

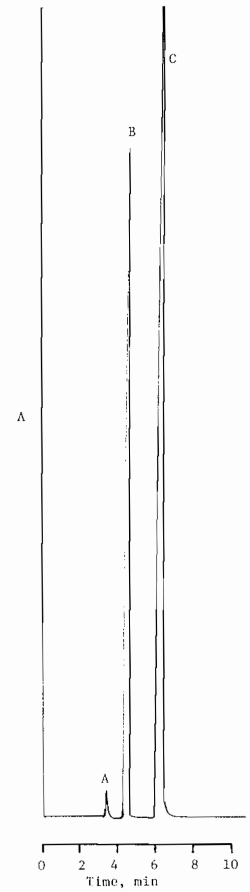


**FIG. 985.08—LC chromatogram of 4-chlorophenol (A), 4-chlorophenylsulfoxide (B), and triadimefon (C)**

## 960.15 Warfarin in Rodenticide Formulations

**Spectrophotometric Method**

**First Action 1960**
**Final Action 1961**

(Applicable to baits contg ca 0.025% and to concs contg ≥0.5% warfarin. Not applicable to pelleted baits or baits consisting of cracked corn treated with alc. warfarin soln and aq. sugar soln, and then dried.)

### A. Reagents

(**a**) *Sodium pyrophosphate soln.*—1%. Dissolve 5 g $Na_4P_2O_7 \cdot 10H_2O$ in 500 mL $H_2O$.

(**b**) *Petroleum ether, purified.*—Ext 200 mL pet ether with three 20 mL portions 1% $Na_4P_2O_7$ soln.

(**c**) *Warfarin std soln.*—10 μg/mL. Dissolve 100 mg pure warfarin (Biocenotics, Inc., 4880 Hudson Rd, Osseo, MI 49266) in 100 mL 1% $Na_4P_2O_7$ soln. Dil. 10 mL to 100 mL with 1% $Na_4P_2O_7$ soln, and dil. 10 mL of second soln to 100 mL with 1% $Na_4P_2O_7$ soln.

### B. Determination

Weigh 10 g sample (0.025%), 0.600 g (0.5%), or equiv. wt of higher concn, into 125 mL g-s flask or 100 mL centrf. tube and add 50 mL Et ether from pipet. Stopper tightly and shake mech. ca 30 min. Transfer 5 or 10 mL to centrf. tube (or centrf. directly), stopper, and centrf. 5 min at high speed or until clear. Take precautions to avoid evapn of ether.

Pipet 10 mL 1% $Na_4P_2O_7$ soln into g-s 16 × 150 mm test tube and add 2 mL centrfd ether ext from pipet. Stopper and shake vigorously 2 min. Centrf. at high speed until aq. layer is clear. Draw off ether layer, including any emulsion that remains, using fine-tip glass tube attached to aspirator. Add ca 2 mL Et ether, shake vigorously, centrf., and completely draw off ether layer. Repeat ether extn, and then ext twice with purified pet ether in same manner.

Prep. blank soln similarly, using 2 mL ether instead of 2 mL ether ext.

Det. $A$ of aq. soln in 1 cm silica cell at 308 nm against blank soln in Beckman spectrophtr, model DU (replaced by models 24/25), or equiv. Det. $A'$ (ca 0.46) of the std warfarin soln against 1% $Na_4P_2O_7$ soln.

$$\% \text{ Warfarin} = (A/A') \times (10^{-5} \text{ g std/mL}) \times [100/(\text{g sample} \times (2/50)(1/10))]$$
$$= (A/A') \times (0.250/\text{g sample})$$

Ref.: JAOAC **43,** 365(1960).

CAS-81-81-2 (warfarin)

## 968.05★ Sulfoxide Pesticide Formulations

### Spectrophotometric Method

**First Action 1968**
**Surplus 1974**

*See* **6.296–6.302**, 11th ed.

**Common and Chemical Names of Pesticides in this chapter**

| Common Name | Chemical Name |
|---|---|
| Alachlor | 2-Chloro-*N*-(2,6-diethylphenyl)-*N*-(methoxymethyl)acetamide |
| Aldicarb | 2-Methyl-2-(methylthio) propionaldehyde *O*-(methylcarbamoyl)oxime |
| Aldrin | 1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-*exo*-1,4-*endo*-5,8-dimethanonaphthalene, not less than 95% |
| Allethrin | *dl*-3-Allyl-2-methyl-4-oxocyclopent-2-enyl *dl-cis/trans* chrysanthemate |
| d-*trans*-Allethrin | *dl*-2-Allyl-4-hydroxy-3-methyl-2-cyclopenten-1-one ester of *d-trans* chrysanthemum monocarboxylic acid |
| Aminocarb | 4-(Dimethylamino)-3-methylphenol methylcarbamate (ester) |
| Amitrole | 3-Amino-1,2,4-triazole |
| Anilazine | 4,6-Dichloro-*N*-(2-chlorophenyl)-1,3,5-triazin-2-amine |
| Azinphos-methyl | *O,O*-Dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)methyl]phosphorodithioate |
| Bendiocarb | 2,2-Dimethyl-1,3-benzodioxol-4-yl methylcarbamate |
| Benfluralin | *N*-Butyl-*N*-ethyl-$\alpha,\alpha,\alpha$-trifluoro-2,6-dinitro-*p*-toluidine |
| Benomyl | Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate |
| $\gamma$-BHC | 1,2,3,4,5,6-Hexachlorocyclohexane, gamma isomer |
| Brodifacoum | 3-[3-(4′-Bromo-(1,1′-biphenyl)-4-yl)-1,2,3,4-tetrahydro-1-napthalenyl]-4-hydroxy-2*H*-1-benzopyran-2-one |
| Bromoxynil | 3,5-Dibromo-4-hydroxybenzonitrile |
| Butachlor | 2-Chloro-*N*-(2,6-diethylphenyl)*N*-(butoxymethyl)-acetamide |
| Butylate | *S*-Ethyl diisobutylthiocarbamate |
| Captan | *N*-[(Trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide |
| Carbaryl | 1-Naphthyl *N*-methylcarbamate |
| Carbofuran | 2,3-Dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate |
| Chloramben | 3-Amino-2,5-dichlorobenzoic acid |
| Chloramine-T | *N*-Chloro-4-methylbenzenesulfonamide sodium salt |
| Chlordane | 1,2,4,5,6,7,8,8-Octachlor-2,3,3a,4,7,7a-hexahydro-4,7-methanoindane |
| Chlordimeform | *N*′-(4-Chloro-2-methylphenyl)-*N,N*-dimethylmethanimidamide |
| Chlorotoluron | *N*′-(3-Chloro-4-methylphenyl)-*N*′*N*-dimethyl urea |
| Chloroxuron | 3-[*p*-(*p*-Chlorophenoxy) phenyl]-1,1-dimethylurea |
| Chlorpyrifos | *O,O*-Diethyl *O*-(3,5,6-trichloro-2-pyridyl)phosphorothioate |
| Cycloate | *S*-Ethylcyclohexylethylthiocarbamate |
| Cyhexatin | Tricyclohexylhydroxystannane |
| Cypermethrin | 3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid cyano(3-phenoxyphenyl)methyl ester |
| 2,4-D | 2,4-Dichlorophenoxyacetic acid |
| Dalapon | 2,2-Dichloropropionic acid |
| DCPA | Dimethyl tetrachloroterephthalate |
| DDT | 1,1′-(2,2,2-Trichloroethylidene)bis[4-chlorobenzene] |
| DDVP | 2-2-Dichlorovinyl dimethyl phosphate |
| Diazinon | *O,O*-Diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothiote |
| Dicamba | 2-Methoxy-3,6-dichlorobenzoic acid |
| Dichlobenil | 2,6-Dichlorobenzonitrile |
| Dichlorvos | 2-2-Dichlorovinyl dimethyl phosphate |
| Dicofol | 4-Chloro-alpha-(4-chlorophenyl)-alpha-(trichloromethyl)benzenemethanol |
| Dieldrin | 3,4,5,6,9,9-Hexachloro-1a,2,2a,3,6,6a,7,7a-octahydro-2,7:3,6-dimethanonaphth(2,3-b)oxirene |
| Diflubenzuron | 1-(4-Chlorophenyl) 3-(2,6 difluorobenzoyl) urea |
| Diquat | 1,1′-Ethylene-2,2′-bipyridylium ion OR 6,7-Dihydrodipyridol (1,2-*a*:2′,1′-*c*) pyrazdiium ion |

(*Continued*)

**Common and Chemical Names of Pesticides in this chapter (*Continued*)**

| Common Name | Chemical Name |
|---|---|
| Disulfoton | *O-O*-Diethyl *S*-[2-(ethylthio)ethyl] phosphorodithioate |
| Dodine | *n*-Dodecylguanidine acetate |
| Endosulfan | 6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahyro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide |
| Endrin | Hexachloroepoxyoctahydro-endo,endo-dimethanonaphthalene |
| EPTC | *S*-Ethyl dipropylthiocarbamate |
| Ethion | *O,O,O,O*-Tetraethyl *S,S*-methylene bisphosphorodithioate |
| Fenitrothion | Phosphorothioic acid *O,O*-dimethyl *O*-(3-methyl-4-nitrophenyl) ester |
| Fensulfothion | Phosphorothioic acid *O,O*-diethyl *O*-[4-(methylsulfinyl)phenyl] ester |
| Fentin | Triphenyltin |
| Ferbam | Ferric dimethyldithiocarbamate |
| Fluazifop-butyl | Butyl 2-{4-(5-trifluoromethyl-2-pyridinyloxy) phenoxy} propanoate |
| Fluometuron | 1,1-Dimethyl-3-($\alpha,\alpha,\alpha$-trifluoro-*m*-tolyl) urea |
| Folpet | *N*-(Trichloromethylthio)phthalimide |
| Formothion | *S*-(2-(Formylmethylamino)-2-oxoethyl) *O-O*-dimethyl phosphorodithioate |
| Glyphosate | Isopropylamine salt of *N*-(phosphonomethyl) glycine |
| Heptachlor | 1,4,5,6,7,8,8-Heptachloro-3*a*,4,7,7*a*-tetrahydro-4,7-methanoindene |
| Isofenphos | 2-[[Ethoxy[(1-methylethyl)amino]phosphinothioyl]oxy]benzoic acid 1-methylethyl ester |
| Lindane | Gamma isomer of 1,2,3,4,5,6-hexachlorocyclohexane |
| Malathion | *O,O*-Dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorodithioate |
| Maneb | Manganese ethylenebisdithiocarbamate |
| MCPA | 4-Chloro-2-methyl phenoxyacetic acid |
| MCPP | 2-(4-Chloro-2-methylphenoxy) propanoic acid |
| Methazole | 2-(3,4-Dichlorophenyl)-4-methyl-1,2,4-oxadiazolidine-3,5-dione |
| Methiocarb | 3,5-Dimethyl-4-(methylthio)phenol methylcarbamate |
| Methyl parathion | *O-O*-Dimethyl *O-p*-nitrophenyl phosphorothioate |
| Metolachlor | 2-Chloro-*N*-(2-ethyl-6-methylphenyl)-*N*-(2-methoxy-1-methylethyl) acetamide |
| Metoxuron | *N'*-(3-Chloro-4-methoxyphenyl)-*N,N*-dimethylurea |
| Metribuzin | 4-Amino-6-(1,1-dimethyethyl)-3-(methythio)-1,2,4-triazin-5(4*H*)-one |
| Molinate | *S*-Ethyl hexahydro-1*H*-azepine-1-carbothioate |
| Nabam | Disodium ethylene-1,2-bisdithiocarbamate |
| Nicotine | 3-(1-Methyl-2-pyrrolidyl) pyridine |
| *N*-Octyl bicycloheptene dicarboximide | Same as common name OR *N*-(2-Ethylhexyl)-5-norborene-2,3-dicarboximide |
| Oxythioquinox | 6-Methyl-1,3-dithiolo[4,5-b]quinoxalin-2-one |
| Paraquat | 1,1'-Dimethyl-4,4'-bipyridinium ion |
| Parathion | *O,O*-Diethyl *O-p*-nitrophenyl phosphorothioate |
| PCNB | Pentachloronitrobenzene |
| Pebulate | *S*-Propyl butylethylthiocarbamate |
| Permethrin | 3-(2,2-Dichloroethenyl)-2,2-dimethylcyclopropanecarboxylic acid (3-phenoxyphenyl)methyl ester |
| Phorate | *O,O*-Diethyl *S*-[(ethylthio)methyl] phosphorodithioate |
| Picloram | 4-Amino-3,5,6-trichloropicolinic acid |
| Piperonyl butoxide | $\alpha$-[2-(2-Butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene |
| Pirimicarb | 2-(Dimethylamino)-5,6-dimethyl-4-pyrimidinyl dimethylcarbamate |
| Propachlor | 2-Chloro-*N*-(1-methylethyl)-*N*-phenylacetamide |
| Propoxur | 2-(1-Methylethoxy) phenol methycarbamate |
| Pyrethrins | Standardized mixture of pyrethrins I and II (Mixed esther of pyrethrolone) |
| Rotenone | 1,2,12,12a-Tetrahydro-8,9-dimethoxy-2-(1-methylethenyl)-(1)benzopyrano(3,4-b)furo(2,3-h)(1)benzopyran-6(6aH)-one |
| Sulfoxide | 1,2-Methylenedioxy-4-(2-(octylsulfidnyl)propyl) benzene |
| Sulprofos | *O*-Ethyl *O*-[4-(methylthio)phenyl] *S*-propyl phosphorodithioate |
| 2,4,5-T | 2,4,5-Trichlorophenoxyacetic acid |
| TCA | Trichloroacetic acid |
| Temephos | *O,O,O',O'*-Tetramethyl *O,O'*-thiodi-*p*-phenylene phosphorothioate |
| TEPP | Tetraethyl diphosphate |
| Terbuthylazine | 4-tert-Butylamino-2-chloro-6-ethylamino-s-triazine |
| Tetradifon | 4-Chlorophenyl 2,4,5-trichlorophenyl sulfone |
| Thiram | Bis(dimethylthiocarbamoyl) disulfide |
| Toxaphene | Chlorinated camphene (67–69% chlorine) |
| Triadimefon | 1-(4-Chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)-2-butanone |
| Trifluralin | $\alpha,\alpha,\alpha$-Trifluoro-2,6-dinitro-*N,N*-dipropyl-*p*-toluidine |
| Vernolate | *S*-Propyldipropylthiocarbamate |
| Warfarin | 3-($\alpha$-Acetonylbenzyl)-4-hydroxycoumarin |
| Zineb | Zinc ethylenebisdithiocarbamate |

Sources: *The Merck Index* (1983) 10th ed., Merck & Co., Inc., Rahway, NJ; *The Agrochemicals Handbook* (1987) 2nd ed., The Royal Society of Chemistry, Nottingham, UK; *Farm Chemicals Handbook (1988) 74th ed., Meister Publishing Co., Willoughby, OH.*

# 8. Hazardous Substances

## 975.05 Cadmium and Lead in Earthenware

*See* **973.32** and **973.33**.

## 911.01 Carbonate and Hydroxide in Soda Lye

### Titrimetric Method

### Final Action

Weigh ca 10 g sample from weighing bottle, dissolve in $CO_2$-free $H_2O$, and dil. to definite vol. Titr. aliquot with 0.5*N* HCl, **936.15A** and **B**, using 0.5% aq. Me orange soln, **930.11A(e)**; note total alky found. Transfer equal aliquot to vol. flask and add enough 10% $BaCl_2$ soln to ppt all carbonate, avoiding any unnecessary excess. Dil. to vol. with $CO_2$-free $H_2O$, stopper, shake, and let stand. When liq. clears, pipet off one-half and titr. with the 0.5*N* HCl, using phthln; mL 0.5*N* acid required for this titrn × 2 = mL 0.5*N* acid equiv. to NaOH present in original aliquot. Difference between this figure and mL 0.5*N* HCl required for total alky = mL 0.5*N* acid equiv. to $Na_2CO_3$ present in aliquot. Calc. % $Na_2CO_3$ and NaOH.

Ref.: Sutton "Systematic Handbook of Volumetric Analysis," 10th ed., p. 61(1911).

CAS-5968-11-6 (sodium carbonate)
CAS-1310-73-2 (sodium hydroxide)

## 975.06 Denaturants (Volatile) in Alcoholic Products

### Gas Chromatographic Method

### First Action 1975

#### *A. Apparatus and Reagents*

(**a**) *Gas chromatograph and integrator.*—With flame ionization detector (F&M Model 400, 402, Hewlett-Packard 7600 series, or equiv.). Column 1.2 m (4′) × 2 mm id glass packed with 100–120 mesh Chromosorb 102; column temp. 160° (isothermal), detector and inlet 200°; He flow rate 50 mL/min; relative retention times: EtOH 1.00 (≤100 sec), n-PrOH 2.06, and tetrahydrofuran 3.04. Integrator: Hewlett-Packard 3370A (new model 3370B), or equiv.

(**b**) *Std solns.*—6% (v/v). Dil. 6.00 mL of each denaturant of interest to 100 mL with anhyd. alcohol in sep. vol. flasks. Approx. slopes and retention times relative to *n*-PrOH are given in Table **975.06**.

#### *B. Determination*

Pipet 25 mL of each expected denaturant std soln into sep. flasks and add 1.00 mL *n*-PrOH as internal std. Cap immediately with rubber stoppers, shake 3 min, and let stand 10 min at room temp. Inject 0.3 μL portions from 1 μL microsyringe. Det. peak areas and calc. slope for each compd as:

$$S_x = (PA_x/PA_i)/6.00$$

where $PA_x$ and $PA_i$ = peak areas of compd X in std soln and of *n*-PrOH internal std, resp., and 6.00 = % compd X in std soln. Slopes and retention times should approximate those of Table **975.06**.

$$\%\ \text{Compd X in sample} = (PA/PA_i) = (1/S_x)$$

where $PA$ = peak area of compd X in sample.

Ref.: JAOAC **57**, 148(1974).

**Table 975.06 Approximate Slopes and Retention Times Relative to *n*-Propyl Alcohol (RT) for Denaturants**

| Compound | Slope | RT |
|---|---|---|
| Acetone CAS-67-64-1 | 0.207 | 0.694 |
| Benzene CAS-71-43-2 | 0.646 | 2.309 |
| *n*-Butyl alcohol CAS-71-36-3 | 0.269 | 2.283 |
| *sec*-Butyl alcohol CAS-78-92-2 | 0.246 | 1.621 |
| Chloroform CAS-67-66-3 | 0.058 | 1.543 |
| Ethyl acetate CAS-141-78-6 | 0.192 | 1.640 |
| Ethylene glycol monoethyl ether CAS-629-14-1 | 0.187 | 3.868 |
| Ethylene glycol monomethyl ether CAS-109-86-4 | 0.151 | 2.071 |
| Isopropanol CAS-67-63-0 | 0.210 | 0.727 |
| Methanol CAS-67-56-1 | 0.130 | 0.266 |
| Methyl isobutyl ketone CAS-108-10-1 | 0.275 | 5.436 |
| Toluene CAS-108-88-3 | 0.454 | 5.302 |

## 973.10 Fluorides in Hazardous Substances

### Potentiometric Method

### First Action 1973
### Final Action 1975

#### *A. Apparatus*

(**a**) *pH meter.*—With expanded mv scale (digital Model 110, Corning Scientific Instruments, 63 North St, Medfield, MA 02052, or equiv.), fluoride ion-selective electrode (Model 94-09, Orion Research Inc., or equiv.), and single junction ref. electrode, plastic sleeve-type (Model 90-01, Orion Research Inc., or equiv.).

(**b**) *Magnetic stirrer.*—With Teflon-coated stirring bar. Use asbestos or foam mat to insulate sample from motor heat.

(**c**) *Beakers.*—4.5 oz (135 mL), polypropylene, or equiv.

(**d**) *Graph paper.*—Linear or semi-antilog, vol. corrected No. 90-00-90 Gran's plot paper (Orion Research Inc., or equiv.).

#### *B. Reagents*

(**a**) *Buffer soln.*—pH 6.0. Add 77.0 g $NH_2OAc$ and 0.452 g $NH_4$ citrate to 1 L $H_2O$. Adjust to pH 6.0 with HOAc.

(**b**) *Fluoride std soln.*—1 mg F/mL. Prep. 2.2108 g NaF (reagent grade, dried 4 hr at 105°)/L buffer soln. Store in leak-proof plastic bottles. Compare with 1 mg F/mL soln prepd from USP Ref. Std; equiv. reading of ±1 mv is satisfactory.

#### *C. Determination*

(Stir all solns constantly at same rate thruout titrns. Let electrodes equilibrate ≥2 min before addn of F std soln and 30 sec after each addn of F std soln.)

(**a**) *Blank.*—Record mv values ($E'$) of 100 mL buffer soln after addn of 4 mL std F soln from 10 mL buret and after each addnl mL up to 10 mL. (Preliminary mv values will not fall on linear range of response curve.) Vol. std soln added = $V'$.

(**b**) *Samples.*—Est. molarity of samples from direct reading. Dil. samples, if necessary, to ca 0.001*M* F. Transfer 50 mL sample soln to beaker and add 50 mL buffer soln. Record initial mv reading, using expanded scale ($E_0$). If initial reading is $<-50$ mv, soln is too concd. Dil. sample to avoid asymptotic slope. Record mv values ($E$) after each mL F std soln is added up to 10 mL. Rinse electrodes with $H_2O$ between samples. Vol. std soln added = $V$.

***D. Calculations***

(**a**) *Linear graph paper.*—For each addn of F std soln and corresponding $E$ value, calc. for blank:

$$Z' = \text{antilog}\ [\log(V_0 + V') - 0.017(E')]$$

where $V_0$ is original vol. soln to which F std soln was added (100 mL) and $E'$ is treated algebraically (+ or − as read). Plot $Z'$ against mL (mg) F std soln added and extrapolate to intersection of mL (mg) F axis to obtain mL (mg) F in blank, $V_e'$. In graph, assign horizontal axis to mL (mg) F, with 0 at center and mL (mg) F increasing in both directions to left and right. Assign $Z$ values to vertical axis. Plot actual readings of mL (mg) F on right portion of horizontal axis so that extrapolation will fall on left portion of axis.

Similarly, for original readings and each addn of F std soln and corresponding $E$ value, calc. for sample:

$$Z = \text{antilog}\ [\log(V_0 + V) - 0.017(E)]$$

where $V_0$ is original vol. soln to which F std soln was added (100 mL). Plot $Z$ against mL (mg) F std soln on same graph as blank and extrapolate to intersection of mL (mg) F axis to obtain mL (mg) F in sample, $V_e$.

(**b**) *Semi-antilog paper.*—Plot $E$ directly for both blank and sample, descending 5 mv for each major line crossing vertical axis. At top of vertical axis place most neg. $E$ reading which still allows extrapolation of $V_e$ on left portion of mL (mg) side of horizontal axis. Obtain $V_e$ and $V_e'$ by extrapolation to left side of 0 mL (mg) F.

$$\% \text{ F} = (V_e - V_e') \times (B \times 100)/[W \times C \times 1000 \text{ (mg/g)}]$$

where $B$ = vol. of diln, $W$ = mL or g sample, and $C$ = aliquot (50 mL max.) buffered to 100 mL.

Ref.: JAOAC **56,** 798(1973).

CAS-7782-41-4 (fluorine)

## 974.02 Lead in Paint

### Atomic Absorption Spectrophotometric Method

**First Action 1974**
**Final Action 1976**

***A. Reagents and Apparatus***

(**a**) *Lead std solns.*—(*1*) *Stock soln.*—1 mg Pb/mL 1% $HNO_3$. Dissolve 159.9 mg $Pb(NO_3)_2$ in $HNO_3$ (1 + 99) and dil. to 100 mL with $HNO_3$ (1+99). (*2*) *Intermediate soln.*—300 µg/mL dil. $HNO_3$. Dil. 15 mL stock soln to 50 mL with 0.5 mL $HNO_3$ and $H_2O$. (*3*) *Working solns.*—To each of seven 100 mL vol. flasks contg 1 mL $HNO_3$, add resp. 0, 1, 2, 3, 4, 5, and 6 mL intermediate soln and dil. to vol. with $H_2O$ (0, 3, 6, 9, 12, 15, and 18 µg Pb/mL).

(**b**) *Atomic absorption spectrophotometer.*—With Pb hollow cathode lamp and 4″ single slot or 3 slot Boling burner head, capable of detecting 0.5 µg Pb/mL, such as Perkin-Elmer Model 403. Operating conditions: 283.3 nm, 0.7 nm band width slit, recorder response (if used) 0.25–1 sec time constant, air-$C_2H_2$ flame, with gas flows adjusted according to directions of manufacturer.

(**c**) *Heater for digestion.*—Drill 7.5 cm Al block to hold ≥16 test tubes, 16 × 150 mm. Place on hot plate capable of maintaining medium at 160–170° (Corning PC 35 (replacement model PC300), or equiv.). Sand bath may be used instead of Al block.

(**d**) *Boiling chips.*—Unglazed boiling chips, 1.5 mm diam., Pb-free.

***B. Determination of Solids***

Thoroly mix samples manually for 10 min or mech. for 5 min. Accurately weigh 0.3–0.4 g into weighed Al dish, 63 mm diam. Add 3–5 mL hexane or pet ether to oil-based paints or $H_2O$ to latex paints and swirl to disperse. Warm on hot plate while swirling until solv. has evapd and film is formed. Heat in oven 4 hr at 105°, cool, and weigh.

$$\% \text{ Solids} = \text{g dried sample} \times 100/\text{g sample}$$

***C. Determination of Lead***

Introduce ca 0.6 g (0.3 mL) thoroly mixed sample near bottom of 16 × 150 mm test tube with syringe and weigh accurately. Add 5 ± 0.2 mL $HNO_3$ and 2 boiling chips to each, including blanks. Place in block or bath at 90–100° so that liq. surface is slightly above heated surface. (Use hood.) After initial fuming has subsided, increase temp. until vapors are condensing in top 1–2 cm of tube (bath temp., 160–170°) and maintain at this temp. 3 hr. Cool to 50–60°, transfer to 25 mL vol. flask, including chips and any ppt, and rinse with four 4 mL portions $H_2O$, transferring as much residue as possible. Dil. to vol. with $H_2O$ and let settle 0.5–1 hr. Floating residue may be removed by aspiration thru disposable pipet.

Aspirate solns and stds into AA spectrophotometer, avoiding introduction of ppt. If $A$ of sample is greater than highest std, dil. sample and re-aspirate. Det. µg Pb/mL from std curve.

$$\begin{aligned} &\% \text{ Pb in paint solids} \\ &= (\mu\text{g Pb/mL}) \times F \times 10^{-2}/(\text{g sample} \times \% \text{ solids in sample}) \end{aligned}$$

$$F \text{ (diln factor)} = 1/[(1/25)\ (b/c)\ (d/e)\ \ldots]$$

where 25 = vol. original sample digest, $b$ = aliquot of original 25 mL dild to $c$ mL; $d$ = aliquot of $c$ (mL) dild to $e$ mL; etc. For dry paint films, % solids in sample = 100.

Ref.: JAOAC **57,** 614(1974).

CAS-7439-92-1 (lead)

## 971.03 Methanol in Hazardous Substances

### Gas Chromatographic Method

**First Action 1971**
**Final Action 1973**

(Applicable in presence of acetone, BuOAc, EtOH, isopropanol, hexane, MeEt ketone, $CH_2Cl_2$, Me Cellosolve, paraffin, toluene, and $H_2O$. This includes many paint removers, fuels, liq. sanders, antifreezes, and paint products.)

***A. Apparatus and Reagents***

(**a**) *Gas chromatograph.*—With flame ionization detector and oven capable of temp. changes ≥5°/min near 160° or preferably temp. programmer. *Column.*—1.8 m (6′) × 4 mm id packed with 120–150 mesh Porapak R (Waters Associates, Inc.); condition 2 hr at 235°. *Conditions:* Temps (°): injection ca 200, column ca 160, detector ca 210; N flow ca 25 mL/min; set electrometer so that 8 µL std soln provides at least half scale peak. Adjust column temp. and N flow so that MeOH retention time is ca 5–7 min.

(**b**) *Methanol std soln.*—0.4% (v/v). Dil. 4.00 mL MeOH to 100 mL with dioxane; dil. 10.0 mL of this soln to 100 mL with dioxane. Rinse pipet into flask before dilg to vol. with dioxane. Prep. fresh daily.

### B. Preparation of Sample

(**a**) *For asphalt-base tar compounds and viscous adhesives.*—Refrigerate unopened sample container ≥3 hr (longer for larger containers) at 1–10°, open container, and mix well; close container and refrigerate 30 min more. Transfer 1.5–3 g sample to tared, 250 mL, wide-mouth g-s erlenmeyer (tared with stopper in place). Let sample reach room temp. in stoppered erlenmeyer and weigh. Refrigerate 30 min and quickly add 100.0 mL dioxane. Stopper and shake mech. 1 hr. Refrigerate 30 min and filter thru rapid paper (S&S sharkskin, or equiv.). Filter as quickly as possible, covering funnel with watch glass and placing funnel against neck of narrow-mouth g-s receiver. Proceed as in **971.03C**, dilg with dioxane, if necessary.

(**b**) *For other less viscous products.*—Prep. soln with pipets and vol. flasks to contain ca 0.4% (v/v) MeOH, dilg with dioxane. Avoid excessive shaking of semiviscous products and do *not* fill pipet above mark. (Use safety pipet filler to draw liq. to mark and hold until transfer.) Wash pipet with dioxane and add washings to soln.

If MeOH concn is unknown, prep. 2% soln. Prep. addnl dilns as needed.

### C. Determination

Inject portion std soln with 10 μL syringe. Note vol. At $R_{MA}$ (retention time relative to MeOH) ca 0.5, inject portion sample soln. Note vol. At $R_{MA}$ ca 2 (from second injection), repeat injection of std soln. At $R_{MA}$ ca 0.5 (from third injection), repeat injection of sample soln. After MeOH from fourth injection elutes, increase column temp. to 235° as rapidly as possible for time ca 4 × $R_{MA}$ until all dioxane ($R_{MA}$ ca 5) is removed from column. Cool column to 160° and repeat sequence for subsequent sample. Modify injection time if necessary to sep. MeOH from other peaks. (*Note:* Injection sequence is used only to save time; it need not be used if desired.)

### D. Calculation

Det. retention areas for each MeOH peak by multiplying peak ht by retention distance. Average retention areas for sample (*RA*) and for std (*RA'*). Presence of solv. in column changes retention times, requiring use of retention areas in calcn.

% MeOH (w/v) in sample soln

$$= F \times (RA/RA') \times (V'/V) \times C \times 0.79$$

where $F$ = diln factor, $C$ = % (v/v) std soln, $V$ and $V'$ = vol. sample and std soln injected, resp., and 0.79 = density of MeOH.

Refs.: JAOAC **54**, 558(1971); **55**, 242(1972).

CAS-67-56-1 (methanol)

## 986.01 *N*-Nitrosodibutylamine in Latex Infant Pacifiers

### Gas Chromatographic Method

### First Action 1986

### A. Principle

Volatile *N*-Nitrosamines are extd from cut-up latex pacifier nipples with $CH_2Cl_2$. Ext is concd and subjected to high temp. purge and trap, and *N*-nitrosamines are eluted from trap and detd by gas chromatgy with thermal energy analysis.

### B. Reagents

Use all glass-distd solvs (Burdick & Jackson Laboratories, Inc., or equiv.).

(**a**) *N-Nitrosamine stock std solns.*—*(1) External stock std soln.*—10 μg/mL each of NDMA (*N*-nitrosodimethylamine), NDEA (*N*-nitrosodiethylamine), NDPA (*N*-nitrosodipropylamine), NDBA (*N*-nitrosodibutylamine), NPIP (*N*-nitrosopiperidine), NPYR (*N*-nitrosopyrrolidine), and NMOR (*N*-nitrosomorpholine) in alcohol. *(2) Internal stock std soln.*—10 μg NDPA/mL alcohol.

*Caution:* Volatile *N*-nitrosamines are extremely hazardous compds. Carry out all manipulations involving handling neat liqs or solns in adequately ventilated and filtered fume hood or glove box.

(**b**) *Mineral oil.*—White, lightwt Saybolt viscosity 125/135 (No. 6358, Mallinckrodt Chemical Works).

(**c**) *Nitrosation inhibitor.*—10 mg α-tocopherol/mL mineral oil.

(**d**) *Keeper solns.*—*(1) For K-D evaporation.*—80 mg mineral oil/mL $CH_2Cl_2$. *(2) For N evaporation.*—20 mg mineral oil/mL isooctane.

### C. Apparatus

(**a**) *ThermoSorb/N™ cartridges.*—Use as received for quant. trapping of volatile *N*-nitrosamines (Thermedics, Inc., Div. of Thermo Electron Corp., 470 Wildwood St, PO Box 2999, Woburn, MA 01801).

(**b**) *Variable temperature oil bath.*—Thermostatically controlled, capable of operating at 150 ± 3° and of moving vertically with aid of laboratory jack (The Lab Apparatus Co., PO Box 42070, Cleveland, OH 44142).

(**c**) *Soxhlet extraction apparatus.*—(Kimble Glass Inc.). Allihn condenser with 34/45 ₮ joint. Extn tube with 34/45 ₮ upper joint and 24/40 ₮ lower joint. Extn thimble, 25 × 85 mm borosilicate glass fitted with coarse porosity frit.

(**d**) *Kuderna-Danish evaporative concentrator.*—(Kontes Glass Co.). 3-ball Snyder column with 24/40 ₮ joints, 250 mL flask with 24/40 ₮ joint and 19/22 ₮ lower joint, and 4 mL graduated concentrator tube with 19/22 ₮ joint.

(**e**) *Gas chromatograph.*—Hewlett-Packard Model 5710A, or equiv., equipped with 6 ft × 4 mm id glass column packed with 10% Carbowax 20M/2% KOH on 80–100 mesh Chromosorb WAW (No. 1-1805, Supelco). Condition column overnight at 215°. Operate at temp. program mode from 150 to 190° at 4°/min. Injection port temp. 250°. Carrier gas prepurified Ar at flow rate 40 mL/min. Interface GC app. to thermal energy analyzer, (**f**), via 1/8 in. od stainless steel tube connected to Swagelok fittings and operate at 170°.

(**f**) *Thermal energy analyzer.*—Model 502, Thermo Electron Corp., 115 Second Ave, Waltham, MA 02154, or equiv. Operate pyrolysis chamber at 500° in GC mode. O flow to ozonator, 10 mL/min. Keep cold trap at −150° using liq. N/2-methylbutane slush bath. Pressure of reaction chamber, ca 0.9 torr. Record TEA detector response on Hewlett-Packard 3380 integrator.

(**g**) *Purge and trap apparatus.*—Fig. **986.01A** contains following parts: *(1)* Ar gas cylinder and gauge (Air Products Specialty Gas, Tamaqua, PA 18252); *(2)* metering valve; *(3)* purge gas manifold, 4-position; *(4)* Nalgene needle valve type CPE (No. 6400-0125, Nalge Co., 75 Panorama Creek Dr, PO Box 20365, Rochester, NY 14602); *(5)* 18/7 g-g outer joints with pinch clamps (No. 772398, The Wheaton Agency, A Div. of Wheaton Industries, 1000 N Tenth St, Millville, NJ 08332); *(6)* impingers, 50 mL graduated glass tubes with 24/40 ₮ clear-seal, grease-free joints 18/7 g-g ball joints, and 1 mm id nozzle ca 5 mm above bottom of impinger (No. 753463, Wheaton Scientific); *(7)* variable scale flow-check, calibrated for purge rate in mL Ar/min (No. 7083, Alltech Associates, Inc.). Bubble meter for measuring gas flow rates for GC may be substituted.

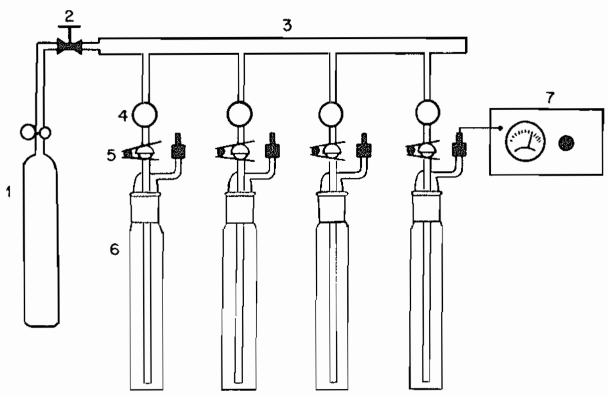


**Fig. 986.01A—Diagram of purge and trap apparatus equipped with 4 impinger tubes**

*Note:* Do not use any rubber tubing, gaskets, O-rings, or other items made of rubber in any part of this method.

***D. Description and Use of Purge and Trap Apparatus***

App. shown in Fig. **986.01A** is designed for high temp. purging and trapping of 7 volatile *N*-nitrosamines from concd sample ext/mineral oil mixt. on 4 samples simultaneously. Cylinder contg prepurified Ar gas equipped with high pressure regulator is used to supply 20 psig to flow-metering valve which regulates final purge flow thru samples. Gas stream is diverted into tubular stainless steel manifold, 250 × 20 mm od, contg 4 exit tubes spaced 50 mm apart and measuring 40 × 10 mm od. Each of these tubes is coupled using $^3/_8$ in. Tygon tubing to Nalgene needle valves which serve dual purposes: as shut-off valve when less than 4 samples are analyzed; and for making minor adjustments in purge rate due to slight differences in flow characteristics of impinger and cartridges. An 18/7 g-g outer spherical joint is attached to Nalgene valve to permit quick gas-tight connection to 18/7 g-g ball joint on impinger inlet, using appropriate pinch clamp. As shown in Fig. **986.01B**, impingers are assembled by inserting glass nozzle (1 mm id

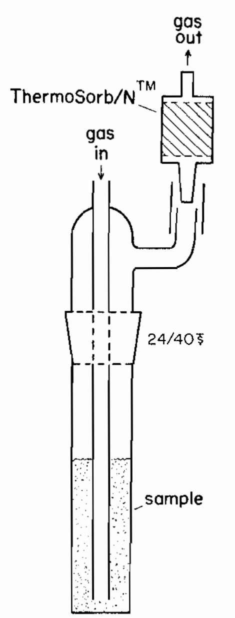


**Fig. 986.01B—Close-up diagram of impinger tube fitted with ThermoSorb/N cartridge**

orifice) into sample mixt. and coupling 24/40 ₸ grease-free male and female joints together to form leak-free seal. Once sealed, Ar gas is allowed to purge thru sample mixt., thru outlet tube of impinger (see Fig. **986.01B**). Tygon tubing is used to connect impinger outlet tube to inlet side marked "AIR IN" of cartridge, which is std male Luer connector. Purged volatile *N*-nitrosamines are then collected on sorbent contained in cartridge with Ar effluent exiting from female Luer connector. Flow rate of Ar is measured directly from cartridge with variable scale flow meter which has been previously calibrated for flow rate of Ar gas (mL/min). Bubble meter can be substituted for variable scale flow meter. Temp. of sample mixt. during purge is controlled by immersing impinger up to sample vol. mark (ca 25 mL line) in thermostatically controlled oil bath capable of operating isothermally up to 150°. Gas manifold, as well as each impinger, is secured by clamps to support grid; therefore, oil bath is moved vertically in and out of position for high temp. purge.

***E. Extraction and Cleanup of Pacifier Samples***

Accurately weigh 5 g from each sample into 250 mL r-b flask and add 100 mL $CH_2Cl_2$. Dil. internal stock std soln to 50 ng/mL with $CH_2Cl_2$ and spike contents of flask with 2 mL dild std. Seal flask and let contents stand overnight (16–21 h) at ambient temp.

Transfer ext and rubber pieces to glass extn thimble fitted with coarse porosity glass frit in Soxhlet extn app. Rinse 250 mL r-b flask with 25 mL $CH_2Cl_2$ and transfer rinse to Soxhlet app. Ext rubber pieces for 1 h in app. at rate of 8 cycles/h.

Let cool and transfer $Ch_2Cl_2$ ext to 250 mL K-D evaporator. Rinse extn flask with two 10 mL portions of $CH_2Cl_2$ and combine rinses with 125 mL ext. Add 1 mL keeper soln *1* and 2 or 3 boiling chips (Boileezers, Fisher Scientific Co.) to ext. Evap. ext in K-D unit using 3-ball Snyder column on 55° water bath until vol. is reduced to 3–4 mL.

Let K-D unit cool to room temp., allowing excess solv. in Snyder column to rinse down walls of unit into 4 mL K-D tube (total = 3–4 mL). Remove 250 mL reservoir and 3-ball Snyder column, reduce vol. of ext to 2 mL in same K-D tube under gentle stream of N (ca 50 mL/min), and transfer 2 mL ext using disposable Pasteur pipet with two 1 mL mineral oil rinses to 50 mL purge and trap app. contg 20 mL mineral oil and 1 mL of 10 mg/mL of α-tocopherol in mineral oil as nitrosation inhibitor.

Assemble purge and trap app. and connect cartridges to exit tubes with Tygon connector. Adjust Ar flow rate to 400 mL/min thru cartridge ± 5% (i.e., 380–420 mL Ar/min). Note: Check flow rate intermittently during purging, especially within first 15 min because of initial increase in temp. of sample. Immerse purge tubes (up to sample line) or to ca 25 mL mark in 150 ± 3° oil bath for 1.5 h. Remove cartridge and tightly cap. (Note: This is good stopping point; cartridge can be eluted on following day if necessary.)

Elute cartridge using 10 or 20 mL glass Luer-Lok syringe connected to female Luer adapter (air exit side) with 20 mL acetone–$CH_2Cl_2$ (1 + 1, v/v). Collect eluate in 30 mL culture tube. (Note: 30 mL tube(s) should be scored with file or piece of tape placed at 5 mL vol. mark.)

Evap. ext to ca 5 mL and then transfer with three 1 mL rinses of $CH_2Cl_2$ to 10 mL graduated tube. Add 0.5 mL keeper soln *2*. Evap. sample (vol. = 8.5 mL) to 2 mL under gentle stream of N. (*Note:* If 2 mL sample cannot be analyzed same day as evapd, it is advantageous to refrigerate sample at larger vol., i.e., 4–5 mL, and evap. next day before analysis by GC-TEA.)

Analyze 2 mL sample by injecting 8 μL aliquot into GC-TEA.

### F. Quantitation

Use internal std technic. Dil. external stock std soln with $CH_2Cl_2$ to 50, 100, and 200 ng/mL to be used as working stds for analysis. Inject 8 µL into GC-TEA to det. responses (peak hts) of NDPA and other nitrosamines for use in internal stdzn calcn. Inject 8 µL of each 2 mL sample ext into GC-TEA. Det. responses (peak hts) of NDPA and any other *N*-nitrosamines detected for use in internal stdzn calcn. Calc. results as follows:

$$\text{ppb } N\text{-Nitrosamine X} = [(PH_X) \times (F_X) \times (100 \text{ ng NDPA})]/[(PH_{NDPA}) \times (F_{NDPA}) \times (\text{g sample})]$$

where $PH_X$ = peak ht in mm of *N*-nitrosamine X in sample; $F_X$ = ng *N*-nitrosamine X/mL in external std soln divided by peak ht in mm of *N*-nitrosamine X in external std soln; 100 ng NDPA = total ng NDPA (internal std) added to sample; $PH_{NDPA}$ = peak ht in mm of NDPA (internal std) in sample; $F_{NDPA}$ = ng NDPA/mL in external std soln divided by peak ht in mm of NDPA in external std soln; g sample = g rubber sample analyzed.

Ref.: JAOAC **69,** 504(1986).

## 930.11 Phenol in Hazardous Substances

### Colorimetric Method

### Final Action

### *Method I*

(Applicable to com. cresols, saponified cresol solns, coal tar dips, and disinfectants, and to kerosene solns of phenols in absence of salicylates or β-naphthol)

### A. Reagents

(*Caution:* See safety notes on nitric acid, formaldehyde, and mercury.)

(**a**) *Dilute nitric acid.*—Aerate $HNO_3$ until colorless and dil. 1 vol. with 4 vols $H_2O$.

(**b**) *Millon reagent.*—To 2 mL Hg in 200 mL erlenmeyer under hood, add 20 mL $HNO_3$. After first violent reaction, shake as needed to disperse Hg and maintain action. After ca 10 min, when action practically ceases even in presence of undissolved Hg, add 35 mL $H_2O$, and if basic salt seps, add enough dil. $HNO_3$ to dissolve it. Add 10% NaOH soln dropwise with thoro mixing until curdy ppt that forms after adding each drop no longer redissolves but disperses as permanent turbidity. Add 5 mL dil. $HNO_3$ and mix well. Prep. fresh daily. Millon reagent is dangerously poisonous and should not be transferred with ordinary pipet and mouth suction unless protective trap is used.

(**c**) *Phenol std soln.*—Dissolve weighed amt pure phenol (congealing point ≥40°) in enough $H_2O$ to make ≥1% soln. On day it is to be used, dil. to make 0.025% aq. soln (final std).

(**d**) *Formaldehyde soln.*—Dil. 2 mL 37% HCHO soln to 100 mL with $H_2O$.

(**e**) *Methyl orange indicator.*—0.5% aq. soln.

### B. Apparatus

(**a**) *Nessler cylinders.*—50 mL tall-form, matched.

(**b**) *Test tubes.*—Approx. 180 × 20 mm, with rubber stoppers, marked at 25 mL.

(**c**) *Water bath for heating test tubes.*—Beaker contg disk of wire gauze raised ca 2.5 cm from bottom may be used.

### C. Preparation of Sample

(**a**) *Commercial cresol.*—Weigh by difference ca 2.5 g sample into 250 mL vol. flask, dissolve in 10 mL 10% NaOH soln, and dil. to vol. with $H_2O$.

(**b**) *Saponified cresol solns, coal tar dips and disinfectants, kerosene solns of phenols, etc.*—Weigh by difference ca 5 g sample (or use 5 mL and calc. wt from density) into 250 mL vol. flask and dil. to vol. with $H_2O$. With products consisting largely of kerosene, bring $H_2O$ level to mark and take aliquots from aq. portion only.

### D. Determination

Transfer 5 mL aliquot prepd soln to 200 mL vol. flask and promptly dil. to ca 50 mL. Add 1 drop Me orange, (**e**), and then dil. $HNO_3$ until soln is practically neut. Dil. to vol. and shake well.

Place 5 mL dild soln in each of 2 marked test tubes; in each of 2 addnl test tubes place 5 mL std phenol soln. Flow 5 mL Millon reagent down side of each tube, mix, and place tubes in boiling $H_2O$ bath; continue boiling exactly 30 min, cool immediately and thoroly by immersion in bath of cold $H_2O$ ≥10 min, and add 5 mL dil. $HNO_3$ to each tube.

Mix well and add 3 mL HCHO soln to one of each pair of tubes. Dil. all tubes to 25 mL mark with $H_2O$, stopper, shake well, and let stand overnight. (Tubes contg HCHO fade to yellow; others show orange or red color.)

Pipet 20 mL from each of the 2 phenol tubes to 100 mL vol. flasks; add 5 mL dil. $HNO_3$ to each, dil. to vol., and mix. (Red flask contains "phenol std," yellow flask "phenol blank.") Transfer these solns to burets. Pipet 10 mL of each sample soln into Nessler tubes. (The orange or red constitutes the "unknown" and the yellow the "sample blank." Mark each Nessler tube distinctly to avoid confusion.) To "sample blank" tube add measured amt of "phenol std" and add same vol. "phenol blank" to "unknown." Agitate thoroly (aided by insertion of rubber stoppers, if necessary), and compare colors. When tubes are brought to match, each mL phenol std used = 1% phenol if sample weighing exactly 5 g was used, or 2% if exactly 2.5 g was used.

*Note.*—Take following precautions: Pair of phenol tubes provides enough final solns to assay several unknowns, but all the latter must have accompanied phenol solns thruout entire process with identical reagents and treatment. If end point is inadvertently overrun it is possible to work back to it, but since mistakes may be made in this operation it is better to repeat comparison on fresh portions from original tubes. Too much delay in matching tubes must be avoided after titrn is started, otherwise excess HCHO present in blanks may have time after mixing to affect intensity of red color.

Refs.: USDA Bull. **1308,** p. 17. JAOAC **13,** 160(1930).

### *Method II*

### E. Determination

(Applicable to detn of phenol in presence of salicylates)

Weigh by difference 10 g sample into separator (or use 10 mL and calc. wt from density of sample). Add 50 mL kerosene and ext with three 100 mL portions $H_2O$. Filter aq. exts thru wet filter into 500 mL vol. flask, dil. to vol. with $H_2O$, and proceed as in **930.11D**.

When tubes are brought to match, each mL phenol std used = 1% phenol if sample weighing exactly 10 g was used.

Ref.: Ind. Eng. Chem. Anal. Ed. **1,** 232(1929).

CAS-108-95-2 (phenol)

# 9. Metals and Other Elements at Trace Levels in Foods

**Stephen G. Capar, *Associate Chapter Editor***
*Food and Drug Administration*

## MULTIELEMENT METHODS

### 986.15 Arsenic, Cadmium, Lead, Selenium, and Zinc in Food

**Multielement Method**

**First Action 1986**
**Final Action 1988**

#### *A. Principle*

Sample is digested with $HNO_3$ in closed system. Cd and Pb are detd by anodic stripping voltammetry (ASV). As, Se, and Zn are detd by atomic absorption spectrophotometry (AAS) after generation of metal hydrides (for As and Se).

(*Caution:* See safety notes on pipets, nitric acid, perchloric acid, sodium hydroxide, and arsenic trioxide.)

#### *B. Apparatus*

(**a**) *Polarograph.*—With anodic stripping accessories. Typical operating parameters for Model 174 with hanging drop Hg electrode are: Scan rate, 5 mv/sec; scan direction, +; scan range, 1.5 v; initial potential, −0.7 v; modulation amplitude, 25 mv; operation mode, differential pulse; display direction, "−"; drop time, 0.5 sec; low pass filter, off; selector, off; pushbutton, initial; output offset, off; and current range, 5–10 μamp, or as needed.

Other instruments and electrodes such as wax impregnated graphite may be used according to manufacturer's directions.

(**b**) *Atomic absorption spectrophotometer.*—Perkin-Elmer Corp. Model 403, or equiv., with Zn, As, and Se hollow cathode lamps or As and Se electrodeless discharge lamps, 3 slot, 10 cm Bolling burner head, air-$C_2H_2$ and H-N-entrained air flames, and deuterium arc background corrector.

(**c**) *Decomposition vessel.*—70 mL. *See* **974.14A**.

(**d**) *Hydride generator.*—*See* Fig. **986.15A**. Constructed from following: (*1*) *Flat bottom flask.*—Borosilicate glass, 50 mL (Corning No. 5160, or equiv.). (*2*) *Stopper fittings.*—Two-hole (1 thru center) No. 9 rubber stopper, fitted with gas outlet tube of 100 mm × $^1/_8''$ (3 mm) id polyethylene tubing thru center hole. Place bottom of gas outlet tube thru cut off bottom 1″ segment of $^5/_8''$ polyethylene test tube with hole in bottom so that 3 mm of tube protrudes thru test tube. Insert thru second hole 75 mm × $^1/_8''$ (3 mm) id polyethylene tubing as N inlet tube. Seal bottom end of tube with burner and then punch several holes at sealed end with 21 gage needle. Alternatively, prep. similarly 500 mm × $^1/_{16}''$ (1.5 mm) id polyethylene tubing and hold in place in stopper with hole-thru septum. Connect other end of tubing to AA spectrophotometer with 500 mm Tygon tubing by cutting auxilliary line at ca 75 mm from mixing chamber and attaching tubing. (*3*) *Generator mount.*—(Optional) 64 mm × 0.5″ id pipe secured to laboratory ring stand by means of clamp holder. Insert extension clamp into pipe and attach another clamp to back of clamp to hold clamp in place and to serve as handle; clamp is now free to rotate ca 180°. Attach rubber stopper of hydride generator to extension clamp with stiff wire and position just at level of clamp jaws. In operation, place flask of generator between jaws of extension clamp, insert stopper firmly into neck of flask, then tighten clamp jaws around neck of flask. Unit can be rapidly and uniformly inverted by rotating handle on extention clamp, thus allowing sample and Na borohydride to mix rapidly and reproducibly.

(**e**) *Pipets.*—50 and 100 μL Eppendorf micropipets, or equiv.

#### *C. Reagents*

(Use double distd $H_2O$. Rinse all glassware with $HNO_3$ (1 + 1) followed by thoro $H_2O$ rinse. Decontaminate digestion vessels by digesting with reagents to be used in digestion. Rinse thoroly with the $H_2O$. Decontamination is necessary to reduce blanks, especially for Pb, to acceptable level.)

(**a**) *Acids.*—(*1*) *Nitric acid.*—Redistd. (*2*) *Perchloric acid.*—70%, double vac. distd (G. Fredrick Smith Chemical Co., or equiv.). (*3*) *Hydrochloric acid.*—*8M*. Dil. 66 mL HCl to 100 mL with $H_2O$.

(**b**) *Nitrate soln.*—*Equimolar soln of $KNO_3$ and $NaNO_3$.* Dissolve 54.3 g $KNO_3$ and 45.7 g $NaNO_3$ (available as Suprapur®, Nos. 5065 and 6546, resp., EM Science) in $H_2O$ in 200 mL vol. flask, dil. to vol., and mix. To further purify, add 1–2 drops $NH_4OH$ to 25 mL aliquot and ext with 2 mL 10 μg dithizone/mL $CCl_4$ until lower solv. layer is colorless.

(**c**) *Magnesium solns.*—(*1*) *Magnesium chloride soln.*—37.5 mg/mL. Dissolve total of 3.75 g MgO, USP, by adding small amts at time to 100 mL 8*M* HCl. (*2*) *Magnesium nitrate soln.*—75 mg/mL. Mix 3.75 g MgO, USP, with ca 30 mL $H_2O$, slowly add $HNO_3$ to dissolve (ca 10 mL), cool, and dil. to 50 mL with $H_2O$.

(**d**) *Sodium borohydride soln.*—4.0 g $NaBH_4$/100 mL 4% NaOH.

(**e**) *Potassium iodide soln.*—Dissolve 20 g KI in $H_2O$ and dil. to 100 mL. Prep. just before use.

(**f**) *Metal powders.*—Purity: 99.99 + % Cd, Pb, Zn; 99.99% Se. Alfa Products, Morton Thiokol, Inc., 152 Andover St, Danvers, MA 01923.

(**g**) *Cadmium std solns.*—(*1*) *Stock soln.*—1 mg/mL. Dissolve 1.000 g Cd powder in 20 mL $HNO_3$ (1 + 1) in 1 L vol. flask, and dil. to vol. with $H_2O$. (*2*) *Working soln.*—2 μg/mL. Pipet 10 mL stock soln into 100 mL vol. flask, and dil. to vol. with $H_2O$. Pipet 2 mL dild soln into 100 mL vol. flask and dil. to vol. with $H_2O$.

(**h**) *Lead std solns.*—(*1*) *Stock soln.*—1 mg/mL. Dissolve 1.000 g Pb powder in 20 mL $HNO_3$ (1 + 1) in 1 L vol. flask, and dil. to vol. with $H_2O$. (*2*) *Working soln.*—5 μg/mL. Pipet 1 mL stock soln into 200 mL vol. flask and dil. to vol. with $H_2O$.

(**i**) *Zinc std solns.*—(*1*) *Stock soln.*—1 mg/mL. Dissolve 1.000 g Zn powder in 20 mL HCl (1 + 1) in 1 L vol. flask, and dil. to vol. with $H_2O$. (*2*) *Working solns.*—0.2, 0.5, 1.0, and 1.5 μg/mL. Pipet 1 mL stock soln into 100 mL vol. flask and dil. to vol. with $H_2O$. Pipet 2, 5, 10, and 15 mL dild soln into sep. 100 mL vol. flasks, each contg 1 mL $HClO_4$, and dil. to vol. with $H_2O$.

(**j**) *Arsenic std solns.*—(*1*) *Stock soln.*—Dissolve 1.320 g $As_2O_3$ in min. vol. 20% NaOH in 1 L vol. flask, acidify with HCl (1 + 1), and dil. to vol. with $H_2O$. (*2*) *Working solns.*—1, 2, 3, 4, and 5 μg/mL. Pipet 10 mL stock soln into 100

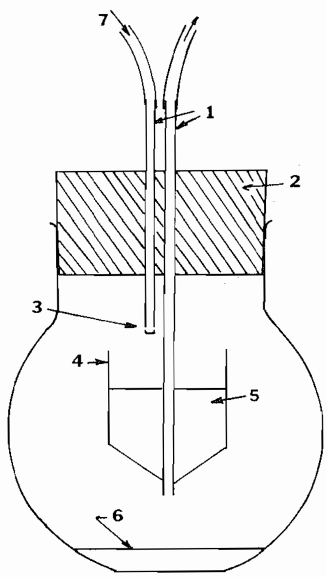


**FIG. 986.15A—Hydride generator: 1, polyethylene tubing; 2, rubber stopper; 3, flame sealed polyethylene tubing with holes punched at one end; 4, reagent cup; 5, sodium borohydride solution; 6, sample solution; 7, nitrogen inlet from "auxiliary" line of AAS**

mL vol. flask, and dil. to vol. with $H_2O$. Pipet 1, 2, 3, 4, and 5 mL dild soln into sep. 100 mL vol. flasks, and dil. to vol. with $H_2O$.

(**k**) *Selenium std solns.*—(*1*) *Stock soln.*—1 mg/mL. Dissolve 1.000 g Se powder in min. vol. $HNO_3$ in 200 mL beaker and evap. to dryness. Add 2 mL $H_2O$ and evap. to dryness. Repeat addn of $H_2O$ and evapn to dryness twice. Dissolve in min. vol. HCl (1 + 9) in 1 L vol. flask, and dil. to vol. with HCl (1 + 9). (*2*) *Working solns.*—1, 2, 3, 4, and 5 μg/mL. Pipet 10 mL stock soln into 100 mL vol. flask and dil. to vol. with $H_2O$. Pipet 1, 2, 3, 4, and 5 mL dild soln into sep. 100 mL vol. flasks and dil. to vol. with $H_2O$.

### *D. Closed System Digestion*

(Do not exceed manufacturer's specifications of 0.3 g solids with 70 mL vessel. Proceed cautiously with new or untried uses. Let such samples stand with $HNO_3$ overnight or heat on hot plate cautiously until any vigorous reaction subsides. Then proceed with closed vessel digestion. Open vessel in hood since N oxides are released.)

Weigh 0.3 g sample (dry basis) into decontaminated decomposition vessel, add 5 mL $HNO_3$, close vessel with lid, and heat in 150° oven 2 hr. Cool in hood, remove vessel from jacket, and transfer contents to 10 mL vol. flask. Add 4 mL $H_2O$ to vessel, cover with lid, and while holding lid tightly against rim, invert several times, and add rinse to flask. Dil. to vol. with $H_2O$ and mix.

### *E. Anodic Stripping Voltammetry*

(For Cd and Pb)

Pipet aliquot of digested sample soln into decontaminated 50 mL Vycor crucible and add 2 mL nitrate soln, (**b**). Conduct reagent blank simultaneously. Heat on hot plate at low heat to dryness; then increase heat to max. (ca 375°). Nitrate salts will melt and digest org. matter in 15–20 min. Place crucibles in 450° furnace to oxidize any remaining carbonaceous matter (10–20 min). Digestion is complete when melt is clear. Let cool, add 1 mL $HNO_3$ (1 + 1) to solidified melt, and heat on hot plate to dryness to expel carbonates and nitrites and to control acidity. Dissolve in 5.0 mL $HNO_3$ (0.5 mL/L), warming on hot plate to speed soln. Transfer to polarographic cell with 5.0 mL $H_2O$. Bubble O-free N thru soln 5 min; then direct N over soln.

Set dial for Hg drops at 4 μm divisions. Stir soln with mag. stirrer at const and reproducible rate so Hg drop is not disturbed. Slide selector switch to "Ext. Cell" and measure time for 120 sec with stopwatch. Turn off stirrer and let stand 30 sec. Press "Scan" button to obtain peaks corresponding to Cd and Pb at ca −0.57 and −0.43 v, resp., against satd calomel electrode.

Add known vols of each std to sample soln in cell from Eppendorf pipet. Amts added should be ca 1×, 2×, etc. of amt metal present initially in cell, and each addn should not change original vol. significantly. After each addn, bubble N thru soln briefly and perform deposition and stripping operations exactly as for original soln. Plot μg metal added on x-axis against peak ht on y-axis. Extrapolate linear line to x axis to obtain μg metal in cell.

$$\mu g\ metal/g\ sample = [(M - M')/g\ sample] \times (10/mL\ aliquot\ taken)$$

where $M$ and $M'$ = μg metal from std curve for sample and blank, resp.

### *F. Atomic Absorption Spectrophotometry*

(For As, Se, and Zn)

(**a**) *Arsenic.*—Pipet aliquot digested sample soln into decontaminated 50 mL round, flat-bottom borosilicate flask, and add 1 mL $Mg(NO_3)_2$ soln, (**c**)(*2*). Heat on hot plate at low heat to dryness; then increase heat to max. (ca 375°). Place flask in 450° furnace to oxidize any carbonaceous matter and to decompose excess $Mg(NO_3)_2$ (≥30 min). Cool, dissolve residue in 2.0 mL 8*M* HCl, add 0.1 mL 20% KI to reduce $As^{+5}$ to $As^{+3}$, and let stand ≥2 min. Conduct reagent blank with sample.

Prep. stds as follows: To six 50 mL flasks (same type as used for sample) add 2.0 mL $MgCl_2$ soln, (**c**)(*1*), and to 5 flasks add 50 μL aliquots of respective working std solns so that series will contain 0, 0.05, 0.1, 0.15, 0.20, and 0.25 μg As. (Other amts may be used depending on sensitivity of system.) Add 0.1 mL 20% KI to each flask, mix, and let stand ≥2 min.

Connect generator to instrument as shown in Fig. **986.15B** and adjust pressures and flows as in Table **986.15**. Operate instrument according to manufacturer's instructions, with lamp in place and recorder set for 20 mm/min.

Add 2.0 mL 4% $NaBH_4$ soln to reagent dispenser of generator, and insert rubber stopper tightly into neck of flask contg sample or std. With single rapid, smooth motion, invert flask, letting soln mix with sample or std. (This operation must be performed reproducibly.) Sharp, narrow *A* peak will appear immediately. When recorder pen returns to baseline, remove stopper from flask, and rinse reagent dispenser with $H_2O$ from squeeze bottle; then suck out $H_2O$. Proceed with next sample or std. When series is complete, rinse glassware thoroly.

Plot calibration curve of μg As against *A*, and obtain μg As in sample aliquot from this curve. Correct for reagent blank.

(**b**) *Selenium.*—Proceed as in (**a**), using Se lamp and stds, but omit addn of KI soln. KI will reduce Se to elemental state and cause loss of signal. Instead, cover flask with small watch glass and place on steam bath 10 min, and cool to room temp.

(**c**) *Zinc.*—Pipet 1 mL aliquot digested sample soln into de-

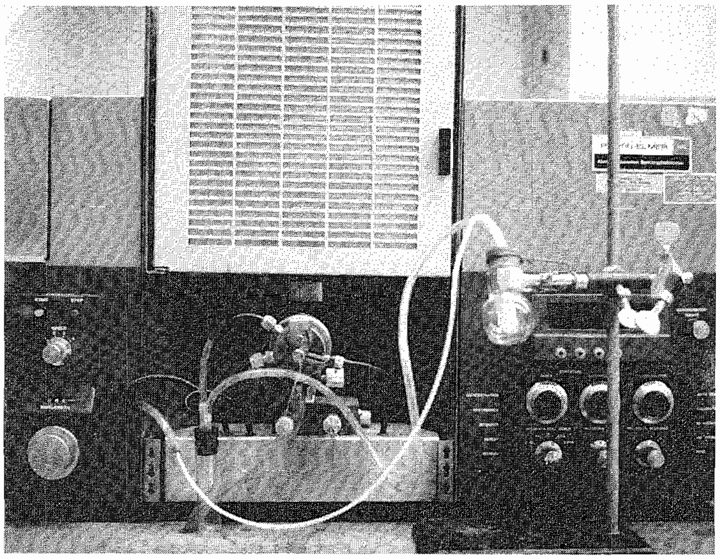

**FIG. 986.15B—Hydride generator and mount connected to auxiliary line of spectrophotometer. Test tube acid trap connected between generator and instrument is not included in method**

**Table 986.15 Flow Rates and Pressures for Arsenic and Selenium Determinations**

| Gas | Tank, psi | AA Control Box, psi | Perkin-Elmer Model 403 Flowmeter, divisions |
|---|---|---|---|
| H | 20 | 10 | 20 (4 L/min) |
| N | 40 | 30 | 25 (10 L/min) |

contaminated 25 mL erlenmeyer, and add 0.1 mL $HClO_4$. Heat on hot plate to white fumes of $HClO_4$. Sample should be completely digested as indicated by clear, practically colorless soln. If sample chars, add 0.5 mL portions $HNO_3$ and again heat to white fumes. Finally, heat just to dryness but do not bake. Cool, and dissolve residue in 3.0 mL $HClO_4$ (1 + 99).

Operate instrument in accordance with manufacturer's instructions, using air-$C_2H_2$ flame, and measure *A* of sample and stds, (**i**)(*2*). Dil. sample soln with $HClO_4$ (1 + 99), if soln is too concd. Plot calibration curve of μg Zn against *A*, and obtain μg Zn in sample aliquot from this curve. Correct for reagent blank.

Ref.: JAOAC **63**, 485(1980).

CAS-7440-38-2 (arsenic)
CAS-7440-43-9 (cadmium)
CAS-7439-92-1 (lead)
CAS-7782-49-2 (selenium)
CAS-7440-66-6 (zinc)

## 982.23 Cadmium and Lead in Food

### Anodic Stripping Voltammetric Method

**First Action 1982**
**Final Action 1988**

(Not applicable to fats and oils)

(*Caution:* See safety notes on pipets and nitric acid.)

### *A. Principle*

Sample is dry-ashed with $K_2SO_4$ and $HNO_3$ at ca 500°. Pb and Cd are detd by anodic stripping voltammetry (ASV). Estd quantitation limits, based on 10 g sample, are 0.005 ppm Cd and 0.010 ppm Pb.

### *B. Apparatus*

(Thoroly soak all glassware and plasticware in 20% (v/v) $HNO_3$ for ≥24 h and rinse with distd, deionized $H_2O$.)

(**a**) *Voltammetric analyzer.*—Capable of ASV and equipped with necessary accessories, i.e., cells, electrodes, recorders, Hg capillaries, micrometer or similar device for adjusting drop size, stirring motor, etc. (EG&G Princeton Applied Research Corp., PO Box 2565, Princeton, NJ 08540, Models 174A, 315A, and 303, or equiv., for differential pulse anodic stripping voltammetry (DPASV) at hanging Hg drop electrode; Environmental Sciences Associates, 45 Wiggins Ave, Bedford, MA 01730, Model 3010A, or equiv., for linear sweep anodic stripping voltammetry (LSASV)).

(**b**) *Ashing vessels.*—150–250 mL quartz, Vycor, or Pyrex beakers equipped with suitable glass covers (Fisher Scientific Co., No. 2-609A, or equiv.). Quartz is preferred. Vycor or Pyrex may be used if quartz beakers are not available. *Note:* For best results, quartz beakers should be fire-polished to retard etching.

(**c**) *Drying oven.*—Controllable within range 50–150° with <5° variation.

(**d**) *Furnace.*—Controllable within range of 100–1000° with <5° variation. Check calibration of oven temp. control to ensure accurate temps. Furnace must be operated in suitable fume hood.

(**e**) *Controllable hot plate.*—Corning Glass Works, Corning, NY, PC-35 (replacement Model PC 300), or equiv.

(**f**) *Micropipets.*—10 thru 100 μL (Eppendorf, or equiv.).

### *C. Reagents*

*Note:* Use only distd, deionized $H_2O$.

(**a**) *Nitric acid.*—J.T. Baker Inc. No. 9598, or equiv.

(**b**) *Potassium sulfate ashing soln.*—10 g/100 mL. Dissolve 50.0 g $K_2SO_4$ (J.T. Baker Inc. No. 3278, or equiv.) in 400 mL $H_2O$ contg 10 mL $HNO_3$. Dil. to 500 mL with $H_2O$.

(**c**) *Nitrogen.*—Prepurified, $H_2O$-pumped.

(**d**) *Electrolyte soln.*—1.7M in HOAc, 1.25M in Na acetate trihydrate, and 0.1M in tartaric acid. Dissolve 170.0 g $NaOAc.3H_2O$ (ACS) in 300 mL $H_2O$. Add 97 mL glacial HOAc and 15 g tartaric acid (ACS). Dil. to 1 L with $H_2O$. pH should be 4.7 ± 0.1.

(**e**) *Cadmium std soln.*—1.0 mg/mL. Dissolve 1.000 g Cd (99.99%) in 10 mL $HNO_3$ in 1 L vol. flask. Dil. to vol. with $H_2O$.

(**f**) *Lead std soln.*—1.0 mg/mL. Dissolve 1.000 g Pb (99.99%) in 10 mL $HNO_3$ in 1 L vol. flask. Dil. to vol. with $H_2O$.

(**g**) *Working std solns.*—Prep. either sep. or mixed working std soln for Cd and Pb in the range 0.1–10 μg/mL from std solns (**e**) and (**f**) by dissolving appropriate aliquots in 1% (v/v) $HNO_3$.

*Note:* Electrolyte soln (**d**) and $K_2SO_4$ soln (**b**) may require further cleanup for sufficiently low reagent blanks. For stated quantitation limits, analyte concns in final cell solns (electrolyte and sample solns) of reagent blank should not be >0.5 ng Cd/mL and >1 ng Pb/mL. Controlled potential electrolysis is recommended means of cleaning reagents.

### D. Preparation of Sample

*Note:* Laboratory contamination control is important. Take all precautions possible to avoid contamination of samples, reagents, and equipment. Prep. at least 3 control reagent blanks which include any addnl $H_2O$ and $HNO_3$ used for sample ashing. Carry control reagent blanks thru entire method.

Weigh 5.0–10.0 g homogenized sample into ashing vessel (**b**). Use 5.0 g for dry materials such as cereals. Add 5.0 mL $K_2SO_4$ ashing soln (**b**) and mix thoroly, using glass stirring rod. If needed, add $H_2O$ to ensure sample and ash aid are well mixed. Cover ashing vessel with glass cover and dry in 110–120° oven (**c**) until thoroly dry (usually 2–3 h or, if desired, overnight). Place vessel in cold furnace (**d**) and set temperature at 500–550°. Caution: Do not heat >500° if using Pyrex beakers, and avoid excessive overshooting of temp. Maintain set temp. ≥4 h (may be ashed overnight). Remove vessel from furnace, and cool. Ash should be white and essentially carbon-free.

Wash down sides of vessel with min. amt. $H_2O$ and add 2.0 mL $HNO_3$. Use glass stirring rod to break up solid particles. Dry thoroly on hot plate (**e**) at low setting. If samples such as sugars and cereals splatter on hot plate during $HNO_3$ treatment, dry under IR lamp instead. Increase hot plate setting to medium for several minutes to ensure dryness. Return vessel to 500° furnace 30 min. Cool; if necessary, repeat $HNO_3$ treatment using 1 mL increments of $HNO_3$, until white, C-free ash is obtained.

Add 1.0 mL $HNO_3$ and 5 mL $H_2O$ to vessel, swirl to dissolve, and let stand 5 min. If residue remains undissolved, warm gently on 80–90° hot plate not >5 min. Minimize possible Sn(II) formation by heating dil. acid soln as little as possible. Small amt of white, siliceous-like ppt may remain undissolved. Cool, and quant. transfer sample to 50 mL vol. flask with aid of $H_2O$. Dil. to vol. with $H_2O$ and mix well. Let stand to allow any ppt present to settle. Do not filter. Use clear supernate to det analytes by either DPASV or LSASV below.

### E. Differential Pulse Anodic Stripping Voltammetry

Transfer 5.0 mL aliquot of sample soln to electrolysis cell containing Teflon-coated stirring bar and add 5.0 mL electrolyte soln (**d**) to cell. (Aliquot vol. may be varied as long as 1:1 ratio is maintained between sample soln and electrolyte.) pH of cell soln should be 4.3 ± 0.3. Room temp. should be constant (±1°/2 h) and between 20 and 30°. Purge soln 5 min with N (**c**). Adjust gas inlet to let N flow gently above and across soln surface. If hanging Hg drop electrode is used, add fresh drop of Hg to capillary tip with micrometer or similar device to ensure reproducibility of drop. Turn on stirrer motor and electrolyze soln at −0.8 V vs satd calomel electrode (SCE) or Ag/AgCl electrode. Deposition time may vary with instrument (see manufacturer's instructions). When using PAR 174 polarographic analyzer, 1–2 min is sufficient, depending on level of analytes of interest in cell soln. Stop stirring and let soln equilibrate 30 s. Linerarly increase applied voltage anodically. Follow manufacturer's instructions for rate of scan, e.g., 2–6 mV/s. Measure wave ht at peak potentials for Cd at −0.62 ± 0.05 V and for Pb at −0.45 ± 0.05 V vs SCE or Ag/AgCl. For widely varying concns of Cd and Pb, change current sensitivity to appropriate range by momentarily stopping stripping scan at end of Cd peak, switching to appropriate sensitivity setting for Pb, and then continuing scan before Pb peak begins.

Quantitate total amts of Pb and Cd in cell soln by using method of std addns in cell as follows: Record voltammogram from known vol. of cell soln. From working std soln (**g**), add known amts of Pb and Cd, using appropriate micropipets (**f**) and being certain to add amt of each element sufficient to generate peak hts ca twice those given by sample cell soln. Repeat with 2 more similar addns of working std soln to cell soln. For each analyte, plot μg added on $x$-axis vs peak ht in μA current on $y$-axis. Extrapolate linear plot to $x$-axis intercept to det. total amt of analyte in sample aliquot. If available, use computer program based on method of least squares to calc. regression line and det. amt of analyte in sample aliquot. Similarly, det. amt of each analyte in reagent blank aliquots, using same vol. of aliquots for reagent blank as for sample.

Calc. ppm analyte in sample as follows:

$$\text{ppm } (\mu g/g) = [(B - C)/A] \times (50/W)$$

where $A$ = mL sample soln taken for analysis; $B$ = μg analyte in sample soln aliquot; $C$ = av. μg analyte in reagent blank soln aliquots; and $W$ = total g sample.

### F. Determination by Linear Sweep Anodic Stripping Voltammetry

Transfer 2.0 mL aliquot of sample soln to electrolysis cell and add 3.0 mL electrolyte (**d**). pH of cell soln should be 4.3 ± 0.3. Deposit elements of interest onto composite Hg graphite electrode (CMGE) at −0.9 V vs Ag/AgCl ref. electrode for 30 min. Bubble N through cell soln during entire deposition period. Linearly increase applied voltage anodically at 60 mV/s from −0.9 to −0.2 V vs Ag/AgCl ref. electrode. Measure peak current (μA) for each analyte.

Run reagent blank in same manner using same size aliquot as for sample and det. peak current (μA) for each analyte. For each analyte, make std addn to cell soln and measure peak current (μA). Calc. conversion factor, μg/μA, for each analyte as μg of addn divided by diff. between peak current before and after addn of analyte std. Verify conversion factors periodically. Multiply sample peak current (μA) by conversion factor to det. μg of each analyte in sample soln aliquot. Calc. ppm, using equation in **982.23E**.

### G. Interference

Tl may interfere with Pb detn, but its occurrence in food is unlikely. If Tl interference is suspected, treat as follows: Transfer 5.0 mL aliquot of sample soln to electrolysis cell and make basic with 3.0 mL NaOH. Det. elements of interest in this soln by ASV in the usual manner. Plating potential is −1.0 V vs SCE or similar ref. electrode. Strip deposited elements by anodically scanning from −1.0 to −0.3 V vs SCE. In this manner, Cd and Pb peaks shift to −0.78 ± 0.05 V and −0.73 ± 0.05 V vs SCE, resp. Tl peak remains at −0.47 V vs SCE.

Ref.: JAOAC **65,** 970, 978(1982); **70,** 295(1987).

CAS-7440-43-9 (cadmium)
CAS-7439-92-1 (lead)

## Leachable Cadmium and Lead from Foodware

The following 2 methods are applicable to detn of Cd and Pb from foodware contact surfaces used to cook, serve, or store food. **973.32** is applicable to ceramic, glass, enameled, or other ware used to serve or store food at room temp. or below. **984.19** is applicable to ceramic, glass, enameled, or other ware in which food is cooked before serving. Decision of which method to use should be based on how ware being tested will be used in practice.

## 984.19 Cadmium and Lead in Cookware

### Hot Leach Atomic Absorption Method

**First Action 1984**
**Final Action 1986**

***WHO-AOAC Method***

#### *A. Principle*

Enameled and ceramic cookware contg 4% acetic acid is heated by elec. hot plate (or by internal heating elements, if present, that are not exposed to the leach solution) to produce slow boil or simmering of solv. soln for 2 h. Pb and Cd in extg solv. are detd by AAS.

#### *B. Apparatus*

See **973.32A** plus the following:

(**a**) *Hot plate*.—Thermolyne Model HP-A1915B (Barnstead/Thermolyne Corp., 2555 Kerper Blvd, Dubuque, IA 52001), or equiv.

(**b**) *Variable transformer*.—Cat No. 09-521-100 (Fisher Scientific Co.), or equiv.

#### *C. Reagents*

See **973.32B**. Use only deionized distd $H_2O$.

Plus (**e**) *Acetic acid*.—Glacial.

#### *D. Cleaning of Laboratory Glassware*

After normal cleaning, soak all glass and plastic ware used to prep., transfer, or store anal. solns in $HNO_3$-$H_2O$ (4 + 6) ≥24 h; thoroly rinse with $H_2O$ before use.

#### *E. Preparation of Standard*

See **973.32B(c)** and (**d**).

#### *F. Extraction*

Samples of ware must be free of grease or other material which could influence test. Gently wash sample with detergent soln, using pad of absorbent cotton. Rinse ware thoroly with $H_2O$ and let drain dry.

Det. total vol. required to fill ware to overflowing or to cover rest, if one is present.

Fill ware to $^2/_3$ total vol. with $H_2O$; cover with self-cover or clean sheet of opaque borosilicate glass to prevent evapn of soln. When leach soln is to be analyzed for Cd, ensure that light is excluded from test surface. Heat on hot plate adjusted by variable transformer to produce simmer or slow boil of leaching soln, or use internal heating element, if present. Also use variable transformer to prevent excessively rapid boiling in ware containing heating elements. If contained heating element is not able to produce temp. high enough to boil soln, then highest temp. reached is test temp.

When boiling or highest temp. has been reached, add sufficient glacial acetic acid to make soln 4% acetic acid, cover, and continue heating 2 h.

At end of 2 h, re-establish initial vol. of solv. with 4% acetic acid. Dip-stick (glass rod marked for depth of soln required) is useful for replacing losses. Stir thoroly and remove test sample at once.

#### *G. Determination*

See **973.32D**.

Ref.: JAOAC **66,** 610(1983).

CAS-7740-43-9 (cadmium)
CAS-7439-92-1 (lead)

## 973.32 Cadmium and Lead in Earthenware

### Atomic Absorption Spectrophotometric Method

**First Action 1973**
**Final Action 1977**

***AOAC-ASTM Method***

#### *A. Apparatus*

*Atomic absorption spectrophotometer*.—Equipped with 4″ single slot or Boling-type burner head and operated as follows: Pb hollow cathode lamp, 283.3 or 217.0 nm; Cd hollow cathode lamp, 228.8 nm; flame, air-$C_2H_2$. (*Caution:* See safety notes on AAS.) App. should have sensitivity of ca 0.5 μg Pb/mL and 0.25 μg Cd/mL for 1% absorption. Use operating conditions specified by manufacturer.

#### *B. Reagents*

(Use glassware of chemically resistant borosilicate glass.)

(**a**) *Acetic acid*.—4%. Mix HOAc and $H_2O$ (1 + 24). Analyze each new batch of reagent for Pb and Cd.

(**b**) *Detergent wash*.—Add 15 g alk. detergent (e.g., Calgonite, Calgon Corp., PO Box 1346, Pittsburgh, PA 15230, or equiv.) to 1 gal. (3.8 L) lukewarm tap $H_2O$.

(**c**) *Lead std solns*.—(*1*) *Stock soln*.—1000 μg/mL. Dissolve 1.5985 g $Pb(NO_3)_2$ in 4% HOAc and dil. to 1 L with same soln. (*2*) *Working solns*.—Dil. 0.0, 1.0, 3.0, 5.0, 10.0, and 15.0 mL stock soln to 1 L with 4% HOAc (0, 1, 3, 5, 10, and 15 μg/mL).

(**d**) *Cadmium std solns*.—(*1*) *Stock soln*.—1000 μg/mL. Dissolve 0.9273 g anhyd. $CdSO_4$ in 250 mL HCl (1 + 37), and dil. to 500 mL with HCl (1 + 37). (*2*) *Intermediate soln*.—10 μg/mL. Dil. 10 mL stock soln to 1 L with 4% HOAc. (*3*) *Working solns*.— Dil. 0.0, 3.0, 5.0, 10.0, 15.0, and 20.0 mL intermediate soln to 100 mL with 4% HOAc (0.0, 0.3, 0.5, 1.0, 1.5, and 2.0 μg/mL).

#### *C. Extraction*

Take, at random, 6 identical units of product and cleanse each with detergent wash. Rinse with tap $H_2O$ followed by distd $H_2O$, and dry. Fill each unit with 4% HOAc from graduate to within 6–7 mm of overflowing. (Measure distance along surface of test unit, not vertical distance.) Record vol. acid required for each unit in sample. Cover each unit with fully opaque glass plate (so that extn is carried out in total darkness) to prevent evapn of soln, avoiding contact between cover and surface of leaching soln (if opaque glass is not available, cover glass with Al foil or other material to prevent exposure to light). Let stand 24 hr at room temp. (22 ± 2°).

If test unit is extremely shallow or has scalloped brim, evapn losses should be anticipated. In those cases, record headspace after filling. After 24 hr leaching period, adjust soln vol. to same recorded headspace, using 4% HOAc.

### D. Determination

(**a**) *Lead.*—Set instrument for max. signal at 283.3 or 217.0 nm, using Pb hollow cathode lamp and air and $C_2H_2$ flow rates recommended by manufacturer. Stir sample soln and decant portion into clean flask. Det. *A* of sample and Pb working std solns. Flush burner with $H_2O$ and check 0 point between readings. Det. Pb from std curve of *A* against μg Pb/mL or calibrate DCR unit in concn mode with Pb working solns, and read and record sample concn directly. Bracket sample soln with next higher and lower working solns.

Dil. samples contg >20 μg Pb/mL with 4% HOAc. Conc. samples contg <1 μg Pb/mL by accurately transferring min. of 50.0 mL of soln to 250 mL beaker, evapg almost to dryness on steam bath (do not use hotplate); then add 1 mL HCl and evap. to dryness. Dissolve residue in 4% HOAc by adding exactly 0.1 vol. of soln taken for concn (i.e., for 50.0 mL soln, add exactly 5.0 mL 4% HOAc), cover with watch glass, and swirl to complete dissoln. Det. Pb as above, except substitute "3.0 mL" and "3 μg/mL" for "20.0 mL" and "20 μg/mL", resp., in **973.32B(c)**(*2*).

(**b**) *Cadmium.*—Proceed as for Pb, setting instrument for max. signal at 228.8 nm, using Cd hollow cathode lamp. Dil. samples contg >2 μg Cd/mL with 4% HOAc. Conc. samples contg <0.1 μg/mL as in (**a**)

Report type of units tested and for each, vol. acid used and Pb and Cd leached in μg/mL.

Refs.: JAOAC **56,** 869(1973); **59,** 158(1976); **62,** 380(1979); **64,** 396(1981); **71,** 92(1988). ASTM C 738–72.

CAS-7440-43-9 (cadmium)
CAS-7439-92-1 (lead)

## 979.16★ Cadmium and Lead in Earthenware

**Rapid Screening Method**
**First Action 1979**
**Surplus 1982**

(Detects 0.3 μg Pb and 0.05 μg Cd/mL 4% HOAc)

*See* **25.028–25.030**, 14th ed.

## 971.20 Copper and Nickel in Tea

**Atomic Absorption Spectrophotometric Method**
**First Action 1971**
**Final Action 1976**

(*Caution:* See safety notes on AAS, wet oxidation, nitric acid, and perchloric acid.)

### A. Principle

Samples are wet ashed and after diln are detd by AA at 232.0 nm (Ni) and 324.7 nm (Cu). Matrix of std solns is matched to that of sample to avoid interference from Na and K.

### B. Apparatus

*Atomic absorption spectrophotometer.*—Capable of measuring content or change of content of 0.05 μg Ni or Cu/mL in aq. soln.

### C. Preparation of Standard Solutions

(**a**) *Copper std soln.*—1000 μg/mL. Dissolve 1.000 g 99.99% Cu in 20 mL $HNO_3$, cool, and dil. to 1 L with $H_2O$.

(**b**) *Nickel std soln.*—1000 μg/mL. Dissolve 1.000 g 99.99% Ni in 20 mL $HNO_3$, cool, and dil. to 1 L with $H_2O$.

(**c**) *Matrix std solns.*—Prep. solns contg 0, 0.2, 0.4, 0.8, 1.6, 2.0, 4.0, 8.0, and 10 μg Ni and Cu/mL and major metal matrix components: (*1*) *For 3 g sample tea.*—To contain 180 μg Ca, 100 μg Mg, and 40 μg Al/mL with final concn of 8% (v/v) $HClO_4$. (*2*) *For 6 g sample instant tea.*—To contain 7000 μg K, 70 μg Na, 700 μg Mg, and 130 μg Ca/mL with final $HNO_3$ concn of (1 + 9).

### D. Preparation of Calibration Curve

Let instrument stabilize. Optimize conditions for Cu or Ni according to manufacturer's instructions.

Aspirate 10 μg/mL std enough times to establish that *A* reading is not drifting. Record 6 readings and calc. std deviation ($\sigma$) = $(x - y) \times 0.40$, where $x$ and $y$ are max. and min. readings, resp., and 0.40 is factor to convert range of 6 values to $\sigma$.

Beginning with soln contg 0 Cu, aspirate each matrix std soln and record *A*. If value for 10 μg/mL soln differs from av. of the 6 values used to calc. $\sigma$ by $>0.01 \times$ (av. of the 6 values), repeat measurements. If these detns indicate drift, det. cause (e.g., deposits in burner or clogged capillary), correct it, and repeat calibration. Repeat for Ni solns. Plot *A* against μg metal/mL.

### E. Determination

Select sample wt to give soln contg ≥0.05 but ≤10 μg Ni/mL, usually 3 g for teas and 6 g for instant teas.

(**a**) *Wet ashing.*—Accurately weigh sample into 400 mL beaker, add 100 mL $HNO_3$, and swirl. Cover, and let react 10 min; then place on hot plate. Evap. to near dryness and cool. Add 50 mL $HNO_3$, and for tea, add 10 mL $HClO_4$. Continue evapn to obtain clear soln.

Transfer to 50 mL vol. flask and dil. to vol. with $H_2O$. (Insol. $KClO_4$ which settles to bottom of flask does not interfere.)

Prep. reagent blank contg same amts of acids taken from same lots, evapd as above.

(**b**) *Photometry.*—Aspirate sample and blank solns, and record *A*. Measure *A* of matrix std soln contg 10 μg/mL. If this value differs from value of av. of the 6 values used to calc. $\sigma$ by $>2\sigma$, repeat measurement. If these values indicate drift, det. cause, correct it, and repeat calibration and sample and blank readings.

(**c**) *Calculations.*—Correct readings of sample soln for blank. Convert corrected *A* to μg/mL from calibration curve.

$$\text{ppm Ni (or Cu)} = (C \times V)/W$$

where $C$ = μg metal/mL from curve, $V$ = final vol. sample soln (50), and $W$ = g sample.

Refs.: JAOAC **53,** 531(1970); **54,** 658(1971).

CAS-7440-50-8 (copper)
CAS-7440-02-0 (nickel)

## SINGLE ELEMENT METHODS

## 964.16 Antimony in Food

**Spectrophotometric Method**
**First Action 1964**
**Final Action 1976**
***American Conference of Governmental Industrial Hygienists-AOAC Method***

### A. Principle

Pentavalent Sb in aq. HCl soln reacts with Rhodamine B to form colored complex extractable with org. solvs. Intensity of extd color is measured spectrophtric at 565 nm.

### B. Reagents

($H_2O$ for aq. reagents should be double distd; final distn from glass.)

(**a**) *Hydrochloric acid soln.*—6*N*. Dil. concd acid with $H_2O$ (1 + 1).
(**b**) *Dilute phosphoric acid.*—3*N*. Dil. 70 mL $H_3PO_4$ (85%) to 1 L with $H_2O$.
(**c**) *Rhodamine B soln.*—0.02% in $H_2O$.
(**d**) *Antimony std solns.*—(*1*) *Stock soln.*—100 μg Sb/mL. Dissolve 0.1000 g pure Sb in 25 mL $H_2SO_4$ with heat; cool, and cautiously dil. to 1 L with $H_2O$. (*2*) *Working soln.*—1 μg/mL. Dil. 2.0 mL stock soln to 200 mL with $H_2O$.

(Cool reagents (**a**), (**b**), (**c**), ca 100 mL benzene, and eight 125 mL separators with Teflon stopcocks in refrigerator before use; maintain temp. of 5–10° during extn and color development. Work in subdued light.)

### C. Preparation of Sample

Digest sample as in **963.21C**. Oxidizing conditions must be maintained.

### D. Determination

(*Caution:* See safety notes on wet oxidation, perchloric acid, and sulfuric acid.)

Transfer digest or aliquot to 125 mL g-s erlenmeyer, add enough $H_2SO_4$ to make total of 5 mL $H_2SO_4$, and evap. to fumes of $SO_3$. Cool flask, add 10 drops 70% $HClO_4$, and again evap. to white fumes. Cool digest in ice bath ≥30 min; then *slowly* add 5 mL precooled 6*N* HCl by pipet. Let stand in ice bath 15 min; then add 8 mL precooled 3*N* $H_3PO_4$. (Until color is extd into benzene, perform subsequent operations as quickly as possible. Color is stable in benzene several hr.) Immediately add 5 mL precooled Rhodamine B soln, stopper, and shake vigorously. Transfer to precooled 125 mL separator. Pipet 10 mL precooled benzene into separator, shake vigorously 1 min, and discard aq. layer. Transfer benzene layer (red if Sb is present) into test tube and let $H_2O$ settle. Rinse 1 cm cell with ext, fill cell, and read at 565 nm against benzene blank taken thru entire detn. Refer readings to std curve.

### E. Preparation of Standard Curve

Pipet 0, 2, 4, 6, 8, and 10 mL Sb working std soln into 125 mL g-s erlenmeyers, add 5 mL $H_2SO_4$ to each, and proceed as in detn. Plot *A* against μg Sb.

Ref.: Manual of Analytical Methods ACGIH, May 1963. JAOAC **47,** 191, 630(1964).

CAS-7440-36-0 (antimony)

## 963.21 Arsenic in Food
### Kjeldahl Flask Digestion
**First Action 1963**
**Final Action 1965**

### A. Reagents

(**a**) *Bromine water.*—Half satd. Dil. 75 mL satd Br-$H_2O$ with equal vol. $H_2O$.
(**b**) *Sodium hypobromite soln.*—Place 50 mL 0.5*N* NaOH in 200 mL vol. flask, and dil. to vol. with half-satd Br-$H_2O$, (**a**).
(**c**) *Ammonium molybdate-sulfuric acid soln.*—Dissolve 5.000 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in $H_2O$ and slowly add 42.8 mL $H_2SO_4$. Dil. to 100 mL with $H_2O$.
(**d**) *Arsenious oxide std solns.*—(*1*) *Stock soln.*—1 mg/mL. Dissolve 1.000 g $As_2O_3$ (*Caution:* See safety notes on arsenic trioxide and toxic dusts.) in 25 mL 20% NaOH soln and dil. to 1 L. (*2*) *Intermediate soln.*—10 μg/mL. Dil. 10 mL stock soln to 1 L. (*3*) *Working soln.*—1 μg/mL. Dil. 100 mL intermediate soln to 1 L.
(**e**) *Hydrazine sulfate soln.*—1.5% $N_2H_4.H_2SO_4$ in $H_2O$.
(**f**) *Potassium iodide soln.*—15%. Keep in dark. Discard when soln turns yellow.
(**g**) *Stannous chloride soln.*—Dissolve 40 g As-free $SnCl_2.2H_2O$ in HCl and dil. to 100 mL with HCl.
(**h**) *Dilute hydrochloric acid soln.*—Dil. 144 mL HCl to 200 mL with $H_2O$.
(**i**) *Lead acetate soln.*—10% $Pb(OAc)_2.3H_2O$ in $H_2O$.
(**j**) *Zinc metal.*—30 mesh.
(**k**) *Sea sand.*—To clean sand ("30 mesh") before use and between detns, mount piece of 3 mm id glass tubing thru rubber stopper in suction flask. Fit piece of rubber or Tygon tubing over top to take bottom of sulfide absorption tube easily and to maintain it upright. Add, in turn, with suction, aqua regia, $H_2O$, $HNO_3$, and $H_2O$ to remove all traces of acid (≥5 washings). Wet sand with $Pb(OAc)_2$ soln and remove excess with suction.
(**l**) *Silver diethyldithiocarbamate.*—Chill 200 mL 0.1*M* $AgNO_3$ soln (3.4 g/200 mL) and 200 mL 0.1*M* Na diethyldithiocarbamate soln (4.5 g/200 mL) to 10° or lower. Add carbamate soln to $AgNO_3$ soln slowly with stirring. Filter thru buchner, wash with chilled $H_2O$, and dry under reduced pressure at room temp. Dissolve salt in pyridine (reagent grade) with stirring, chill, and add cold $H_2O$ slowly until completely pptd. Filter thru buchner, and wash with $H_2O$ to remove all pyridine. Dry pale yellow crystals under reduced pressure (mp 185–187°; recovery 85–90%). Store in amber bottle in refrigerator. (Second recrystn may be necessary to obtain correct mp.)
(**m**) *Silver diethyldithiocarbamate soln.*—Dissolve 0.5000 g salt, (**l**), in colorless pyridine in 100 mL vol. flask, and dil. to vol. with pyridine. Mix, and store in amber bottle. Reagent is stable several months at room temp.

### B. Generators and Absorption Tubes

*See* Fig. **963.21**. Use 2 oz (60 mL) wide-mouth bottles of uniform capacity and design as generators, and fit each by means of perforated stopper with glass tube 1 cm diam. and 6–7 cm

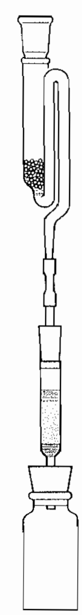

**FIG. 963.21—Arsenic apparatus**

long, with addnl constricted end to facilitate connection. Place small wad of glass wool in constricted bottom end of tube and add 3.5–4 g sand, taking care to have same amt in each tube. Moisten sand with 10% $Pb(OAc)_2$ soln and remove excess by light suction. Clean sand when necessary by treatment (do not remove sand from tube) with $HNO_3$ followed by $H_2O$ rinse and suction. Treat with $Pb(OAc)_2$ soln. If sand has dried thru disuse, clean and remoisten it as directed. Connect tube by means of rubber stopper, glass tube, and rubber sleeve to bent capillary tubing (7 mm od, 2 mm id) tapered at end to slide easily into connecting tube and later into neck of 25 mL vol. flask. Other end of capillary is sealed to Pyrex ₮ 19/38 female joint. To transfer contents of trap, attach bulb aspirator to male ₮ 19/38 joint and place in top of trap.

Clean traps between detns without removing beads by flushing with $H_2O$, followed by $HNO_3$, soaking for 30 min or until $HNO_3$ becomes colorless. Remove all traces of acid with $H_2O$, rinse with acetone, and dry with air current applied by suction to tip of trap.

### C. Preparation of Sample

(*Caution:* See safety notes on wet oxidation, nitric acid, and sulfuric acid.)

(For details of convenient churn-type washer that will remove arsenical spray residues from firm fruits or vegetables with an aq. $NH_4NO_3$-$HNO_3$ soln, *see* JAOAC **26,** 150(1943). Digest aliquot of "strip" soln and proceed as in (**a**).

All digestions can be greatly facilitated by following optional method, JAOAC **47,** 629(1964): Proceed as in (**a**) until mixt. no longer turns brown or darkens. Cool, add 0.5 mL 70% $HClO_4$ (*Caution:* See safety notes on perchloric acid.) and heat until fuming occurs and digest is clear. Cool, and add 2 addnl 0.5 mL portions $HClO_4$, heating each time as above. Finish digestion with $H_2O$ and satd $NH_4$ oxalate as in (**a**).

Conduct $\geq 1$ blank detn with samples. Blanks should not show $>1$ µg As.)

(**a**) *For fresh fruits (apples, pears, or similar products).*—Weigh and peel representative sample (1–5 lb; 0.5–2 kg). At blossom and stem ends cut out all flesh thought to be contaminated with As compds and include with peelings, if desired. Place peelings in 1 or more 800 mL Kjeldahl flasks. (As-free Pyrex glassware and "wet ashing" app. of Duriron are available.) Add 25–50 mL $HNO_3$; then cautiously add 40 mL $H_2SO_4$ (20 mL if Gutzeit method is used). Place each flask on asbestos mat with 5 cm hole. Warm slightly and discontinue heating if foaming becomes excessive.

When reaction has quieted, heat flask cautiously and rotate occasionally to prevent caking of sample upon glass exposed to flame. Maintain oxidizing conditions in flask at all times during digestion by cautiously adding small amts of $HNO_3$ whenever mixt. turns brown or darkens. Continue digestion until org. matter is destroyed and $SO_3$ fumes are copiously evolved. (Final soln should be colorless, or at most light straw color.) Cool slightly, and add 75 mL $H_2O$ and 25 mL satd $NH_4$ oxalate soln to assist in expelling oxides of N from soln. Evap. again to point where fumes of $SO_3$ appear in neck of flask. Cool, and dil. with $H_2O$ to 500 or 1000 mL in vol. flask.

(**b**) *For dried fruit products.*—Prep. sample by alternately grinding and mixing 4–5 times in food chopper. Place 35–70 g portions in 800 mL Kjeldahl flasks, and add 10–25 mL $H_2O$, 25–50 mL $HNO_3$, and 20 mL $H_2SO_4$. Continue digestion as in (**a**). Dil. digested soln to 250 mL.

(**c**) *For small fruits, vegetables, etc.*—Use 70–140 g sample and digest as in (**a**) or (**b**).

(**d**) *For materials other than* (**a**), (**b**), *or* (**c**).—Digest 5–50 g, according to moisture content and amt of As expected, as in (**a**) or (**b**). Dil. to definite vol. detd by As concn expected.

(**e**) *For products containing stable organic As compounds, products liable to yield incompletely oxidized organic derivatives that inhibit arsine evolution, or products that are difficult to digest.*—Shrimp, tobacco, oils, and some other products require special treatment to complete oxidn of org. As to inorg. $As_2O_5$, or to destroy org. interferences previous to As detn. For details consult: Ind. Eng. Chem., Anal. Ed. **5,** 58(1933); **6,** 280, 327 (1934); JAOAC **20,** 171(1937); **47,** 629(1964).

Dil. As solns obtained by these special methods of prepn to definite vol.

(**f**) *For ultra-micro quantities of As, very labile forms of As, and vacuum-accelerated Gutzeit reduction system for mercuric bromide spot filtration.*— Consult Ind. Eng. Chem., Anal. Ed. **16,** 400(1944).

### D. Isolation of Arsenic

Before making detns, isolate As, when interfering substances are present in digests (*e.g.*, pyridine from tobacco), or when samples contain excessive amts of salts, or $H_2SO_4$ from digestions. Consult first ref. of **963.21C(e)** for method of isolating As after digestion, or isolate As by $AsCl_3$ distn (JAOAC **16,** 75, 325(1933); **17,** 202(1934). Gelatin may be hydrolyzed with HCl and As isolated as in first ref. of **963.21C(e)**.

Ref.: JAOAC **46,** 245(1963).

CAS-7440-38-2 (arsenic)

## 912.01★ Arsenic in Food

**Gutzeit Method**

**Final Action**

**Surplus 1970**

*See* **25.006–25.009**, 11th ed.

## 942.17 Arsenic in Food

**Molybdenum Blue Method**

**Final Action**

### A. Determination

Transfer 20 mL aliquots of sample and blank digest solns to generator bottles. Add, swirling after each addn, 10 mL $H_2O$, 5 mL dil. HCl, (**h**), 5 mL KI soln, (**f**), and 4 drops $SnCl_2$ soln, (**g**), **963.21A**. Let stand $\geq 15$ min.

Place 4 g sea sand over small glass wool wad in sulfide absorption tube and cap with glass wool. Place 3 mm diam. solid glass beads in trap over small glass wool pad until 1/4 full and add 3.0 mL NaOBr soln, (**b**). Assemble app. except for generator bottle. Add 4 g Zn, (**j**), to generator bottle, attach immediately, and let react 30 min.

Disconnect trap and transfer contents to 25 mL vol. flask with aspirator assembly. Rinse trap with six 2 mL portions $H_2O$ and aspirate into flask. Add, with swirling, 0.5 mL $NH_4$ molybdate-$H_2SO_4$ soln, (**c**), and 1.0 mL $N_2H_4.H_2SO_4$ soln, (**e**). Dil. to vol., mix, and let stand 75 min. Mix, and read in spectrophtr or colorimeter at 845 nm against blank prepd similarly. Alternatively, heat vol. flask and contents 10 min at 50° and cool in tap $H_2O$ to room temp. before reading. Det. $As_2O_3$ (or As) in aliquot from std curve.

### B. Preparation of Standard Curve

Place 0.0, 1.0, 2.0, 3.0, 5.0, 6.0 mL std soln contg 10 μg $As_2O_3$/mL in 25 mL vol. flasks. Add 3.0 mL NaOBr soln, (**b**), and $H_2O$ to 15 mL. Add, with swirling, 0.5 mL $NH_4$ molybdate-$H_2SO_4$ soln, (**c**), and 1.0 mL $N_2H_4.H_2SO_4$ soln, (**e**). Dil. to vol., mix, and let stand 75 min or heat 10 min at 50° as for samples. Mix, and read at 845 nm. Plot *A* against μg $As_2O_3$ (or As).

Refs.: Ind. Eng. Chem. Anal. Ed. **14,** 442(1942). JAOAC **46,** 245(1963).

CAS-7440-38-2 (arsenic)

## 952.13 Arsenic in Food

**Silver Diethyldithiocarbamate Method**

**Final Action**

### A. Determination

Transfer aliquot of sample digest, **963.21C** (usually 2–5 mL), and same vol. blank to generator bottles. Add $H_2O$ to 35 mL; then add, with swirling, 5 mL HCl, 2 mL KI soln, (**f**), and 8 drops $SnCl_2$ soln, (**g**), and let stand ≥15 min. Evolve $AsH_3$ as in **942.17A**, except add 4.0 mL Ag diethyldithiocarbamate soln, (**m**), to trap.

Disconnect trap and mix trapping soln by gently drawing back and forth 5 times with aspirator assembly. Transfer soln directly to spectrophtr cell (g-s preferred) and read at 522 nm. Det $As_2O_3$ (or As) in aliquot from std curve.

### B. Preparation of Standard Curve

Place 0.0, 1.0, 3.0, 6.0, 10.0, and 15.0 mL std soln contg 1.00 μg $As_2O_3$/mL in generator bottles. Add $H_2O$ to 35 mL and proceed as in **952.13A**. Read at 522 nm and plot *A* against μg $As_2O_3$ (or As).

Refs.: Chem. Listy **46,** 341(1952). Anal. Chem. **31,** 1589(1959). JAOAC **46,** 245(1963).

CAS-7440-38-2 (arsenic)

## 973.33 Arsenic in Meat and Poultry

**Molybdenum Blue Method**

**First Action 1973**

**Final Action 1975**

### A. Principle

Sample is ashed in presence of $Mg(NO_3)_2$ at 600°. Ash is dissolved in dil. HCl; Zn is added to generate $AsH_3$, which is trapped with I soln in cell. Heteropoly blue compd is developed and read at 840 nm in same cell. Chief source of error is often contamination. Always perform reagent blank and, when possible, std sample.

### B. Reagents

(Glassware should not be subjected to routine washing with soap or detergents, which are often source of As contamination. When soap or detergent is used, clean with aqua regia before use. Rinse delivery tubes by holding in slanted position with crook up and squirting jet of $H_2O$ up and over inside crook until tube is filled; then rinse outside while tube drains. Repeat rinsing 3 times. Rinse funnels in each direction alternately by filling end that is up and placing funnel on 1-hole rubber stopper in mouth of vac. flask to pull $H_2O$ thru frit by vac.)

(**a**) *Tissue solvent.*—$CHCl_3$ (or benzene)-acetone-absolute alcohol (1 + 1 + 2).

(**b**) *Dilute hydrochloric acid.*—Mix 175 mL HCl and 280 mL $H_2O$.

(**c**) *Potassium iodide soln.*—15%. *See* **963.21A(f)**.

(**d**) *Stannous chloride soln.*—40% in dil. HCl, (**b**). Store in contact with metallic Sn.

(**e**) *Zinc.*—Shot of uniform size and shape, ca 0.5 g each.

(**f**) *Lead acetate soln.*—Prep. satd aq. $Pb(OAc)_2.3H_2O$ soln in dropping bottle. Prep. fresh weekly or when soln becomes cloudy.

(**g**) *Iodine solns.*—*(1) 0.02N.*—Dissolve 8 g KI and 2.54 g I in small amt $H_2O$ and dil. to 1 L with $H_2O$. Store in dark bottle. *(2) 0.001N.*—Dil. 5 mL 0.02*N* I to 100 mL with $H_2O$. Prep. fresh daily.

(**h**) *Ammonium molybdate soln.*—Dissolve 7.0 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in warm mixt. of 70 mL $H_2SO_4$ and 300 mL $H_2O$, cool, and dil. to 500 mL with $H_2O$.

(**i**) *Hydrazine sulfate soln.*—Dissolve 0.3 g $N_2H_4.H_2SO_4$ in $H_2O$ and dil. to 200 mL.

(**j**) *Arsenious oxide std solns.*—*(1) Stock soln.*—1 mg As/mL. Dissolve 0.1320 g $As_2O_3$ in 50 mL $H_2O$ contg 0.7 mL 50% NaOH. Neutze with 50% $H_2SO_4$ and dil. to 100 mL. *(2) Working solns.*—Dil. sep. 1.0 mL portions stock soln to 100, 200, and 500 mL with $H_2O$ (10, 5, and 2 μg As/mL, resp.).

(**k**) *Arsanilic acid std solns.*—*(1) Stock soln.*—1 mg As/mL. Dissolve 0.2897 g arsanilic acid (based on label assay) in $H_2O$ and dil. to 100 mL with $H_2O$. *(2) Working solns.*—Prep. in same concns as in (**j**).

### C. Apparatus

(**a**) *Cell rack.*—Metal rack capable of holding eight 19 × 105 mm cells in 600 mL beaker.

(**b**) *Distilling apparatus.*—Kingsley-Schaffert As distg app. (Corning Glass Works, No. 33680), consisting of 125 mL flask, funnel trap, and bent dispersion tube.

(**c**) *Absorbent cotton.*—*See* **945.58B(i)**.

### D. Preparation of Sample

Assure absence of interferences arising from laboratory and reagent contamination. Recoveries thru method of added compds should be ≥90%. Conduct one or more reagent blanks and std samples along with samples.

To obtain representative aliquot of large sample (≥100 g), grind entire sample ≥2 times, using fine plate (grind liver sample only once or blend). Mix thoroly and weigh calcd amt into 50 mL Vycor crucible. Add 4 g $Mg(NO_3)_2.6H_2O$/10 g sample and mix, using stainless steel spatula or glass rod, until all $Mg(NO_3)_2$ is dissolved. Spread mixt. in even layer around sides of crucible.

For smaller samples (<100 g), weigh known amt or entire sample into homogenizer or blender, add 4 g $Mg(NO_3)_2.6H_2O$/10 g sample and enough tissue solv. to aid blending, weigh, and blend 1 min. (*Caution:* Use explosion-proof blender when benzene-acetone-alcohol is used as tissue solv.) Weigh aliquot equiv. to desired amt sample into 50 mL Vycor crucible and carefully evap. excess solv. and $H_2O$ from tissue on steam bath or in 95° oven.

### E. Determination

Place crucible in cool furnace, gradually increase temp. to 600°, and ash sample at 600° until most visible C is burned. Cool crucible and cool furnace. Dampen ash with $H_2O$ and add 3 mL $HNO_3$ (1 + 4). Place in cooled furnace (100°) and heat gradually to 600°. Hold at 600° ca 1 hr until all $HNO_3$ fumes are evolved. Repeat dil. $HNO_3$ treatment if necessary to obtain white ash.

Remove crucibles and let cool. Dampen ash with $H_2O$ and dissolve in 10 mL dil. HCl, (**b**), delivered from all-glass hypodermic syringe without needle. Quant. transfer to 125 mL flask, **973.33C(b)**, with aid of two 10 mL portions dil. HCl and wash sides of flask with fourth 10 mL portion. (Use same vol. liq. for each sample because vol. of air space above liq. affects efficiency of distn of H and $AsH_3$.) Cool to room temp. Add 2.0 mL 15% KI soln and mix thoroly by swirling. Add 1.0 mL 40% $SnCl_2$ soln and mix thoroly by swirling. Let stand $\geq$15 min (but <30 min). Place 7.0 mL 0.001*N* I soln in cell. Place small ball absorbent cotton in top of funnel and dampen with satd $Pb(OAc)_2$ soln. Lubricate ground glass joint with $H_2O$ and join delivery tube to funnel firmly. This union must be firm enough to hold together under wt of funnel, flask, and contents. Union must not be so firm as to prevent disassembly. Do not dry parts by heating before use. Teflon sleeves are not satisfactory.

Fill 600 mL beaker with finely crushed ice layered between levels of cell holder. Add ice-$H_2O$ to ca $^2/_3$ ht of beaker and place cell in holder. (It may be necessary to make path thru ice for cell.) Lubricate lower ground glass joint of funnel with $H_2O$, add ca 12.5 g Zn to flask, join flask and funnel firmly, and place delivery tube in cell as quickly as possible. Let distn continue 1 hr (without added heat).

Carefully and slowly remove delivery tube from cell, letting liq. drain as tube is removed. Add 0.5 mL $NH_4$ molybdate soln and mix thoroly. Add 0.3 mL $N_2H_4.H_2SO_4$ soln and mix thoroly. Place cell (in cell holder) in moderately boiling $H_2O$ bath or on medium (not vigorous) steam bath 10 min. Remove from bath, wipe cell dry with soft lintless material, and place in cool, dark place ca 1 hr (to ensure that samples reach same temp. and full color development). Read in precalibrated spectrophtr or colorimeter at 840 nm against $CO_2$-free $H_2O$. Correct for blanks.

### *F. Preparation of Standard Curve*

Prepare stds of 10 g As-free liver, 4 g $Mg(NO_3)_2$, and suitable amts arsanilic acid working solns in definite progression, such as 2, 4, 6, 8, and 10 µg As. Repeat analysis of stds $\geq$3 times. Det. mean for each level and prep. curve; or fit line by method of least sqs, in *Definitions of Terms and Explanatory Notes,* if desired.

Ref.: JAOAC **56,** 1144(1973).

CAS-7440-38-2 (arsenic)

## 945.58 Cadmium in Food
### Dithizone Method
### Final Action 1976

### *A. Principle*

Sample is digested with $H_2SO_4$ and $HNO_3$. All reactive metals are extd from soln (after adjustment to pH ca 9) with dithizone-$CHCl_3$. Cu, Hg, and most of any Ni or Co present are removed by stripping $CHCl_3$ soln with dil. HCl. Aq. layer, adjusted to 5% NaOH, is extd with dithizone-$CCl_4$. At this alky, Zn, Pb, and Bi do not ext, whereas Cd dithizonate is relatively stable. Stripping with dil. HCl and development of Cd dithizonate in 5% NaOH are repeated. Cd is finally estd photometrically as dithizonate. Zn constitutes chief interference.

### *B. Reagents*

(**a**) *Citrate.*—Diammonium salt or citric acid.

(**b**) *Chloroform.*—Distil from hot $H_2O$ bath, collecting distillate in absolute alcohol in proportion of 10 mL alcohol to 1 L distillate. Intermittently shake receiver during distn.

(**c**) *Diphenylthiocarbazone (dithizone), twice purified.*—Purify as in **934.07A(e)**, but make only 3 dil. $NH_4OH$ extns of $CHCl_3$ soln. Carry thru, including $H_2O$-washing steps, and then repeat purification with 3 $NH_4OH$ extns, pptn with dil. acid, etc. Instead of heating ext to dryness, evap. spontaneously, and complete drying under vac. in bell jar overnight.

(**d**) *Carbon tetrachloride.*—(*Caution:* See safety notes on distillation, and carbon tetrachloride.) Reflux vigorously on steam bath 1 hr with $^1/_{20}$ vol. 20% KOH in MeOH. Cool, add $H_2O$, drain off $CCl_4$ layer, and wash $\geq$3 times with copious vols of $H_2O$ until alkali-free. Dry over $CaCl_2$, filter, and distil on hot $H_2O$ bath. (Unless reagent is so purified, erratic Cd results may be obtained with some lots of $CCl_4$.)

(**e**) *Dithizone in carbon tetrachloride.*—20 mg/L $CCl_4$, (**d**). Prep. daily, as dil. solns of dithizone are unstable. (When many detns are to be made, dithizone reagent may be prepd by diln from 300 mg/L soln. Store concd reagent under 0.1*M* $SO_2$ soln in refrigerator.)

(**f**) *Dithizone in chloroform.*—1000 mg/L $CHCl_3$, (**b**), prepd as needed.

(**g**) *Sodium hydroxide soln.*—28%. Dissolve 28 g NaOH pellets in $H_2O$ and dil. to 100 mL.

(**h**) *Thymol blue indicator.*—Triturate 0.1 g indicator in agate mortar with 4.3 mL 0.05*N* NaOH. Dil. to 200 mL in g-s flask with $H_2O$.

(**i**) *Absorbent cotton.*—Metal-free. If traces of metal are present, remove by digesting cotton several hr with 0.2*N* HCl, filtering on buchner, and finally washing with copious vols of redistd $H_2O$ until acid-free.

(**j**) *Cadmium std solns.*—(*1*) *Stock soln.*—1 mg/mL. Dissolve 1 g Cd (Fisher Chemical Co., certified 99.9% pure, C-565, or equiv.) in 20–25 mL $HNO_3$ (1 + 9), evap. to dryness, add 5 mL HCl (1 + 1), evap. to dryness, and then add several mL $H_2O$ and again evap. to dryness. Dil. to 1 L. (*2*) *Intermediate soln.*—100 µg/mL. Dil. 10 mL stock soln to 100 mL. (*3*) *Working soln.*—2 µg/mL. Transfer 20 mL intermediate soln to 1 L vol. flask, add 15 mL HCl, and dil. to vol. to give final acidity of ca 0.2*N*.

### *C. Preparation of Standard Curve*

Prep. in duplicate 6 stds contg 0, 5, 10, 15, 20, and 25 µg Cd as follows: Add appropriate vols std soln to Squibb-type separators (125 mL size is convenient), adjust to 40 mL with 0.2*N* HCl, add 10 mL NaOH soln (soln is then 5% with respect to NaOH) and 25 mL dithizone soln, (**e**), shake vigorously exactly 1 min, let stand exactly 3 min, and filter org. layer thru pledget of absorbent cotton, discarding first 5 mL. Fill absorption cell (1 cm length is convenient) and det. *A* at 510 nm. Plot std curve or calc. ref. equation by method of least squares, *Definitions of Terms and Explanatory Notes*, Item (24).

### *D. Preparation of Sample*

Use sample equiv. to 5–10 g of product, calcd to dry basis. (Sample size is of concern only when comparatively large amts of Mg and P are present.) Digest with 10 mL $H_2SO_4$ (1 + 1) and $HNO_3$ as needed. If sample tends to char rather than to oxidize evenly, add 5 or 10 mL addnl $H_2SO_4$. Continue digestion, adding $HNO_3$ as required, until digestion is complete and $SO_3$ is evolved. Cool, add 15 mL satd $NH_4$ oxalate soln, and again heat to fumes.

Fat in biological materials, such as liver and kidney, may cause bumping and frothing during digestion. If comparatively large samples of such materials are available, make partial digestion with warm $HNO_3$ until only fat remains undissolved. Cool, filter free of solid fat, wash residue with $H_2O$, make combined filtrate to suitable vol., and digest appropriate aliquots as above.

### *E. Determination*

Dil. digest, **945.58D**, with 25 mL $H_2O$, filter free from excessive insol. matter (sulfates or silica) if present, and transfer to separator marked at 125 mL, using addnl 10 mL portions $H_2O$ for rinsing and completing transfer. Add 1–2 g citrate reagent, (**a**), and 1 mL thymol blue indicator, (**h**), and adjust to ca pH 8.8 by adding $NH_4OH$ slowly, while cooling intermittently, until soln changes from yellowish green to greenish blue. Dil. to 125 mL mark with $H_2O$. Ext vigorously with 5 mL portions dithizone soln, (**f**), until $CHCl_3$ layer remains green. Then ext with 3 mL $CHCl_3$.

Transfer all $CHCl_3$ exts to second separator previously wetted with 2–3 mL $CHCl_3$. Add 40 mL 0.2*N* HCl to combined dithizone exts, shake vigorously ≥1 min, and after layers sep., carefully drain $CHCl_3$ phase contg any Cu, Ni, Co, or Hg that may be present, and discard. Remove remaining droplets of dithizone by extg with 1–2 mL $CCl_4$, (**d**), carefully conducting draining operation so that no acid enters bore or stem of separator, as its presence there would in part decompose Cd dithizonate subsequently formed and extd in next step.

Adjust aq. phase to 5% alky by adding 10 mL NaOH soln, (**g**). Ext Cd with 25 mL dithizone soln, (**e**), shaking vigorously ≥1 min, and transfer to third separator previously wetted with 2–3 mL same dithizone soln. Repeat extn with addnl 10 mL portions dithizone soln until $CCl_4$ layer becomes colorless. Amts of Cd usually found in foods or biological materials (ca 100 μg) are completely removed by third extn.

To verify assumption that pale pink persisting after third extn is due to Zn, transfer questionable ext to fourth separator contg 5% NaOH soln, add several mL dithizone soln, (**e**), and shake vigorously. If $CCl_4$ layer becomes colorless, original pink was due to Zn and no further extns are necessary. If, however, pink persists, indicating presence of Cd, add ext to contents of third separator, and continue extn.

Convert Cd and Zn dithizonates in third separator to chlorides by adding 40 mL 0.2*N* HCl and shaking vigorously ≥1 min. Carefully drain $CCl_4$ layer, which may contain traces of Co and Ni not removed in second step, and discard. Remove droplets of dithizone from aq. phase by rinsing with 1–2 mL $CCl_4$ and drain off as completely as possible, but do not permit any acid to pass bore of separator. Again adjust alky to 5% by adding 10 mL NaOH soln, (**g**). Wipe separator stems dry with cotton, (**i**). Det. Cd present by adding exactly 25 mL dithizone soln, (**e**), shaking vigorously exactly 1 min, permitting layers to sep. exactly 3 min, and continuing as in **945.58C**, beginning ". . . filter org. layer . . ." Calc. Cd in μg by substituting *A* in linear equation or from std curve.

*Note:* If photometric measurement indicates >25 μg Cd, make first approximation by dilg dithizonate soln with $CCl_4$ and evaluating *A*. For best results repeat analysis with wts or aliquots of samples contg ≤25 μg Cd; 30 μg is upper limit of solubility of Cd dithizonate in 25 mL $CCl_4$. Therefore amts >30 μg are incompletely extd.

Refs.: JAOAC **28,** 257(1945); **32,** 349(1949). Anal. Chem. **21,** 300(1949).

CAS-7440-43-9 (cadmium)

## 973.34 Cadmium in Food

### Atomic Absorption Spectrophotometric Method

**First Action 1973**
**Final Action 1974**

(*Caution:* See safety notes on AAS, wet oxidation, nitric acid, sulfuric acid, and peroxides.)

### *A. Principle*

Sample is digested with $HNO_3$, $H_2SO_4$, and $H_2O_2$. All reactive metals are extd from soln, after adjustment to ca pH 9, with dithizone-$CHCl_3$. Cd is removed by stripping $CHCl_3$ soln with dil. HCl and detd by AA spectrophotometry at 228.8 nm.

### *B. Reagents and Apparatus*

(Thoroly wash all new glassware and glassware which has contained high Cd concn with 8*N* $HNO_3$, and rinse with $H_2O$. Cover beakers with watch glasses during all operations.)

(**a**) *Nitric acid.*—Low in Pb and Cd (G. Frederick Smith Chemical Co., No. 63).

(**b**) *Hydrogen peroxide.*—50% (Fisher Scientific Co., No. H-341).

(**c**) *Citric acid.*—Monohydrate, fine crystal.

(**d**) *Thymol blue indicator.*—*See* **945.58B(h)**.

(**e**) *Dithizone solns.*—(*1*) *Concentrated soln.*—1 mg/mL. Prep. 200 mL in $CHCl_3$. (*2*) *Dilute soln.*—0.2 mg/mL. Dil. concd soln 1 + 4 with $CHCl_3$. Prep. fresh daily.

(**f**) *Cadmium std solns.*—(*1*) *Stock soln.*—1.0 mg/mL. Dissolve 1.000 g Cd, **945.58B(j)**, in 165 mL HCl in 1 L vol. flask. Dil. to vol. with $H_2O$. (*2*) *Intermediate soln.* —10 μg/mL. Dil. 10 mL stock soln with 2*N* HCl to 1 L. Prep. just before use. (*3*) *Working solns.*—Dil. 0, 1, 5, 10, and 20 mL intermediate soln to 100 mL with 2*N* HCl (0, 0.1, 0.5, 1.0, and 2.0 μg Cd/mL, resp.).

(**g**) *Atomic absorption spectrophotometer.*—With hollow-cathode Cd lamp and 10 cm burner head for air-$C_2H_2$ flame; wavelength 228.8 nm, range 0–2.0 μg/mL.

### *C. Digestion*

Weigh 50.0 g sample into 1.5 L beaker. Add several boiling chips or beads, and cover. Carefully add 25 mL $HNO_3$, cover, and warm gently with flame to initiate reaction. (Meker-type burners are preferred thruout for their versatility and speed.) When reaction subsides, add 25 mL $HNO_3$, warm again, and continue until 100 mL $HNO_3$ has been added. (Alternatively, add 100 mL $HNO_3$ all at once, with caution, and let stand at room temp. overnight.) Heat until most NO fumes have evolved; control excessive frothing by cooling or quenching with $H_2O$ from wash bottle. Only some cellulose and fatty materials, if any, remain undissolved.

To remove any fat visible in hot soln, proceed as follows: Cool beaker in ice, and decant clear, aq. soln from coagulated oils and solids thru glass wool pad into 1 L beaker. Add 100 mL $H_2O$ to 1.5 L beaker with fat, heat, swirl vigorously to rinse fat, chill, and filter as before. Wash funnel and glass wool pad with ca 20 mL $H_2O$.

Add 20 mL $H_2SO_4$ to sample, dil. to ca 300 mL with $H_2O$, and evap. over flame until charring begins. When charring becomes extensive, cautiously add 50% $H_2O_2$, 1 mL at time. Let reaction subside before adding next portion of oxidant, and never add >1 mL at a time. Continue addns of $H_2O_2$ until soln is colorless. Heat vigorously to $SO_3$ fumes, adding more $H_2O_2$ as required to remove char. Heat vigorously to expel excess $H_2O_2$. Cool colorless digest to room temp.

Prep. reagent blank of 100 mL $HNO_3$, 20 mL $H_2SO_4$, and *same amts* of $H_2O$ as added to sample. Cautiously add *same amts* 50% $H_2O_2$, as above, and remove all $HNO_3$ from blank. Carry blank thru same operations as sample.

### *D. Extraction*

Add 2 g citric acid to cooled digest and cautiously dil. to ca 25 mL with $H_2O$. Add 1 mL thymol blue indicator and adjust to ca pH 8.8 by slowly adding $NH_4OH$ while cooling in ice bath, until soln changes from yellowish green to greenish blue. Transfer quant. to 250 mL separator, using $H_2O$, and dil. to ca 150 mL.

Cool soln, and ext with two 5 mL portions concd dithizone soln, shaking 1–2 min each time. Continue extn with 5 mL portions dil. dithizone soln until last 5 mL portion dithizone ext shows no change in color. Combine dithizone exts in 125 mL separator; wash with 50 mL $H_2O$, and transfer solv. to another 125 mL separator. Ext $H_2O$ wash with 5 mL $CHCl_3$ and add this to dithizone exts. Add 50 mL 0.2$N$ HCl to combined dithizone exts, shake vigorously 1 min, and let layers sep.; discard dithizone layer. Wash aq. soln with 5 mL $CHCl_3$ and discard $CHCl_3$. Quant. transfer aq. soln to 400 mL beaker, add boiling chips, and evap. carefully to dryness. Carefully rinse down sides of beaker with 10–20 mL $H_2O$ and again evap. to dryness.

### *E. Determination*

Set instrument to previously established optimum conditions, using air-$C_2H_2$ oxidizing flame and 228.8 nm resonant wavelength. Dissolve dry residue in 5.0 mL 2$N$ HCl and det. $A$ of sample and std solns against 2$N$ HCl as blank. Flush burner with $H_2O$ between readings. Use scale expansion controls to obtain 4–10× expansion, as convenient. Det. Cd from curve of $A$ against µg Cd/mL:

$$\text{ppm Cd} = (\mu g\ \text{Cd/mL}) \times (\text{mL } 2N\ \text{HCl/g sample})$$

For concn >2.0 µg Cd/mL, dil. soln with 2$N$ HCl.

Ref.: JAOAC **56,** 876(1973).

CAS-7440-43-9 (cadmium)

## 960.40 Copper in Food

**Colorimetric Method**

**First Action 1960**
**Final Action 1965**

***International Union of Pure and Applied Chemistry-AOAC Method***

### *A. Principle*

Sample is digested with $HNO_3$ and $H_2SO_4$. Cu is isolated and detd colorimetrically at pH 8.5 as diethyldithiocarbamate in presence of chelating agent, EDTA. Bi and Te also give colored carbamates at pH 8.5 but are decomposed to colorless compds with 1$N$ NaOH. Cu complex is stable. Range of color development is 0–50 µg. Blank is ca 1 µg Cu.

### *B. Precautions*

Clean glassware with hot $HNO_3$. Use white petrolatum to lubricate stopcocks of separators, and do not use brass chains. Purify $H_2O$ and $HNO_3$ by distn in Pyrex.

### *C. Reagents*

(**a**) *Sodium diethyldithiocarbamate (carbamate soln).*—Dissolve 1 g of the salt in $H_2O$, dil. to 100 mL, and filter. Store in refrigerator and prep. weekly.

(**b**) *Citrate-EDTA soln.*—Dissolve 20 g dibasic $NH_4$ citrate and 5 g $Na_2EDTA$ (Eastman Kodak Co.) in $H_2O$ and dil. to 100 mL. Remove traces of Cu by adding 0.1 mL carbamate soln and extg with 10 mL $CCl_4$. Repeat extn until $CCl_4$ ext is colorless.

(**c**) *Copper std solns.*—(*1*) *Stock soln.*—1 mg/mL. Place 0.2000 g Cu wire or foil into 125 mL erlenmeyer. Add 15 mL $HNO_3$ (1 + 4), cover flask with watch glass, and let Cu dissolve, warming to complete soln. Boil to expel fumes, cool, and dil. to 200 mL. (*2*) *Intermediate soln.*—100 µg/mL. Dil. 20 mL stock soln to 200 mL. (*3*) *Working soln.*—2 µg/mL. Prep. daily by dilg 5 mL intermediate std soln to 250 mL with 2.0$N$ $H_2SO_4$.

(**d**) *Ammonium hydroxide.*—6$N$. Purify as in (**b**).

(**e**) *Thymol blue indicator.*—0.1%. Dissolve 0.1 g thymol blue in $H_2O$, add enough 0.1$N$ NaOH to change color to blue, and dil. to 100 mL.

### *D. Preparation of Sample*

(*Caution:* See safety notes on nitric acid and sulfuric acid.)

Weigh sample contg ≤20 g solids, depending upon expected Cu content. If sample contains <75% $H_2O$, add $H_2O$ to obtain this diln. Add initial vol. $HNO_3$ to equal ca 2 times dry sample wt and 5 mL $H_2SO_4$, or as many mL $H_2SO_4$ as g dry sample, but ≥5 mL. Digest as in **963.21C**.

When sample contains large amt of fat, make partial digestion with $HNO_3$ until only fat is undissolved. Cool, filter free of solid fat, wash residue with $H_2O$, add $H_2SO_4$ to filtrate, and complete digestion as above. After digestion, cool, add 25 mL $H_2O$, and remove nitrosylsulfuric acid by heating to fumes. Repeat addn of 25 mL $H_2O$ and fuming. If after cooling and dilg, insol. matter is present, filter thru acid-washed paper, rinse paper with $H_2O$, and dil. to 100 mL.

Prep. reagent blank similarly.

### *E. Isolation and Determination of Copper*

Pipet 25 mL sample soln into 100 or 250 mL short-stem separator and add 10 mL citrate-EDTA reagent. Add 2 drops thymol blue indicator, (**e**), and 6$N$ $NH_4OH$ dropwise until soln turns green or blue-green. Cool, and add 1 mL carbamate soln and 15 mL $CCl_4$. Shake vigorously 2 min. Let layers sep. and drain $CCl_4$ through cotton pledget into g-s tube or flask. Det $A$ or $T$ in suitable instrument at ca 400 nm.

If >50 µg Cu is present in 25 mL aliquot, use smaller aliquot and dil. to 25 mL with 2.0$N$ $H_2SO_4$. Highest accuracy is obtained at ca 25 µg Cu level ($A$ ca 0.3 in 1 cm cell).

To test for Bi and Te, return $CCl_4$ soln to separator, add 10 mL 5% KCN soln, and shake 1 min. If $CCl_4$ layer becomes colorless, Bi and Te are absent.

If test is pos., develop color in another 25 mL aliquot as above (without KCN). Drain $CCl_4$ layer into second separator, add 10 mL 1$N$ NaOH, and shake 1 min. Let layers sep. and drain $CCl_4$ into third separator. Again wash $CCl_4$ ext with 10 mL 1$N$ NaOH. Det. $A$ or $T$ of $CCl_4$ layer and convert to µg Cu.

### *F. Preparation of Standards and Calibration Curves*

Transfer 0, 1, 2.5, 5, 10, 15, 20, and 25 mL of Cu std soln (2 µg/mL) to separators and add 2.0$N$ $H_2SO_4$ to make total vol. of 25 mL.

Add 10 mL citrate-EDTA reagent and proceed as in **960.40E**, beginning "Add 2 drops thymol blue indicator, . . ."

Plot $A$ against µg Cu on ordinary graph paper. If readings are in % $T$, use semilog paper, and plot $T$ on log scale. Since there is usually some deviation from linearity, read sample values from smoothed curve.

Ref.: JAOAC **43,** 695(1960).

CAS-7440-50-8 (copper)

## 944.07 Fluorine on Apples and Pears

**Colorimetric Method**

**Final Action**

### *A. Principle*

Add filtrate from strip soln of apples and pears prepd with HCl rinse and acidification, **935.51A**, is used. Aliquot of filtrate is oxidized colorless with $KMnO_4$, soln is then reduced with $H_2NOH$, and subaliquot is backtitrd in Nessler tubes; $Zr(NO_3)_4$ is used in titrn, with purpurin (1,2,4-trihydroxyanthraquinone) as indicator (Ind. Eng. Chem., Anal. Ed. **6,** 118(1934)). Principle of back-titrn, as applied here, is similar to that used in general method where $Th(NO_3)_4$ and alizarin occupy similar roles. Provision is made for removal of interfering anions, and high acidity used in titrn minimizes interference of metals that would otherwise lake with indicator.

### B. Apparatus

*Nessler tubes.*—50 mL g-s, tall-form, matched for ht and color (*see* **944.07E**).

### C. Reagents

(**a**) *Mixed nitrate soln.*—Dissolve 3.0 g $Ba(NO_3)_2$ and 2.0 g $Th(NO_3)_4.4H_2O$ in $H_2O$, and dil. to 100 mL.

(**b**) *Potassium permanganate soln.*—Satd; ca 6%.

(**c**) *Hydroxylamine hydrochloride soln.*—5%.

(**d**) *Ferrous chloride soln.*—Dissolve ca 1.0 g Fe powder or wire in 50 mL HCl (1 + 1), dil., and filter into 500 mL vol. flask. Add few mL 5% $H_2NOH.HCl$ soln and dil. to vol. Dil. still further before use, if desired.

(**e**) *Purpurin indicator.*—0.01% in alcohol. Dissolve 25 mg pure 1,2,4-trihydroxyanthraquinone in alcohol, heating if necessary, and dil. to 250 mL with same solv. Prep. fresh weekly.

(**f**) *Zirconium nitrate soln.*—Dissolve 1.50 g $Zr(NO_3)_4.5H_2O$ in $H_2O$, acidify with 20 mL HCl, and dil. to 1 L. Filter if not clear.

(**g**) *Fluorine std soln.*—54.5 μg F/mL. Dissolve 0.1464 g NaF in $H_2O$ and dil. to 1 L.

### D. Determination

Place 20 mL well mixed acid strip filtrate, **935.51A**, in 50 mL vol. flask. Add 2.0 mL mixed nitrate soln, then 4.0 mL $KMnO_4$ soln. Rinse down neck of flask with little $H_2O$ and place on active steam bath 5 min. Remove flask, and while still hot, add 5% $H_2NOH.HCl$ soln from buret, slowly and with swirling, until $MnO_2$ is dissolved and soln is colorless; then add ca 0.5 mL in excess. (Appreciable phosphate is revealed as flocculent $Th_3(PO_4)_4$, and sulfate as ppt with Ba. Sometimes $KMnO_4$ is occluded in sulfate and/or phosphate ppt, and pink color tends to persist but does not interfere.) Cool, dil. to vol., and filter. (Filtrate must be clear. If there is perceptible turbidity, return filtrate thru filter several times if necessary, until filtrate is *brilliant.*) Pipet 25 mL clear filtrate into Nessler tube and mark "S."

For blank or comparison tube use 25 mL "blank" soln, contg reagents used in method, prepd as follows:

Dil. 50 mL 10% Na oleate soln, **934.07A(k)**, 50 mL 30 g/100 mL NaOH soln, and 15 mL HCl to 1 L. Acidify portions with $^1/_{10}$ vol. HCl as if soln were an actual "strip," and filter, refiltering until filtrate is perfectly clear. (Chilling soln and shaking vigorously will "churn" pptd oleic acid and aid in obtaining clear filtrate.) Carry 20 mL portions of acidified filtrate thru method exactly as above. (In order more closely to duplicate conditions of actual detn, use 50 mL vol. flasks and 20 mL aliquots in preference to using larger aliquots with correspondingly larger amts of reagents. After being dild to vol. and filtered, blank soln may be combined to form supply of "blank"; 10 portions worked up as above yield ca 500 mL "blank," or enough for ca 20 detns.)

Add 25 mL of this "blank" to second Nessler tube, "B," and to both tubes "S" and "B" add 15.0 mL HCl measured as carefully as possible from graduate. (Always add acid to soln instead of vice versa.) Mix, and match tubes for color. "S" tube will usually be found to have slight greenish tint in comparison with "B" tube, due presumably to traces of Fe. Balance both tubes to same shade by adding $FeCl_2$ soln dropwise to appropriate tube and mixing. *This operation must be done carefully.* When tints are indistinguishable, add exactly 1.00 mL purpurin indicator to each tube. Mix; then add 1.50 mL Zr soln to each tube from 10 mL buret, and mix. Do not shake tubes violently when mixing in reagents; 4 or 5 gentle inversions are enough. Observe color difference, if any, between tubes when looking down their length toward white reflecting surface. If there is no appreciable difference *after 5 min,* F content of sample is negligible. If color of tube "S" is yellower, presence of F is indicated. In this case, add addnl amts of $Zr(NO_3)_4$ soln to tube "S" until its color about matches that of tube "B" (to nearest 0.5 mL Zr soln). Dil. "S" to mark and mix.

Now add to "B" exactly same total vol. Zr soln as was added to tube "S," mix, and let tube stand 2 min for lake to develop fully. Back-titr. std F soln into "B" from 10 mL buret until tubes match, mixing frequently, and dilg nearly to vol. as end point approaches. Add NaF soln in increments of ca 0.1 mL at this stage, and observe usual precautions of transposing and letting bubbles subside when making comparisons. Dil. to mark for final comparison. Check end point by adding 0.1–0.2 mL std F soln in excess. Distinct overbleach should develop.

For sample wt of 1 kg and aliquots specified above, each mL std F soln consumed in back-titrn is equiv. to F content on fruit sample, *removable by solv. treatment,* of 3.0 ppm. Correct result obtained in titrn by sample wt ratio. (Thus, titer of 3.27 mL std F soln, with 1.40 kg sample (ca 10 fruit), represents F content of 7.0 ppm. Vol. restrictions of 50 mL Nessler tube will allow estn of spray residue content up to ca 11 ppm F.) If calibration mark is exceeded in back-titrn, use 10 mL aliquot of acid filtrate in tube "S," and dil. to 25 mL with "blank" soln, correcting titer of std F soln by appropriate factor.

### E. Notes on Rapid Method

G-s Nessler tubes are almost essential with concd acid prescribed in this detn. Analysts familiar with Th-alizarin back-titrn method should have no difficulty with Zr-purpurin titrn. With latter, however, color changes are not so apparent and titrn is less sensitive. However, with careful work, results accurate to $\geq$0.5 ppm may be expected.

Indicator color at prescribed acidity is yellow, and fully laked indicator is orange-red. This contrasts with Th titrn where corresponding range is from yellowish green to reddish purple. Hence, in rapid method, choice of end point involves discrimination between varying shades of orange. Addn of 1.50 mL Zr soln to tube "B" at start is merely to provide intermediate shade of orange to guide analyst in amt of Zr to be added to tube "S." Analysts may prefer to work with redder or yellower end point shade. In any event, make number of titrns by adding varying amts of std F soln as unknowns to Nessler tubes and carrying thru back-titrn as above, for purpose of learning color changes involved. Pure aq. solns instead of "blank" may be used, with acidities of 20 mL HCl/50 mL.

Accuracy of results with rapid method presupposes complete removal of spray residue F by solv. process and good accuracy (not necessarily precision) in titrn. These conditions may not always hold; unless carefully done, solv. method may not be entirely effective, and result on strip solns contg known amts of F have tended to be slightly low. Hence accuracy >95% is not to be expected with this method.

Refs.: JAOAC **27,** 90, 246(1944); **28,** 277(1945); **33,** 587(1950).

CAS-7782-41-4 (fluorine)

## 944.08 Fluorine in Food
### Distillation Method
### Final Action

### A. Principle

Sample is ashed with $Ca(OH)_2$ as F fixative; F is isolated by Willard-Winter distn (Ind. Eng. Chem., Anal. Ed. **5**, 7(1933)) from $HClO_4$, and estd in distillate by $Th(NO_3)_4$ back-titrn method (JAOAC **27**, 246(1944)). Technic and reagent concns are designed to handle $\leq$10.0 mg F conveniently. Modifications applicable to specific products are described.

### B. Precautions and Interferences

Control magnitude of detn blank by careful choice and purification of reagents (*see* **944.08D**). With care, blank will be low (1–3 μg F), but with low-F foods it may represent considerable part of total F detd; hence, it must be stable. Large part of it will be "distn blank" apparently resulting from F leached from glassware of still during distn. This blank can be minimized by preliminary treatment of stills, **944.08F**, and av. distn blank detd if stills of same material and design are routinely used; otherwise, each still must bear its special blank. New, unused stills will usually be found to exhibit high blank, which will diminish to const low figure after several detns. They should not be used until several consecutive blank detns yield const, low amt of F.

Check ashing utensils by blank detns with fixative soln to det. if they contribute appreciable F. Even Pt vessels may become contaminated (owing presumably to slight Ca content) if they have been used recently for HF volatilization of $SiO_2$. In addn, such blank detns are useful for testing reagents and app. used in method and also evaporators, hoods, furnaces, and laboratory atm. for presence of F fumes and dust. If HF bottles are permitted in same laboratory, seal immediately after use; avoid contamination from roach powders.

Ordinary tap $H_2O$ may be source of F contamination, since 1 mL $H_2O$ contg 2 ppm F will contribute 2 μg F if allowed to remain or to dry in still. Therefore, routinely rinse all glassware (stills, flasks, burets, etc.) with $H_2O$, preferably redistd from alk. $KMnO_4$. Filter papers may contribute μg amts of F, and glass filters are preferred if filtration is required in micro detns.

*Interferences* are gelatinous $SiO_2$, Al, and B compds, which repress evolution of F as $H_2SiF_6$ in distn; materials such as nitrates, nitrites, peroxides, Cl, $SO_2$, and $H_2S$, which act upon indicator in titrn or otherwise interfere; halides (Cl), which distil to give excessive acidity in distillate; and phosphates and sulfates, which react with Th in titrn to give high results. Method is so designed that most of these interferences are automatically eliminated, but analyst should be on guard against their possible occurrence under unusual circumstances.

### C. Apparatus

(**a**) *Fluorine still*.—Claisen 100–125 mL distg flask is most practical for general work. It must be of Pyrex glass with auxiliary neck sealed off immediately above side arm to prevent pocketing and refluxing of distillate. Still should be as small and simply designed as practicable; ordinary distg flasks can be used for some work and they are slightly more efficient than Claisen type, except that danger of spraying over of distg acid is greater.

Equip still with dropping funnel and 0–150° thermometer, latter extending to within 6 mm of bottom of flask, so that bulb is immersed in boiling acid mixt. Acid-alkali washed beads, preferably Pyrex, should be on hand. Clean rubber stoppers by boiling in 10% NaOH soln. All-glass app. with ₸ accessories is convenient, especially in routine work, and eliminates need for rubber stoppers.

While not entirely necessary for heating still, use of Wood metal (50 Bi, 25 Pb, 12.5 Sn, 12.5 Cd) bath, adequately shielded, will prevent undue decomposition of $HClO_4$ and aid materially in securing low blank and low-acid distillate; hence, its use is strongly urged. If metal bath is used, do not immerse flask so deeply that bath level is above that of liq. in flask; if bath is not used, transite or asbestos shielding boards are essential, and flask should be heated thru small hole in such shield by low "clean" flame. (Bath and shielding boards prevent overheating of upper still walls.)

At analyst's option, distg $H_2O$ may be added as steam instead of thru dropping funnel; elec. boiler, Fig. **938.09**, is convenient steam generator. If steam is used, inlet tube should dip below surface of liq. in still. One advantage in adding distg $H_2O$ thru funnel is that last portions of rinse $H_2O$ used in transferring an ash can be used in distn. If funnel plug is thinly notched with sharp file on either side of bore, dropping rate can be more easily controlled, and end of funnel stem need not extend into liq. in still. Still is used in conjunction with clean straight-tube condenser no longer than necessary for adequate cooling. (Vertical arrangement of condenser will conserve bench space.)

(**b**) *Nessler tubes*.—Tall-form, 100 and 50 mL, g-s type preferred. Matched in sets of $\geq$6. (100 mL size is used most frequently in general method.)

(**c**) *Additional apparatus*.—(*See* **944.08B**.) Carefully cleaned and tested Pt, or well-glazed porcelain, dishes of $\geq$100 mL size; 150 mL vol. flasks, or if these are not available, 200 mL size; and 10 mL burets (conveniently automatic) to deliver various solns required in distn and titrn. Overhead radiant heater will be found invaluable for drying and preliminary charring of samples, especially those of high-sugar type.

### D. Reagents

(*Caution:* See safety notes on distillation, hydrofluoric acid, perchloric acid, and sulfuric acid.)

(**a**) *Lime suspension*.—Carefully slake ca 56 g (1 mole) low-F CaO (ca 2 ppm F) with ca 250 mL $H_2O$, and *slowly* add 250 mL 60% $HClO_4$ with stirring. Add few glass beads and boil down to copious fumes of acid; cool, add 200 mL $H_2O$, and boil down again. Repeat diln and boiling down once more; cool, dil. considerably, and filter thru fritted glass filter, if pptd $SiO_2$ appears. Pour clear soln, with stirring, into 1 L NaOH soln (100 g/L), let ppt settle, and siphon off supernate. Remove Na salts from ppt by washing 5 times in large centrf. bottles, shaking mass thoroly each time. Finally, shake ppt into suspension and dil. to 2 L. Store in paraffined bottles. (100 mL of this suspension should give no appreciable F blank when evapd, distd, and carried thru titrn, (**l**).) Always shake suspension well before use.

(**b**) *Perchloric acid soln*.—60%. Dil. $HClO_4$ with 3–4 vols $H_2O$ and boil down to original vol. Do not fume strongly. Repeat, and store in Pyrex. (Prepd acid should be Cl-free by test.)

(**c**) *Sulfuric acid soln*.—Carefully mix equal vols $H_2SO_4$ and $H_2O$, boil down to fumes, cool, dil. *carefully*, boil down once more, and dil. to 1 + 1 vol.

(**d**) *Silver perchlorate soln*.—50 g/100 mL.

(**e**) *p-Nitrophenol indicator*.—0.5% alc. soln.

(**f**) *Potassium hydroxide soln*.—Exactly 0.05*N*.

(**g**) *Potassium chloride soln*.—0.05*N*. 3.728 g/L.

(**h**) *Hydroxylamine hydrochloride soln*.—1.0%.

(**i**) *Hydrochloric acid soln*.—Exactly 0.05*N*.

(**j**) *Alizarin indicator*.—0.01% aq. soln of sodium alizarin sulfonate (Alizarin Red S).

(**k**) *Potassium fluosilicate std solns*.—(*1*) *Stock soln*.—0.5 mg F/mL. Dissolve and dil. 0.9661 g (corrected for purity as indicated below) $K_2SiF_6$ to 1 L (much more will not dissolve). Soln keeps indefinitely in paraffined bottle. (*2*) *Working soln.*—10 µg F/mL. Prep soln used in titrn, **944.08G**, by dilg 20 mL stock soln to 1 L. Soln is stable several weeks in ordinary volumetric ware.

If pure $K_2SiF_6$ is not obtainable, prep. as follows: Add, thru dropping funnel, satd soln of NaF, or suspension of crude $K_2SiF_6$, into 500 mL Claisen distg app. contg 60 mL $H_2SO_4$ (1 + 1), some glass beads, and 10–20 g powd $SiO_2$ (or glass) kept at boiling temp. of 120–125°. Distil into 25% soln of KCl, held at simmering temp. on hot plate so that vol. of distillate does not become excessive. If necessary, add more $H_2O$ to mixt. from dropping funnel in side-neck of still. Regulate rate of addn of NaF to still and temp. of condensing $H_2O$ so that side arm and condenser do not become clogged with evolved $H_2SiF_6$, which tends to lodge as gelatinous mass. $K_2SiF_6$ is formed in receiver, and altho entirely cryst. it assumes appearance of gelatinous mass.

When substantial amt collects, pour contents of receiver into large centrf. bottle and wash repeatedly by centrfg (shaking up ppt thoroly each time), until washings are Cl-free by test. Collect on buchner and either air dry or bring to const wt *in vacuo* at 50–70°.

Det. purity by Travers titrn, **921.04B**, at boiling temp. with 0.2 *N* NaOH (1 mL = 0.01101 g $K_2SiF_6$); also by conversion to $K_2SO_4$ by treating 0.3–0.4 g in deep Pt dish with little $H_2O$, then $H_2SO_4$ plus little HF, fuming off excess acid *carefully* (if overheated, mixt. has tendency to spatter), and heating to const wt of $K_2SO_4$ at 650°. With glass app., entirely pure product is not usually obtained, as some contamination with $SiO_2$ results from leaching effect of vapors on condenser. Pure product can be obtained by use of Pt still. Prep. stock soln, correcting wt of 0.9662 by purity factor of the $K_2SiF_6$ (figure for purity obtained from av. of 2 above methods of assay).

(**l**) *Thorium nitrate soln.*—0.25 g $Th(NO_3)_4.12H_2O$ or 0.20 g $Th(NO_3)_4.4H_2O$/L. Check titer against std (10 µg/mL) F soln as follows: Measure 10, 20, 30, etc., up to 80 µg F into 100 mL Nessler tubes, and add 4.00 mL 0.05*N* HCl (2.00 mL if 50 mL Nessler tubes are used, and limiting range to only 50 µg F) (JAOAC **24,** 350(1941)). Dil. mixt. to ca 80 (or 40) mL mark and add 1.00 mL 1.0% $NH_2OH.HCl$ soln. Mix; then add exactly 2.00 mL alizarin indicator (or 1.00 mL for smaller tube) and add Th soln from buret, mixing frequently until, when sighting down tube toward white reflecting surface, incipient pink or salmon pink color is observed. Add little $H_2O$ occasionally so that soln is nearly to mark as end point is approached. Finally, dil. exactly to mark and mix thoroly before checking final end point. Do not shake tube vigorously (5–6 gentle inversions are enough).

Make effort to secure end point shade intermediate between yellowish green of acid indicator and reddish purple of fully developed Th lake. Complete series and plot mL Th soln against mL std fluoride to obtain rough equivalence curve for 2 solns. Depending upon amt of F known to be present, add Th soln in 1–2 mL portions at first, with final addns of 0.25 mL.

### E. Preparation of Sample

(*Caution:* See safety notes on distillation and perchloric acid.)

Methods of sample prepn are designed to furnish representative sample in workable amt of material and to obtain sample in condition for final distn. Mineralization by ashing is usually involved. Some mineral food products can be dissolved in and distd from $HClO_4$, **944.08F**, provided no interferences appear in final distillate.

In general, ≥20 g dry material, 50–100 mL liq. samples, and 50–100 g undried food products or plant material can be taken for analysis, depending upon expected F content and interferences, such as excessive Cl, which use of large samples may introduce. For reasonable precision in analysis of low-F foods, sample should be sufficient to yield titer of ≥0.5 mL for aliquot taken in final titrn. However, it may not always be possible to handle this amt of material. If adequate grinding and mixing equipment is available, it is often feasible to prep. large amts of material (vegetables, mixed foods) and to take aliquot portions for analysis (Ind. Eng. Chem., Anal. Ed. **13,** 93(1941)).

Dry plant materials, feeds, bone meal, etc., can be ground to convenient size in Wiley mill and thoroly mixed before sample is taken. Following special methods for certain products are indicated:

(**a**) *Direct ashing.*—Applicable to fibrous (not highly fatty) foods, liq. samples and, in general, to all foods that can be thoroly wet with aq. fixative soln. This method will apply to majority of food products.

Weigh suitable portion of prepd sample into clean Pt dish and add 25 mL $Ca(OH)_2$ suspension. (Porcelain casseroles or dishes are second choice because they may contribute small amts of F and $Al_2O_3$ to sample.) Mix in $Ca(OH)_2$ suspension with glass rod, adding addnl $H_2O$ if necessary; rinse and remove rod. Dry *thoroly* on steam bath or in hot air oven; then slowly char sample by heating over low flame or elec. plate with thermostat. Overhead radiant heater is convenient for both drying and charring sample. Control excessive swelling of high sugar foods by playing small flame over surface of sample from time to time, and char these products *slowly* so that excessive acidity is not generated. When sample is charred past danger of catching fire, ash in furnace at 600°. (For very small samples and min. blanks, it may be advisable to cover ashing vessel with inverted Pyrex petri dish while ashing.)

For plants high in silica, fusion with NaOH may be necessary (Anal. Chem. **25,** 450, 1061(1953)).

When clean ash is obtained, cool dish and wet ash with ca 10 mL $H_2O$. (Small amt of unburned C does not interfere but if much is apparent, dry down and repeat ashing.) Cover dish with watch glass and cautiously introduce under cover just enough $HClO_4$ soln to dissolve ash. Rinse down cover with little $H_2O$ and transfer soln to freshly prepd F still, **944.08F**, thru long-stem funnel. Rinse dish with remainder of distg acid, using ca 20 mL in all, and adding and transferring in several small portions. *Do not prolong transferring operation.* Finally rinse funnel and stirring rod into dish, assemble still, and complete rinsing of dish with several small portions $H_2O$, pouring these into dropping funnel of still. If distg $H_2O$ is added as steam, **944.08C(a)**, rinse dish with little addnl $H_2O$ and add directly to acid mixt. in still, but avoid excessive initial vol. Add ca 6 Pyrex beads and enough $AgClO_4$ soln, **944.08D(d)**, to ppt all Cl. (Reasonable excess of $AgClO_4$ does no harm; enough solid $Ag_2SO_4$ may also be used.) Proceed as in **944.08F**.

(**b**) *Preliminary distillation.*—(Necessary with certain products high in phosphate, such as Ca phosphate and bone meal, in order to eliminate distd $H_3PO_4$ that may be present in appreciable amts in first distillates. Also advisable with certain excessively fatty materials that may not be thoroly wet with $Ca(OH)_2$ fixative, thus causing F loss in direct ashing method.)

(*1*) *For inorganic phosphatic materials, such as Ca phosphate.*—Weigh sample, usually 10 g, into still; add few glass beads, enough $AgClO_4$ to ppt possible Cl, and ca 20 mL $HClO_4$ soln. (If inorg. phosphatic material does not contain excessive Ca (enough to cause heavy ppt of $CaSO_4$ in still), use similar amt of 1 + 1 $H_2SO_4$.) Distil at 135–140°, collecting ca 200 mL distillate. (For this preliminary distn, extreme care in securing low-acid distillate is not essential.) Evap. distillate to dryness in Pt dish after addn of excess $Ca(OH)_2$ suspension, assuring alk. conditions by testing with drop of phthln. (If $H_2SO_4$ is used in this preliminary distn, add few drops *F-free 30% $H_2O_2$* to distillate to oxidize possible sulfites.) Heat dried residue at 600° few min to destroy indicator residues and possible Cl-contg compds. Transfer contents of dish to freshly prepd still, **944.08F**, with 20 mL distg $HClO_4$ soln as in (**a**), and proceed with final distn as in **944.08F**.

Take 20 mL samples of sirupy $H_3PO_4$ and collect ≥300 mL first distillate at 135°, letting $H_3PO_4$ function as its own distg acid. (More distillate is necessary because $H_3PO_4$ is less effective as F distg acid.) Neutze with $Ca(OH)_2$ suspension, evap. to dryness, transfer to prepd still as above, and proceed as in **944.08F**.

(*2*) *For organic phosphatic materials, such as bone meal, feed supplements, etc.*—As preliminary ashing treatment to destroy most org. matter, moisten sample with enough $Ca(OH)_2$ suspension, dry, char, and heat 2–3 hr at 600°. Transfer ashed material to still, which contains several beads and enough $AgClO_4$ to ppt Cl, with 20 mL distg acid ($HClO_4$ or $H_2SO_4$, depending on Ca content of sample) as in (**a**), and continue as in (**b**)(*1*), beginning, "Distil at 135–140°, . . ."

*Certain org. phosphatic materials* (small samples of bone, 2–5 g, such as entire bones of small test animals) *in which amt of org. matter is not excessive*, may be distd directly as in (**b**)(*1*) without preliminary ashing. If sample contains appreciable Ca (bone samples), use $HClO_4$ with reasonable precaution; if org. phosphatic material does not contain excessive Ca, use 1 + 1 $H_2SO_4$. In either case, add more $Ca(OH)_2$ to first distillates and ash for longer periods to completely destroy distd org. matter (fatty acids). Transfer contents of dish to freshly prepd still, **944.08F**, with 20 mL $HClO_4$ soln as in (**a**) and proceed with final distn, **944.08F**.

*Baking powders* (Ca phosphate and combination types): Place 10 g sample in deep, covered Pt dish or casserole and slake cautiously with ca 20 mL $Ca(OH)_2$ suspension. After action subsides, rinse cover, dry contents of dish *thoroly*, and ash 2–3 hr at 600°. Cool dish and, because of excess of carbonate in ash, treat it with several small portions of warm $H_2O$, breaking up with flat-end stirring rod, and transfer leachings to still. Transfer remaining contents of dish with 20 mL $HClO_4$ soln, avoiding excessive effervescence when acid is added to carbonate soln in still. Add several glass beads and enough $AgClO_4$ soln, and proceed as in (**b**)(*1*), beginning, "Distil at 135–140°, . . ." With *combination* or *Na Al sulfate* baking powders, collect ≥400 mL preliminary distillate, (**b**)(*4*).

Use of special still trap makes possible analysis of highly phosphatic *inorg. or thoroly ashed* materials, and phosphoric acids, with single distn. Special trap, or scrubber, consists of 12–15 g small, hollow glass beads supported in side-neck of the 125 mL Claisen flask by several indentations punched in side wall, and capped by glass disk or inverted bottom of 15 mm test tube. After construction of glass-bead scrubber, side-neck is sealed off immediately above outlet tube. (Beads in scrubber must be wet with little $H_3PO_4$ (by tipping flask) before distn to furnish liq. acid phase.) Take 20 mL sirupy $H_3PO_4$, by itself, and 10 g samples Ca phosphate with 20 mL $HClO_4$ soln, for distn, and collect ≥400 mL distillate at 135°. With single distn, observe precautions in **944.08C(a)**, and also in **944.08F**, regarding neutzn of final distillates. (Distillates should show practically no acidity.) Presence of only *traces* of distd $H_3PO_4$ will vitiate titrn; as little as 20 μg $P_2O_5$ will definitely interfere. Accordingly, if single distn procedure is to be applied with confidence, it is necessary to test distillates obtained from phosphatic materials, by means of the special still, for presence of this interference.

For convenient test utilizing Schricker reagent (JAOAC **22,** 167(1939)), add 5 mL of 1 + 9 diln of this reagent to 45 mL distillate in 50 mL graduate or Nessler tube, mix, and immerse in steam bath 5–10 min. Compare against blank by sighting down tube. Blue or blue-green color indicates phosphate, and as little as 5 μg (as $P_2O_5$) is readily detected. If distillate shows traces, make sure that such amts are below interference level of 15 μg in titrn aliquot before titrg addnl portions of distillate. (Test with Schricker reagent is also useful in usual double distn where phosphate interference is possible. Use of special trap will save time where highly phosphatic materials are handled routinely, but it is not justified in ordinary work because of poor efficiency owing to excessive refluxing in distn.)

(*3*) *For excessively fatty and oily food materials* (*oil-packed foods, certain meats, etc.; also entire undried and unground organs of test animals*).—If there is danger of F loss thru incomplete wetting with $Ca(OH)_2$ fixation soln, handle as follows: Weigh appropriate amt of sample, usually 10–25 g, into still, and add Ag (preferably 0.1–0.2 g solid $Ag_2SO_4$), several glass beads, and 20–25 mL $H_2SO_4$ (1 + 1). Distil at 130–135° and collect 200–250 mL distillate in beaker or open vessel. If foaming is excessive, increase vol. of distg acid, and where necessary, use larger (250–300 mL) still. If larger still or more acid is used, collect proportionately more of first distillate. (Oil or fat of many of these products will tend to prevent foaming, and, in some instances, use of ca pea-size piece of pure paraffin is addnl aid.)

Oxidize distillate in cold by cautious addn of 2–3 mL *F-free 30% $H_2O_2$* to remove sulfites, let stand few min, and evap. portionwise in Pt dish contg excess (10–15 mL) $Ca(OH)_2$ suspension. Ash residue at 600° until clean. Proceed as in (**b**)(*1*), beginning "Transfer contents of dish to freshly prepd still, . . ."

Handle pure oils by similar procedure: Use 10 g sample with 25 mL $H_2SO_4$ (1 + 1) and carry temp. at first to ca 170° to saponify; then carefully bring temp. down to 140° with distg $H_2O$ and collect ≥250 mL distillate. (It will probably be necessary to use higher reading thermometer for this procedure.) Oxidize distillate with 30% $H_2O_2$ and evap. to dryness after adding excess $Ca(OH)_2$ suspension. Ash at 600° and after brief preliminary ash period remove dish, add little $H_2O$ plus addnl 1–2 mL of the $H_2O_2$ to remove sulfides, dry, and complete ashing. Proceed as in (**b**)(*1*), beginning "Transfer contents of dish to freshly prepd still, . . ."

(*4*) *For aluminum and boron compounds.*—Al and B repress evolution of F. Isolate F by preliminary distn at elevated temp. For this purpose, weigh sample, usually 5–10 g, into still, add 25 mL $H_2SO_4$ (1 + 1), and conduct first distn at 160–165° (special thermometer), collecting 300 mL distillate. Oxidize distillate with 30% $H_2O_2$ as above, evap. in Pt dish with excess $Ca(OH)_2$ suspension, ash briefly at 600°, and proceed as in (**b**)(*1*), beginning, "Transfer contents of dish to freshly prepd still, . . ."

### *F. Final Distillation*

(*Caution:* See safety notes on distillation and perchloric acid.)

Always make final distn from $HClO_4$, and take precautions to secure low-acid distillate, **944.08C(a)**. Since interferences, such as org. matter, phosphate, sulfate, etc., must be absent from distillate, make distn with careful temp. control in presence of enough Ag salt to repress HCl evolution (**944.08B**). It is well to check distillates for presence of possible phosphate as in **944.08E(b)**(*2*), and where advisable, as in (**b**)(*4*), to test for sulfate with little dil. $BaCl_2$ soln. $HClO_4$ used in final distn is usually used in transferring ash to still, **944.08E(a)**. Few acid-alkali washed beads are used to control bumping. (Use of powd $SiO_2$ does not appear necessary for microdetn.)

To promote better recoveries, and to minimize and render const distn blank discussed in **944.08B** and **944.08G**, prep. still by special cleaning process before this transfer by treating it with *hot* 10% NaOH soln after each detn, flushing out with tap $H_2O$, and then rinsing with distd $H_2O$. Occasionally (at least once daily, and especially after it has stood idle for any length of time), give still addnl treatment by boiling down 15–20 mL $H_2SO_4$ (1 + 1) until still is filled with fumes. Cool, pour off acid, treat with the 10% NaOH soln, and *thoroly* rinse out. (Cleaning should be especially meticulous after high-F or high-$SiO_2$ samples have been distd, and in such cases condenser should also be cleaned.)

At this stage, prepd sample has been transferred to specially treated still, as directed above, for final isolation of F. Begin distn, and when temp. reaches 137°, keep at this point (±2°) by adding $H_2O$ from dropping funnel, **944.08C(a)**. Heat still at such rate that all distns require ca same time. (Time promotes uniformity in blank correction.) Collect distillate in 150 or 200 mL vol. flask. After few mL distillate collects, add 1–2 drops *p*-nitrophenol indicator, (**e**), and keep distillate alk. to this indicator (faintest perceptible yellow) by occasionally adding 1–2 drops 0.05*N* KOH from 10 mL buret during distn while swirling receiver. So regulate this addn of alkali that distillate is neutzd (within 1 drop of alkali) as it approaches mark. Carefully note vol. alkali used. Dil. distillate to vol. and mix thoroly. Do not let F distillate stand more than few min before neutzg.

If sample contains such large amts of Cl that bumping in still cannot be controlled, dissolve ash of another sample, and acidify *slightly* with $HClO_4$. Dil. considerably and ppt Cl in dish with $AgClO_4$ soln, avoiding large excess. Filter thru glass filter, wash ppt *thoroly* with hot $H_2O$, and evap. filtrate and washings to dryness after adding excess (to alky) of $Ca(OH)_2$ suspension. Transfer residue to still with $HClO_4$ soln and repeat distn as above.

### G. Titration

Place aliquot of final distillate in Nessler tube and mark "S" (sample). (Optimum F content for titrn is 60–70 μg for 100 mL Nessler tubes and 30–40 μg for 50 mL size, and it is well to make exploratory titrn on small aliquot to check approx. F content of distillate. Larger tubes are necessary for precise results on low-F foods.)

Add 0.05*N* HCl, 4.00 mL for 100 mL tubes and 2.00 mL for 50 mL size, and 1.00 mL $H_2NOH.HCl$ soln. (For routine work *with 100 mL tubes*, dissolve 1.0 g $H_2NOH.HCl$ in 500 mL 0.04*N* HCl and dil. to 500 mL. Then proper amt of both reagents can be added in single operation with 5 mL pipet.) Dil. to ca 90 (or 40) mL, mix well, then add alizarin indicator (2.00 or 1.00 mL), and mix again. Always add and mix in $H_2NOH.HCl$ before adding indicator.

Prep. blank tube "B" by adding proper amt HCl and $H_2NOH.HCl$, and amt 0.05*N* KCl soln representing same proportion of total vol. of 0.05*N* KOH used to neutze distillate as aliquot vol. taken for sample tube represents of total distillate vol. (Thus, if 1.50 mL 0.05*N* KOH was used to neutze distillate of 150 mL and aliquot taken for tube "S" was 75 mL, add 0.75 mL 0.05*N* KCl to tube "B.") Dil. and mix, allowing slightly more headspace than in sample tube. Then add proper vol. alizarin indicator and mix.

Measure Th soln into tube "S," mixing between addns, until end point of about proper shade is reached. Dil. to mark, mix, and check this end point shade. Note from curve, **944.08D(l)**, approx. vol. std F soln corresponding to this vol. Th soln, and add ca 0.5 mL *less* than this amt of std F soln to "B." Mix; then add exactly same vol. Th soln as was added to "S," duplicating approx. increments in which it was added and number of mixings. Dil. nearly to mark and compare colors of "S" and "B." (If vol. std F soln added to "B" was properly chosen, this tube should be only slightly pinker in shade than sample tube.)

Bleach "B" tube to exact match with tube "S" by adding more std F soln to "B" in increments of 1–2 drops, mixing gently between addns. Dil. to mark for final comparison and observe usual precautions of letting bubbles subside and of transposing tubes when final comparisons are made. (At matchpoint, F content of tube "S" equals amt added to tube "B.") Check this end point by adding 1–2 drops excess std F soln to tube "B." Distinct overbleach should develop.

Repeat titrn on aliquots of different size to obtain total amt of F distd. If time is available, repeat entire detn with different wt sample.

*For precise work*, evaluation of reagent and of distn blank is necessary, **944.08B**. Det. distn blank by making several distns with prescribed amts $HClO_4$ and $AgClO_4$ solns from freshly cleaned still, titrg distillate as above with as large aliquot as practicable. Av. of values found should be ≤3 μg F. If amts found by individual blank detns are too small to be detd accurately, make ≥5 sep. distns and evap. distillates, 150 mL each time, successively in same Pt dish for final distn and average blank figure. Distn and total detn blanks can usually be *combined* by carrying run (with same amts of reagents and similar evapn and ashing treatment) thru entire detn. Reagents and manipulations should increase distn blank but little.

Calc. total amt F distd from amt found in aliquot titrd, subtract proper blank, and refer net figure to wt sample taken. If double distn procedure was used, make appropriate blank correction.

Refs.: JAOAC **27,** 90, 246(1944); **28,** 277(1945); **33,** 587 (1950).

CAS-7782-41-4 (fluorine)

## 935.50 Lead
### Suitability of Methods and Precautions

### A. Principle

Instrumental methods, polarography and atomic absorption (AA) spectrophotometry, are generally more reliable than colorimetric method at lower concns. Method **972.25** is particularly applicable to samples contg high Ca concn. Special instrumental methods optimized for evapd milk and fish are given in **973.35**, **974.13**, **979.17**, **972.23**, and **972.24**.

General colorimetric method calls for ashing, **934.07B**, sepn of Pb, either as dithizone complex, **934.07D**, or as sulfide, **934.07E**, followed by colorimetric dithizone detn, **934.07F**, in comparator tubes, or with spectrophtr. Interference is treated sep., **934.07G–I**, and analyst should become familiar with details of these sections before applying method. Special methods of sample prepn are given in **934.07J** and **K**.

### B. Precautions

Analyst should decide whether nature of detn requires unusual care in purification of reagents, or whether blank detn will suffice. Smaller the amt of Pb to be detd, greater the care required in reduction of blank (*see also* **934.07F**).

To test suitability of reagents, place 10–15 g solid reagents dissolved in redistd $H_2O$ or 15–20 mL concd acids previously neutzd with redistd $NH_4OH$ in separator and add enough Pb-free citric acid to prevent pptn by $NH_4OH$ of Fe, Al, alk. earth phosphates, or other substances. Make soln ammoniacal and add 2–3 mL 10% KCN soln. Shake soln with ca 5 mL dithizone soln, **934.07A(e)** (5–10 mg/L). If lower layer is green, transfer it to another separator and ext excess dithizone with $NH_4OH$ (1 + 99) to which has been added drop of KCN soln. If $CHCl_3$ layer is colorless, consider test neg. for use with dithizone methods.

When special purification becomes necessary, redistil $H_2O$ (distd $H_2O$ stored in Sn-lined tanks usually contains Pb and Sn), $HNO_3$, HCl, HBr, Br, and $CHCl_3$ in all-glass (pyrex) or quartz stills (preferably quartz). Prep. $NH_4OH$ by distg ordinary reagent into ice-cold redistd $H_2O$. If stills are new, steam them out with hot HCl or $HNO_3$ vapors to remove "surface" Pb. (Subsequent distillates may not be totally Pb-free.)

$Pb(NO_3)_2$ may be purified as follows: Dissolve 20–50 g in min. of hot $H_2O$ and cool with stirring. Filter crystals with suction on small buchner, redissolve, and recrystallize. Dry crystals at 100–110° to const wt. Cool in desiccator and store in tightly stoppered bottle. (Product has no $H_2O$ of crystn and is not appreciably hygroscopic.)

Purify citric acid, NaOAc or $NH_4OAc$, $Al(NO_3)_3$, $Ca(NO_3)_2$, and $Na_2SO_4$ by pptg Pb from their aq. solns with $H_2S$ (*Caution:* See safety notes on hydrogen sulfide.), using 5–10 mg $CuSO_4$ as coprecipitant (citric acid and $Al(NO_3)_3$ solns require adjustment with $NH_4OH$ to pH 3.0–3.5, bromophenol blue indicator). Filter (fritted glass filter is most convenient), boil filtrates 20 min to expel excess $H_2S$, and refilter if necessary to obtain brilliantly clear solns. Purify other reagents by recrystn.

Store redistd acids or purified solns of reagents in Teflon or conventional polyethylene containers carefully cleaned of surface Pb with hot $HNO_3$. Paraffin-lined bottles may be used for alk. reagents.

Carefully clean new glass, plastic, and chemical ware with hot 10% NaOH soln followed by hot $HNO_3$, and use only for Pb detns.

In prepn of samples for analysis, avoid Pb contamination. If mixing or grinding is necessary, use porcelain mortar if possible. Avoid use of metal food grinders unless previous experiment has shown that no contamination of sample with Pb or Sn results. If product to be analyzed cannot be thoroly mixed in its own container, or if composite sample of number of containers is desired, empty into large glass jar or porcelain dish and mix thoroly with wooden spoon or porcelain spatula. If liq. portion of sample cannot be incorporated into ground solid material to obtain homogeneous mixt., analyze sep. If food is packed in tins having soldered seams (sardines and meats), open tins from bottom to avoid contaminating sample with bits of solder. Avoid sifting in prepn of samples to prevent metallic contamination or segregation of Pb.

CAS-7439-92-1 (lead)

## 935.51 Lead on Apples and Pears

### Colorimetric Method

### Final Action

(Efficiency of 95% expected)

(For rapid detn of Pb spray residue on apples and pears; ppm × 0.007 = grains/lb; (grains/lb) × 143 = ppm)

### *A. Preparation of Sample*

Weigh ≥10 units and pull or cut out stems with narrow-blade knife, cutting no more of flesh than necessary. Trim off sepals (dried residue of blossom) and discard sepals and stems. To 25 mL 30% NaOH soln in 600 mL beaker, add 175 mL $H_2O$ and 25 mL Na oleate soln, (**k**), and bring to gentle boil. Have ready in wash bottle 250 mL hot $HNO_3$ (2 + 98) or hot HCl (3 + 97). (Reasonably accurate figure for $As_2O_3$ can be obtained by using the HCl rinse and applying Gutzeit As detn, **912.01★**, to portion of filtrate, after acidifying part of the 500 mL alk. strip soln with $^1/_{10}$ vol. HCl instead of $HNO_3$ (*see* later in this section). Rapid method for F, **944.07D**, likewise specifies HCl rinse and acidification.)

Impale each fruit in turn upon pointed glass rod; immerse in the alk. soln, with occasional rotation, until skin begins to check; then remove to large funnel inserted in 500 mL vol. flask and rinse with stream of the hot acid, being careful to flush out stem and calyx ends thoroly. When all fruit has been so treated, cool alk. soln and add it thru funnel to acid soln in flask. Rinse beaker and funnel with any remaining acid and with $H_2O$, using entire 250 mL rinse acid. Cool, and dil. to vol.

In dry 200 mL erlenmeyer place exactly 10 mL $HNO_3$ (10 mL HCl for As or F). Thoroly mix contents of vol. flask and immediately add 100 mL to acid in erlenmeyer while swirling vigorously. Filter on rapid paper. If first portion of filtrate is cloudy, refilter until clear. Det. Pb as in **935.51B** or **C**.

(*See* JAOAC **26,** 150(1943)) for details of churn-type washer for removing Pb spray residues from apples and pears.)

### *B. Determination with Nessler Tubes*

(At least 15 tall-form tubes matched for uniformity in color *and diam.* are necessary. *Caution:* See safety notes on cyanides.)

(**a**) *Stds.*—To each of two 1 L vol. flasks add 47.5 mL 30% NaOH soln. If $HNO_3$ was used in rinsing and acidification, **935.51A**, add 100 mL $HNO_3$ to each flask. If HCl (3 + 97) was used in rinsing, add 91 mL $HNO_3$ and 13.6 mL HCl to each flask. Do not mix in the acids unless solns are cold and dil. To one flask add stock reagent, (**a**), equiv. to 25.45 mg Pb. Mark this flask "std" and other "blank." Dil. both solns to vol. at room temp. and mix. These 2 solns contain reagents as they occur in acidified and filtered sample soln. The "std" is equiv. in Pb content to acidified soln from sample of 1400 g carrying Pb load (removable by "stripping" operation) of 10 ppm. By combination of the 2 solns in suitable proportions, equiv. of any Pb load from 0 to 10 ppm may be obtained.

Std tubes made up in intervals corresponding to 1.0 ppm may be interpolated to 0.5 ppm. Following table gives vols of "std" and "blank" to be added to Nessler tubes for each interval; measure into tube by burets:

| Pb, ppm | "Standard," mL | "Blank," mL |
|---|---|---|
| 0.0 | 0.0 | 10.0 |
| 1.0 | 1.0 | 9.0 |
| 2.0 | 2.0 | 8.0 |
| 3.0 | 3.0 | 7.0 |
| 4.0 | 4.0 | 6.0 |
| 5.0 | 5.0 | 5.0 |
| 6.0 | 6.0 | 4.0 |
| 7.0 | 7.0 | 3.0 |
| 8.0 | 8.0 | 2.0 |
| 9.0 | 9.0 | 1.0 |
| 10.0 | 10.0 | 0.0 |

Working with 1 tube at time, add 10 mL $NH_3$-cyanide-citrate soln, (**l**), to each tube followed by 30 mL std dithizone soln, 30 mg/L, **934.07A(e)**. Shake vigorously 1 min and let sep. The pH of aq. phase should be ca 9.4 regardless of whether HCl or $HNO_3$ is used in rinsing. Stopper each std tube securely with new cork stopper. It is unnecessary to make up entire series of stds if only portion of range, e.g. 5.0–10.0 ppm, is of quant. interest.

(**b**) *Comparison*.—Transfer 10 mL portions of clear filtrate from **935.51A** to each of 3 Nessler tubes. First add 10 mL $NH_3$-cyanide-citrate soln, (**l**), to each tube; to one tube add 30 mL std dithizone soln, 30 mg/L, **934.07A(e)**, and to other 2 tubes 30 mL clear $CHCl_3$. Shake vigorously 1 min and let sep. With tube of clear $CHCl_3$ backing sample tube (contg the dithizone) and 1 sample tube contg $CHCl_3$ backing each of 2 std tubes, compare color in lower layer of sample with that of stds, looking thru tubes at right angles to their lengths toward strong diffused light. (Comparator box similar to boxes used in colorimetric pH measurements but of larger size is convenient. When working with apple strip solns, slight turbidity is produced in sample tube, which slightly changes color observed. To compensate for this effect, same turbidity is introduced in field of view of std tubes made up exactly as sample, except that $CHCl_3$ is substituted for the dithizone soln.)

If color produced by sample is redder than 10 ppm std, repeat with smaller aliquot of filtrate, dilg to 10 mL with "blank" soln. If, for example, 5 mL aliquot is taken, indicated reading must be doubled. After match is obtained, calc. result to basis of 10 mL aliquot and 1400 g sample.

### C. Determination with Photometer

(This method is suitable for photometric measurement of "mixed color," **934.07F(b)**. Changes in **935.51B** are introduced here to prevent formation of colors too dense for measurements. Use 5 mL instead of 10 mL aliquots of acidified wash soln, **935.51A**.)

(**a**) *Stds*.—Measure following proportions of "std" and "blank" solns, **935.51B(a)**, into separators:

| Pb, ppm | "Standard," mL | "Blank," mL |
|---|---|---|
| 0.0 | 0.0 | 10.0 |
| 2.0 | 1.0 | 9.0 |
| 4.0 | 2.0 | 8.0 |
| 6.0 | 3.0 | 7.0 |
| 8.0 | 4.0 | 6.0 |
| 10.0 | 5.0 | 5.0 |

Add 10 mL $NH_3$-cyanide-citrate soln, (**l**), and working with 1 separator at time, immediately develop color by shaking 1 min with 50 mL pure dithizone soln of 10 mg/L strength. Let stand few min to cool, filter $CHCl_3$ layers thru specially washed papers, (**m**), and fill cell of appropriate length (1 cm is convenient). Det. *A* and plot against ppm Pb to obtain std curve.

(**b**) *Comparison*.—Place appropriate size aliquot of acidified strip soln in separator and dil. to 10 mL with "blank" soln. Add 10 mL $NH_3$-cyanide-citrate soln, (**l**), and ext with 50 mL 10 mg/L std dithizone soln. Let stand few min to cool, filter, and read as above. Det. amt of Pb from std curve prepd as in (**a**) and calc. to basis of 5 mL aliquot and 1400 g sample.

CAS-7439-92-1 (lead)

## 973.35 Lead in Evaporated Milk

### Atomic Absorption Spectrophotometric Method

**First Action 1973**
**Final Action 1974**

(*Caution:* See safety notes on AAS.)

### A. Principle

Sample is dry-ashed; Pb is extd as the 1-pyrrolidinecarbodithioate into BuOAc, and detd by AA spectrophotometry at 283.3 nm.

### B. Apparatus

(**a**) *Atomic absorption spectrophotometer*.—Equipped with 4″ single slot burner head.

(**b**) *Ashing vessels*.—Approx. 100 mL, flat-bottom Pt crucible or dish, Vycor or quartz tall-form beaker, or evapg dish (Corning Glass Works, No. 13180, or equiv.). Discard Vycor vessels when inner surfaces become etched.

(**c**) *Centrifuge*.—Capable of holding 15 mL conical tubes and centrfg at 2000 rpm.

(**d**) *Furnace*.—With pyrometer to control range of 250–600° with variation ≤10°.

### C. Reagents

(**a**) *Nitric acid*.—1*N*. *See* **972.25C(c)**.

(**b**) *Butyl acetate*.—Spectral grade, $H_2O$-satd.

(**c**) *Ammonium 1-pyrrolidinecarbodithioate* (*APDC*).—2%. Dissolve 2.00 g APDC in 100 mL distd or deionized $H_2O$. Remove insol. free acid and other impurities normally present by 2–3 extns with 10 mL portions BuOAc.

(**d**) *Lead std solns*.—(*1*) *Stock soln*.—1 mg Pb/mL 1*N* $HNO_3$. *See* **972.25C(d)**(*1*). (*2*) *Intermediate soln*.— 5.0 μg Pb/mL. Pipet 5 mL stock soln into 1 L vol. flask, add 1 mL $HNO_3$, and dil. to vol. with $H_2O$. (Soln is stable several months if stored in polyethylene bottle.) (*3*) *Working solns*.—Pipet 20, 10, 5, and 2 mL intermediate soln into sep. 100 mL vol. flasks, and dil. to vol. with 1*N* $HNO_3$ (1.0, 0.50, 0.25, and 0.10 μg Pb/ mL, resp.). Pipet 10 and 5 mL soln contg 0.50 μg Pb/mL into sep. 100 mL vol. flasks, and dil. to vol. with 1*N* $HNO_3$ (0.05 and 0.025 μg Pb/mL, resp.).

(**e**) *Citric acid soln*.—10%. Weigh 10.0 g Pb-free citric acid into 100 mL vol. flask, dissolve in $H_2O$, and dil. to vol. Stopper flask and shake thoroly. If necessary, remove Pb impurity as in **972.24D**.

(**f**) *Bromocresol green*.—0.1%. pH range, 3.8 (yellow) to 5.4 (blue). Transfer 0.100 g bromocresol green, Na salt, to 100 mL vol. flask, and dil. to vol. with $H_2O$. Use 1 drop/10 mL anal. soln.

### D. Ashing

(Clean all glassware thoroly in $HNO_3$ (1 + 1).)

Weigh ca 25 g (to nearest 0.1 g) sample into ashing vessel. Dry samples overnight in 120° forced-draft oven. (Sample must be absolutely dry to prevent flowing or spattering in furnace.) Place sample in furnace set at 250°. *Slowly* (50° increments) raise temp. to 350° and hold at this temp. until smoking ceases. Increase temp. to 500° in ca 75° increments (sample must not ignite). Ash 16 hr (overnight) at 500°. Remove from furnace and let cool. Ash should be white and essentially C-free. If ash still contains excess C particles (i.e., ash is gray rather than white), proceed as follows: Wet with min. amt $H_2O$ followed by dropwise addn of $HNO_3$ (0.5–3 mL). Dry on hot plate. Transfer to furnace at 250°, slowly increase temp. to 500°, and continue heating 1–2 hr. Repeat $HNO_3$ treatment and ashing if necessary to obtain C-free residue. (*Note:* Local overheating or deflagration may result if sample still contains much intermingled C and especially if much K is present in ash (*see* **934.07B**).)

Dissolve residue in 5 mL 1*N* $HNO_3$, warming on steam bath or hot plate 2–3 min to aid soln. Filter, if necessary, by decantation through S&S 589 black paper into 50 mL vol. flask. Repeat with two 5 mL portions 1*N* $HNO_3$, filter, and add washings to original filtrate. Dil. to vol. with 1*N* $HNO_3$.

Prep. duplicate reagent blanks for stds and samples, including any addnl $H_2O$ and $HNO_3$, if used for sample ashing. *Note:* Do *not* "ash" $HNO_3$ in furnace, since Pb contaminant will be lost. Dry $HNO_3$ in ashing vessel on steam bath or hot plate, and then proceed as above.

### E. Extraction

(Complete analysis on same day.)

Pipet 20 mL each working soln, reagent blank for stds (if different from that used for samples), sample soln, and appropriate reagent blank(s) for samples into sep. 60 mL separators. Treat each soln as follows: Add 4 mL citric acid soln, (**e**), and 2–3 drops bromocresol green indicator, (**f**). (Color of soln should be yellow.) Adjust pH to ca 5.4, using $NH_4OH$ initially and then $NH_4OH–H_2O$ (1 + 4) in vicinity of color change (first permanent appearance of light blue). Add 4 mL APDC soln, (**c**), stopper, and shake 30–60 sec. Pipet in 5 mL BuOAc, (**b**). Stopper separator and shake vigorously ca 30–60 sec. Let stand until layers sep. cleanly; drain and discard lower aq. phase. If emulsion forms or solv. layer is cloudy, drain solv. layer into 15 mL centrf. tube, cover with Al foil or Parafilm, and centrf. ca 1 min at 2000 rpm.

### F. Determination

Set instrument to previously detd optimum conditions for org. solv. aspiration (3–5 mL/min), using 283.3 nm Pb line and air-$C_2H_2$ flame adjusted for max. Pb absorption. Flame will be somewhat fuel-lean. Optimum position in flame for max. absorption should be just above burner top. If using recorder, DCR, etc., adjust to manufacturer's specifications. Depending upon signal-to-noise ratio, scale expansion up to 10× may be used. Check 0 point while aspirating $H_2O$-satd BuOAc. Aspirate sample and std solns, flushing with $H_2O$ and then BuOAc between measurements. Record *A* of each soln.

Prep. std curve by plotting *A* of each std corrected for blank against concn of that std in μg Pb/mL BuOAc. Concn of std in BuOAc is 4 times that in aq. std. Det. Pb concn from std curve, using *A* corrected for sample reagent blank, if used.

$$\text{ppm Pb} = [(\mu g\ \text{Pb/mL from curve}) \times 5\ (\text{mL BuOAc})]/(\text{g sample} \times 20/50)$$

Ref.: JAOAC **56,** 1246(1973).

CAS-7439-92-1 (lead)

## 974.13 Lead in Evaporated Milk

### Anodic Stripping Voltammetric Method

**First Action 1974**
**Final Action 1976**

### A. Principle

Evapd milk is dry-ashed and residue is dissolved in dil. $HNO_3$. Pb is electroanal. concd on hanging Hg drop or Hg film electrode, and detd by reversing potential sweep and measuring anodic current peak.

### B. Apparatus

*See* **982.23B(a)**, (**b**), (**c**), and (**d**).

### C. Ashing

Proceed as in **973.35D**.

### D. Preparation of Standard Curve

Prep. stds as in **973.35C(d)** and 1*N* $HNO_3$ reagent blank. Transfer 10–20 mL std soln to cell, depending on cell capacity. Adjust to 25 ± 1°, and bubble N thru soln 5 min. Adjust gas inlet tube to let N flow gently above and across soln surface. If hanging drop Hg electrode is used, add fresh drop Hg to capillary tip with micrometer (Hg drop must be reproducible for each measurement), turn on stirrer motor, and electrolyze soln 1–10 min at −0.6 v against satd calomel electrode (time depends on manufacturer's instructions). Stop stirring and let soln stand 30 sec. Linearly increase applied voltage (pos. voltage scan). Use manufacturer's instructions for rate of scan, e.g., 2–6 mv/sec. Measure wave ht at half-wave potential (−0.45 ± 0.05 v against satd calomel electrode). Plot μg Pb/mL std soln against wave ht × sensitivity factor (*SF*). Repeat for each std soln and reagent blank soln. Prep. new curve with each batch of samples.

### E. Determination

Transfer to cell same vol. sample soln as used in stdzn. Bubble N, add Hg drop, and apply voltage as in stdzn. Measure wave ht at appropriate potential and det. Pb concn by comparing wave ht of sample soln with that of std curve or analyze std soln immediately before or after sample soln (preferable when instrument scale factor must be changed).

Prep. reagent blank soln as in **973.35D**, last par., and analyze as above.

### F. Calculations

Calc. μg Pb/mL as follows:

$$C = C'\{[(WH \times SF) - (WH_B \times SF_B)]/[(WH' \times SF') - (WH'_B \times SF'_B)]\}$$

where $C$ and $C'$ = μg Pb/mL sample and std, resp.; $SF$, $SF'$, $SF_B$, and $SF'_B$ = sensitivity factor settings of sample, std, and reagent blank (may be different for sample ($SF_B$) and std ($SF'_B$)) solns, resp.; and $WH$, $WH'_B$, $WH_B$, and $WH'$ = wave hts.

$$\text{ppm Pb} = (C \times 50)/\text{g sample}$$

Ref.: JAOAC **56,** 1246(1973).

CAS-7439-92-1 (lead)

## 979.17 Lead in Evaporated Milk and Fruit Juice

### Anodic Stripping Voltammetric Method

**First Action 1979**
**Final Action 1984**

### A. Apparatus

(**a**) *Voltammetric analyzer.*—With staircase anodic stripping ramp and graphite electrode coated with thin film of Hg. Capable of measuring 5 ng Pb in presence of dissolved O. (Solns cannot be deaerated). Peak area integration desirable. ESA Model 3010A Trace Metals Analyzer (ESA, Inc., 45 Wiggins Ave, Bedford MA 01730), or equiv.

(**b**) *Micropipets.*—50, 100, 200, and 300 μL, pos. displacement type. (SMI or Drummond, available from supply houses, or equiv.).

### B. Reagents

(Use deionized $H_2O$ to prep. std solns. Prep. and store solns in same Pyrex vol. flasks. Do not wash flasks with strong acids between use; just rinse 3 times with deionized $H_2O$. Always prep. same soln in same flask.)

(**a**) *Lead releasing reagent.*—Contg <1 ppb Pb. Acid soln of cation able to displace Pb from sample. Metexchange Reagent (ESA, Inc.), or equiv.

(**b**) *Lead std solns.*—(*1*) *Stock soln.*—1 mg/mL. Prep. as in **972.25C(d)**(*1*). (*2*) *Intermediate soln.*—10 μg/mL. Pipet 1 mL stock soln into 100 mL vol. flask contg 1.0 mL $HNO_3$ and ca 50 mL $H_2O$. Mix, and dil. to vol. Prep. each week. After soln is prepd 6 times in same flask, it is stable 1 month. (*3*) *Working soln for fruit juice detn.*—3 μg/mL. Pipet 30 mL soln (*2*) into 100 mL vol. flask contg 0.7 mL $HNO_3$ and ca 50 mL $H_2O$. Mix, and dil. to vol. (*4*) *Working soln for evap-*

*orated milk detn.*—1 μg/mL. Prep. as in (*3*), using 10 mL soln (*2*) and 1.0 mL $HNO_3$. Working solns are stable 3 days. After being prepd 5 times in same flask, they are stable 2 weeks. (*5*) *Calibration solns.*—Evapd milk or fruit juice of type being detd, and contg ≥ 0.5 ppm added Pb.

### C. Determination

Calibrate instrument according to manufacturer's directions. Mix aliquot of sample with releasing reagent, (**a**), and perform detn according to manufacturer's instructions. Data for ESA analyzer are as follows:

| Calibration | Juice | Milk |
|---|---|---|
| Initial potential, v | −1.025 ± 0.005 | −1.090 ± 0.005 |
| Final potential, v | −0.100 ± 0.005 | −0.100 ± 0.005 |
| Sweep rate, mv/step | 14.0 ± 0.05 | 10.50 ± 0.05 |
| Integration set points, v | −0.490 ± 0.005 | −0.490 ± 0.005 |
| Sample size, μL | 300 | 200 |

Run control or spiked sample with each 15–20 analyses in a series.

Refs.: JAOAC **66,** 1409, 1414(1983).

CAS-7439-92-1 (lead)

## 972.23 Lead in Fish

### Atomic Absorption Spectrophotometric Method

**First Action 1972**
**Final Action 1976**

(*Caution:* See safety notes on AAS.)

### A. Apparatus

(**a**) *Atomic absorption spectrophotometer.*—*See* **973.35B(a)**; range 0–10 μg/mL.
(**b**) *Lead lamp.*—Hollow cathode Pb lamp.
(**c**) *Crucible.*—Porcelain, ca 50 mL capacity and 5 cm deep; or tall-form Vycor or quartz beaker, 100 mL.

### B. Reagents

(**a**) *Hydrochloric acid.*—1*N*. Dil. 82 mL HCl to 1 L with $H_2O$.
(**b**) *Lead std solns.*—(*1*) *Stock soln.*—1 mg Pb/mL 1*N* $HNO_3$. *See* **972.25C(d)**. (*2*) *Working soln.*—10 μg Pb/mL. Pipet 10 mL stock soln into 1 L vol. flask, add 82 mL HCl, and dil. to vol. with $H_2O$.
(**c**) *Buffer soln.*—Disperse 163 g EDTA in 200 mL $H_2O$ in 2 L vol. flask and add enough $NH_4OH$ to dissolve. Dil. 60 mL 70.5% $HClO_4$ (*Caution:* See safety notes on perchloric acid) by pouring carefully into ca 500 mL $H_2O$ and cool. Dissolve 50 g $La_2O_3$ in $HClO_4$ soln. Add 8 drops Me orange indicator to ammoniacal EDTA soln and add $La_2O_3$ soln to EDTA soln while stirring vigorously. If necessary, add $NH_4OH$ to maintain alky of above soln to Me orange. Dil. to 2 L.

### C. Reagent Blank

(*Caution:* See safety notes on nitric acid.)

Before proceeding with analysis, test purity of reagents as follows: Evap. 4 mL $HNO_3$ in crucible to dryness on hot plate or steam bath, dissolve residue in 1*N* HCl, and transfer to 25 mL vol. flask. Heat residue again successively with two 5 mL portions 1*N* HCl and add to flask. Cool, dil. to vol. with 1*N* HCl, and mix. Proceed with detn. Total reagent blank should be ≤10 μg Pb (equiv. to 0.4 ppm in sample) for detns at levels ≥1 ppm. For detns at <1 ppm, purify reagents as in **973.50B** to attain blank <50% of limiting level of concern.

### D. Preparation of Sample

Weigh ca 25 g (to nearest 0.1 g) sample into crucible, (**c**), and dry 2 hr at 135–150°. Transfer to cold, temp.-controlled furnace and slowly raise temp. to 500°. Set control and check for maintenance of 500°. (Temp. as low as 550° may cause loss of Pb.) Ash overnight (16 hr). Remove sample, let cool to room temp., cautiously add 2 mL $HNO_3$, and swirl. Evap. carefully *just* to dryness on *warm* hot plate or steam bath. Transfer to cooled furnace, slowly raise temp. to 500°, and hold at this temp. 1 hr. Remove dish and cool. Repeat $HNO_3$ ashing, if necessary, to obtain clean, practically C-free ash. Add 10 mL 1*N* HCl and dissolve ash by heating cautiously on hot plate. Transfer to 25 mL vol. flask. Heat ash residue again successively with two 5 mL portions 1*N* HCl and add to flask. Cool, dil. to vol. with 1*N* HCl, and mix.

### E. Preparation of Standard Curve

Transfer 0, 1, 3, 5, 15, 25, and 50 mL Pb working soln, **972.23B(b)**(*2*), to sep. 50 mL vol. flasks and dil. to vol. with 1*N* HCl (0, 0.2, 0.6, 1.0, 3.0, 5.0, and 10.0 μg Pb/mL, resp.). Set spectrophtr to previously established optimum conditions for max. signal at 283.3 nm. Use air-$C_2H_2$ flow rates recommended by manufacturer for std conditions for Pb. For digital concn readout, calibrate in concn mode with solns contg 0.2 and 10.0 μg Pb/mL. Record concn directly after calibration of instrument. For strip chart readout, set amplification to give ≥1% absorption reading for 0.2 μg/ mL working soln and prep. std curve of *A* against μg Pb/mL.

### F. Determination

Use aliquot of sample soln, **972.23D**, and proceed as in (**a**) or (**b**). Treat reagent blank, **972.23C**, as sample and subtract reading from *A* of samples.
(**a**) *Clear solns.*—Det. *A* of sample and std solns as in **972.23E**, using following sequence 3 times: Read std soln first, then sample soln, alternating until all sample and std solns have been read. When many samples are to be analyzed, std solns may be read after series of 3 samples instead of after each.

$$\text{ppm Pb} = [(\mu g \text{ Pb/mL sample soln}) \times 25]/g \text{ sample}$$

(**b**) *Cloudy solns.*—Proceed as in (**a**), but add 1 mL buffer soln, (**c**), to aliquots of sample and std solns before reading.
If addnl dilns are necessary or if buffer is added:

$$\text{ppm Pb} = (\mu g \text{ Pb/mL dild sample}) \times (\text{mL dild sample/mL original aliquot}) \times (25/g \text{ sample})$$

Ref.: JAOAC **55,** 727, 733(1972); **56,** 406(1973).

CAS-7439-92-1 (lead)

## 972.24 Lead in Fish

### Polarographic Method

**Final Action 1974**

### A. Apparatus

*Polarograph.*—Any voltammetric or polarographic instrument with necessary accessories (cells, electrodes, Hg, capillaries, etc.) capable of effectively scanning up to 3.0 volts in either pos. or neg. direction, starting at selected initial potential, and of measuring ≥1.0 ppm Pb.

### B. Preparation of Standard Curve

Transfer 0, 1, 3, 5, 15, 25, and 50 mL Pb std working soln, **972.23B(b)**(*2*), to sep. 50 mL vol. flasks and dil. to vol. with 1*N* HCl (0, 0.2, 0.6, 1.0, 3.0, 5.0, and 10.0 μg Pb/mL, resp.). Transfer 5 mL soln to polarographic cell, adjust to 25 ± 1°, and bubble N thru soln 5 min. Polarograph between $-0.2$ and $-0.7$ v against Hg pool ref. electrode.

Peak potential for Pb at 25° is $-0.45 \pm 0.05$ v. Plot μg Pb/mL cell soln against wave ht × sensitivity factor.

### C. Determination

Transfer 5 mL sample soln to polarographic cell, adjust to 25 ± 1°, bubble N thru soln 5 min, and polarograph as in **972.24B**. Measure ht of wave whose potential corresponds to that of Pb and det. concn from newly prepd std curve or, preferably, by comparing wave ht of sample soln with that of std soln polarographed immediately before or after sample. Use latter method for greater accuracy, particularly when it is necessary to change instrument scale factor.

$$C_u\,(\mu g\ Pb/mL) = (C_s \times WH_u \times SF_u)/(WH_s \times SF_s)$$

where subscripts s and u refer to std and sample, resp.; $C$ = μg Pb/mL cell soln; $WH$ = wave ht; and $SF$ = sensitivity factor setting.

$$ppm\ Pb = (C_u \times 25)/g\ sample$$

### D. Interference from Tin

Sn polarographs at same peak potential as Pb. If presence of Sn is suspected, add 1 mL $NH_4OH$ and 0.4 g *tartaric acid* to cell soln, bubble N thru soln, and polarograph as in **972.24B**. Treat std in same manner. Peak potential for Pb is 0.54 v. Sn does not polarograph at this peak potential.

Ref.: JAOAC **55,** 727, 733(1972).

CAS-7439-92-1 (lead)

## 972.25 Lead in Food

**Atomic Absorption Spectrophotometric Method**
**First Action 1972**
**Final Action 1976**

### A. Principle

(*Caution:* See safety notes on AAS, wet oxidation, nitric acid, perchloric acid, and sulfuric acid.)

Org. matter is digested and Pb released co-ppts with $SrSO_4$. Sol. sulfate salts are decanted, and ppt is converted to carbonate salt, dissolved in acid, and detd by AAS at 217.0 or 283.3 nm.

### B. Apparatus

(**a**) *Atomic absorption spectrophotometer.*—Operated at 217 or 283.3 nm.)

(**b**) *Stirring motor.*—With eccentric coupling for stirring centrf. tubes (Sargent-Welch Scientific Co. Model S-76509-21C (Vortex, Jr.), or equiv.).

### C. Reagents

(Age all new glassware and all glassware which has contained high Pb concn in boiling $HNO_3$ before washing. *Never* let used glassware dry before washing, and *always* include final $HNO_3$ rinse followed by deionized $H_2O$ rinse.)

(**a**) *Strontium soln.*—2%. Dissolve 6 g $SrCl_2.6H_2O$ in 100 mL $H_2O$.

(**b**) *Ternary acid mixture.*—Add 20 mL $H_2SO_4$ to 100 mL $H_2O$, mix, add 100 mL $HNO_3$ and 40 mL 70% $HClO_4$, and mix.

(**c**) *Nitric acid.*—Add 128 mL redistd $HNO_3$ to 500–800 mL distd or deionized $H_2O$ and dil. to 2 L. Redistd $HNO_3$ (G. Frederick Smith Chemical Co., No. 63) may be dild and used without redistn.

(**d**) *Lead std solns.*—*(1) Stock soln.*—1000 μg/mL. Dissolve 1.5985 g $Pb(NO_3)_2$, recrystd as in **935.50B**, in ca 500 mL 1*N* $HNO_3$ in 1 L vol. flask and dil. to vol. with 1*N* $HNO_3$. *(2) Working solns.*—Prep. 100 μg Pb/mL by dilg 10 mL stock soln to 100 mL with 1*N* $HNO_3$. Dil. 1, 3, 5, 10, 15, and 25 mL aliquots of this soln to 100 mL with 1*N* $HNO_3$ (1, 3, 5, 10, 15, and 25 μg Pb/mL, resp.).

### D. Separation of Lead

Accurately weigh sample contg ≤10 g dry matter and ≥3 μg Pb. Place in 500 mL boiling or Kjeldahl flask and add 1 mL 2% Sr soln, (**a**), and several glass beads. Prep. reagent blank and carry thru same operations as sample. Add 15 mL ternary acid mixt., (**b**), for each g dry matter and let stand ≥2 hr. Heat under hood or $H_2O$ vac. manifold system until flask contains only $H_2SO_4$ and inorg. salts. (*Note:* Take care to avoid sample loss from foaming when heat is first applied, and when foaming occurs soon after sample chars. Remove heat and swirl flask before continuing digestion. Add $HNO_3$, if necessary.)

Cool digest few min. (Digest should be cool enough to add ca 15 mL $H_2O$ safely, but hot enough to boil when $H_2O$ is added.) Wash while still hot into 40–50 mL tapered-bottom centrf. tube and swirl. Let cool, centrf. 10 min at 350 × *g*, and decant liq. into waste beaker. (Film-like ppt on surface may be discarded.) Dislodge ppt by vigorously stirring with eccentric-coupled stirring motor. To complete transfer, add 20 mL $H_2O$ and 1 mL 1*N* $H_2SO_4$ to original flask and heat. *Do not omit this step* even though it appears transfer was complete in first wash. Wash hot contents of original digestion flask into centrf. tube contg ppt. Swirl to mix, cool, centrf., and decant liq. into waste beaker.

Dislodge ppt by stirring vigorously, add 25 mL *satd* $(NH_4)_2CO_3$ *soln* (ca 20%), and stir until all ppt is dispersed. Let stand 1 hr, centrf., and decant liq. into waste beaker. Repeat $(NH_4)_2CO_3$ treatment.

After decanting, invert centrf. tube on paper towel and drain all liq. Add 5 mL 1*N* $HNO_3$ (use larger vol. 1*N* $HNO_3$ in both sample and blank if >25 μg Pb is expected), stir vigorously to expel $CO_2$ or use ultrasonic bath 2–3 min, let stand 30 min, and centrf. if ppt remains. (Use same technic for all samples.)

### E. Determination

Set instrument to previously established optimum conditions, using air-$C_2H_2$ oxidizing flame and 217 or 283.3 nm resonant wavelength. Det. $A$ of sample and blank solns and ≥5 stds within optimum working range (10–80% $T$) before and after sample readings. Flush burner with 1*N* $HNO_3$ and check 0 point between readings. Det. Pb from std curve of $A$ against μg Pb/mL:

$$ppm\ Pb = [(\mu g\ Pb/mL) \times (mL\ 1N\ HNO_3)]/g\ sample$$

Ref.: JAOAC **55,** 737(1972).

CAS-7439-92-1 (lead)

## 934.07 Lead in Food

**General Dithizone Method**
**Final Action**

(Sn and Bi Absent)

(Applicable to such materials as carbohydrates, cereals and cereal products, cacao and dairy products, feeds, meats, fish, plant material, fruit and fruit products, fresh vegetables, etc., and in general to all org. materials (except fats) in which no Sn and Bi are encountered. For products contg Sn (canned foods) or Bi, proceed as in **934.07G–I**.)

### A. Reagents

(*Caution:* See safety notes on bromine.)

(**a**) *Lead std solns*.—(*1*) *Stock soln*.—2 mg Pb (3.197 mg $Pb(NO_3)_2$)/mL in 1% $HNO_3$. Prep. from $Pb(NO_3)_2$ purified as in **935.50B**. (*2*) *Working solns*.—Prep. as needed by dilg stock soln with 1% $HNO_3$.

(**b**) *Nitric acid*.—1%. Dil. 10 mL fresh, colorless $HNO_3$ (sp gr 1.40) to 1 L with redistd $H_2O$. If acid has been redistd, boil off nitrous fumes before dilg.

(**c**) *"Ash-aid" soln*.—Dissolve 40 g $Al(NO_3)_3.9H_2O$ and 20 g $Ca(NO_3)_2.4H_2O$ in 100 mL $H_2O$.

(**d**) *Citric acid soln*.—Concd Pb-free soln. 1 mL = 0.5 g citric acid (reagent partially neutzd with $NH_4OH$ during purification, **935.50B**, fifth par.).

(**e**) *Diphenylthiocarbazone* (*dithizone*).—Dissolve ca 1 g com. reagent in 50–75 mL $CHCl_3$ and filter if insol. material remains. Ext in separator with four 100 mL portions metal-free (redistd) $NH_4OH$ (1 + 99). (Dithizone passes into aq. phase to give orange soln.) Filter aq. exts into large separator thru cotton pledget inserted in stem of funnel. Acidify slightly with dil. HCl and ext pptd dithizone with two or three 20 mL portions $CHCl_3$. Combine exts in separator and wash 2 or 3 times with $H_2O$. Drain $CHCl_3$ into beaker and evap. with gentle heat on steam bath, avoiding spattering as soln goes to dryness. Remove last traces of moisture by heating 1 hr at ≤50° *in vacuo*. Store dry reagent in dark in tightly stoppered bottle. Prep. reagent solns for extn to contain 100, 50, and 10 mg/L in freshly redistd $CHCl_3$ (JAOAC **21,** 695(1938); **26,** 26(1943)) and store in dark at 5–10°. (Stock soln of dithizone in $CHCl_3$ contg 1 mg/mL will keep long time and is convenient for use in making dilns.) Soln of 30 mg/L $CHCl_3$ stored in dispensing app. is required for use in rapid method, **935.51B**.

(**f**) *Ammonia-cyanide mixture*.—To 100 mL 10% recrystd, $PO_4$-free KCN (JAOAC **20,** 191(1937)) in 500 mL vol. flask, add enough redistd $NH_4OH$ to introduce 19.1 g $NH_3$, and dil. to vol. with redistd $H_2O$. (Concn of redistd $NH_4OH$ can be detd by sp gr or titrn.)

(**g**) *Pure metallic tin*.—Purest obtainable, such as NIST Sample No. 42. Granulate Sn as finely as possible by melting and pouring very slowly into $H_2O$. Det. Pb content as follows: Dissolve 1–2 g sample in HBr or HCl and volatilize Sn by evapg soln to dryness and treating with several 5 mL portions of the HBr-Br mixt., (**h**), evapg to dryness on steam bath after each treatment. Take up with 2–3 mL $HNO_3$, evap. to dryness to expel Br, and take up with ca 50 mL hot $H_2O$. Filter, and proceed as in **934.07D** and **F**.

(**h**) *Hydrobromic acid-bromine mixture*.—To 250 mL 40% redistd HBr add 35 mL redistd liq. Br.

(**i**) *Sodium polysulfide soln*.—Dissolve 480 g $Na_2S.9H_2O$ and 40 g NaOH in $H_2O$, add 16 g powd S, shake until S dissolves, filter, and dil. to 1 L.

(**j**) *Hydrochloric-citric acid soln*.—Add vol. reagent (**d**) equiv. to 50 g citric acid to 50 mL HCl and dil. to 250 mL.

(**k**) *Sodium oleate soln*.—10%. To 45 mL 30% NaOH soln and 400 mL $H_2O$ in 1.5 L beaker, add slowly, while heating and stirring, 90 g (by difference from separator) oleic acid. Heat mixt. on steam bath until soap is entirely dissolved. (Small flocculent ppt of impurities may remain.) Cool, dil. to 1 L, mix, and filter.

(**l**) *Ammonia-cyanide-citrate soln*.—Dissolve 10 g phosphate-free KCN and 10 g citric acid in 250 mL $NH_4OH$ (sp gr 0.90) and dil. to 1 L. Reagent is conveniently preserved in dispensing app. that causes min. volatilization of $NH_3$.

(**m**) *Washed filter paper*.—Soak 9 cm quant. papers overnight in 1% $HNO_3$. Wash with large vols $H_2O$ on buchner to remove acid and any traces of Pb.

### B. Preparation of Sample (Ashing)

(*Caution:* See safety notes on hydrofluoric acid and perchloric acid.)

Accurately weigh representative sample of 5–200 g, depending upon amt sample available and expected Pb content, into suitable porcelain dish or casserole. Dry wet samples on steam bath or in oven. Add 2–5 mL "ash-aid" soln, (**c**), to products difficult to ash (meats), or to furnish ash bulk to low-ash products (candies, and jellies low in fruit content); mix well, and dry.

Char gelatin, carbohydrate foods such as jam, and other products that tend to swell excessively by carefully heating over burner. (Swelling can be controlled by playing small flame from glass jet over surface of material in dish, but metallic burner must not be used because of possible metallic contamination.) Do not let material ignite. Milk, candies, etc., may be charred without ignition by adding sample little at time to casserole heated over burner or hot plate. (Overhead radiant heater is often very convenient.) When samples are dry or charred, place in temp.-controlled furnace and raise temp. *slowly* to 500° without ignition.

If sample contains fat, "smoke" it away by heating long enough at ca 350°. Cover floor of furnace with piece of asbestos board or $SiO_2$ plate so that sample receives most of its heat by radiation from sides and roof and not by conduction from hotter floor of furnace.

If furnace has automatic control, ash overnight at ≤500°. If sample is not completely ashed next morning or if day-time ashings at 500° are not proceeding satisfactorily, remove casserole, cool, and moisten char with 2–5 mL ash-aid. Dry contents of casserole past danger of spattering (no free liq.) and replace in furnace. If ashing is not complete or proceeding rapidly after 30 min, remove casserole, cool, and cautiously add 2–3 mL $HNO_3$. Dry, place in furnace, and continue ashing until practically C-free. Avoid excessive use of ash-aid, and particularly $HNO_3$, if sample still contains much intermixed C, because local overheating or deflagration may result, especially if much K is present in ash.

When clean ash is obtained, cool, cover casserole with watch glass, and cautiously add 15–20 mL HCl. Rinse down watch glass with $H_2O$ and heat on steam bath. If *clear* soln is not obtained, evap. again to dryness and repeat addn of HCl. If insol. matter persists, evap. HCl and dehydrate $SiO_2$ by heating to fumes with 5–10 mL 60% $HClO_4$ (double distd preferred). If $HClO_4$ is used, considerable $H_2O$ (200 mL) may be necessary to completely dissolve $KClO_4$ later, as when KCN is used in dithizone extn of Pb, **934.07D**.

Dil. with $H_2O$ and filter soln when necessary with suction thru fine fritted glass filter. Catch filtrate in 500 mL g-s erlenmeyer under bell jar. Leach insol. material on filter successively with few mL hot HCl, hot HCl-citric acid soln, and hot 40% $NH_4OAc$ soln.

In certain instances take following special precautions:

(*1*) If amt of insol. material ($SiO_2$) remaining on filter is abnormal, flush it into Pt dish with $H_2O$, evap., and treat residue with one or two 5 mL portions HF. Evap. to dryness, take up residue with $H_2O$ and few drops of HCl or $HClO_4$, and add to bulk of ash filtrate.

(*2*) When ashing is of long duration, no ash-aid has been used, or natural ash is low with little ash bulk, Pb may be baked on dish. To remove this Pb, add few pellets (2–3 g) of NaOH and dissolve in few mL hot $H_2O$. Tilt dish so that sirupy soln completely wets that portion of interior originally occupied by sample; then heat short time on steam bath, but do not bring to dryness. (Overheating with concd NaOH may result in extg few μg Pb from casserole. Porcelain retains Pb to less extent than does $SiO_2$ but may contain very small amts of Pb.) Take up residue with $H_2O$ and add directly to filtrate. Finally rinse dish with few mL hot HCl followed by hot $H_2O$.

### C. Isolation of Lead: Principle

Method **934.07D**, while rapid and convenient, is limited to those materials that, with aid of citric acid, yield clear ammoniacal soln required for quant. extn of Pb with dithizone. Pb is readily occluded by many alk. ppts (Mg and Ca phosphates, Al and Fe hydroxides and silicates). Many food materials may be handled in this way because the naturally occurring amts of these substances are not excessive. However, some materials contain more of these substances than can be kept in soln under alk. conditions with any reasonable amt of citric acid (JAOAC **26,** 26(1943)). In these cases proceed as in **934.07C**. Difficulty of ammoniacal pptn may sometimes be overcome by limiting sample size in cases where sampling is no problem.

### D. Dithizone Extraction

(Applicable to most carbohydrates and cereal foods, fruit and fruit products, milk, fresh vegetables, plant materials, etc.)

Transfer ash soln to 300 mL short-stem separator and add citric acid reagent, (**d**), equiv. to 10 g citric acid. Make slightly alk. to litmus with $NH_4OH$, keeping soln cool, and let stand 1–2 min. If ppt forms, redissolve with HCl and isolate Pb as in **934.07C**. If no ppt forms, add 5 mL 10% KCN soln (more may be necessary if large amts of Zn, Cu, Cd, etc., are present), and check pH of soln by adding drop of *thymol blue soln* and observing color of drop (pH should be ≥8.5, blue-green to blue with thymol blue).

If ash was highly colored with Fe, keep pH of soln comparatively low, because pH of ≥10 in presence of Fe may cause oxidn of dithizone. Immediately ext with 20 mL portions dithizone reagent, using more dil. solns unless exceptionally large amts of Pb are present. Shake 20–30 sec, let layers sep., and note color of $CHCl_3$ phase. (Pb dithizone complex is red, but color may be masked by excess green dithizone, giving intermediate hues of purple and crimson. Color of $CHCl_3$ ext gives first indication of amt of Pb present, and progress of extn can be followed by noting color of successive exts.)

Drain exts directly into small separator contg 25 mL 1% $HNO_3$, (**b**). When extn is complete, shake combined exts in smaller separator and drain green dithizone layer into another separator contg addnl 25 mL portion 1% $HNO_3$. Shake, let layers sep., and discard $CHCl_3$ fraction. Filter acid exts contg Pb in succession thru small pledget of wet cotton inserted in stem of small funnel, into 50 mL flask or g-s graduate, using second acid ext to rinse separator in which first acid extn was made. (This procedure removes $CHCl_3$ globules.) Make up any slight deficiency in vol. with 1% $HNO_3$ and mix. Proceed as in **934.07F**.

### E. Sulfide Separation

(Applicable to all products and usually necessary in case of cacao products, tea, sardines, and all food products contg high proportion of alk. earth phosphates, especially those of Mg, which promote formation of ppts in ammoniacal citrate solns.)

Cool acid soln of ash, add citric acid soln, (**d**), equiv. to 10 g citric acid, and adjust to pH 3.0–3.4 (bromophenol blue) with $NH_4OH$. If enough Fe is present to color soln strongly, make final adjustment with help of spot plate. (Phosphates pptd by local action of $NH_4OH$ may usually be redissolved by shaking and cooling.) If amt of Pb is small, add 5–10 mg pure $CuSO_4.5H_2O$ to soln to act as coprecipitant. Ppt sulfides by passing in $H_2S$ until soln is satd (3–5 min). (*Caution:* See safety notes on hydrogen sulfide.) Immediately filter with suction into flask in bell jar (fine fritted glass filter is preferred).

Dissolve sulfides, without previous washing, with 5 mL hot $HNO_3$, drawing soln thru into original flask; wash with hot $H_2O$, stopper, shake, and boil to remove $H_2S$. Transfer to 200 mL separator, add citric acid soln equiv. to 5 g citric acid, make ammoniacal, ext, and det. Pb as in **934.07D** and **F(a)** or (**b**).

### F. Colorimetric Dithizone Determination

(Pb 0.001–0.200 mg)

Limiting factor in detn of minute amts of Pb by colorimetric dithizone method is size of reagent blank, particularly when amts of Pb of order of 1–5 μg are being detd. With special care in purification of reagents and by use of carefully cleaned Pyrex ware, including separators, it is possible to reduce reagent blank to ≤1 μg. Owing to Pb-bearing dust, vapors, etc., it is necessary to expose blank detn in furnace or on steam bath for same length of time as sample is exposed, and to use exactly same amts of reagents (even $H_2O$) for blank and actual detns.

Pb is extd from aq. soln, under std conditions of vol. and pH, with definite vol. of $CHCl_3$ soln of dithizone of std concn. Optimum pH of operation is 9.5–10.0. Dithizone strengths are so chosen that excess dithizone is always present in reaction mixt. Pb is brought into $CHCl_3$ phase in form of red complex, and uncombined green dithizone partitions between aq. and $CHCl_3$ phases and modifies color of ext according to relative amts of Pb and dithizone. Thus, series of colors from red to green may be arranged with intermediate crimsons, purples, and blues. Vols and strengths of $CHCl_3$ solns depend upon Pb range it is desired to cover and are so chosen as to give same general color progression from red to green for each range. Limiting range increases accuracy at expense of flexibility. Colors produced with std amts of Pb furnish basis for quant. estn by comparison. Vols and concns of std dithizone for various ranges are as follows when 1 cm cell is used:

| Pb Ranges, μg | Concn, mg/L | Volume, mL |
|---|---|---|
| 1–10 | 8 | 5 |
| 0–50 | 10 | 25 |
| 0–200 | 20 | 40 |

*See* Anal. Chem. **19,** 684(1947), for modification operated at pH 11.5.

(**a**) *Simple color matching.*—Prep. 10 stds covering in equal steps the desired concn range, as follows: Use std Pb soln, **934.07A(a)**, in 1% $HNO_3$, 1 mL of which equals some simple fraction or multiple of 1 μg Pb. Measure vols representing various steps of range into series of separators and add 1% $HNO_3$ so that total vol. is always 50 mL. (Add acid first so that Pb soln is not lost around stopcock of separator.) Add 10 mL $NH_3$-cyanide mixt., (**f**), and mix. Resultant pH will be ca 9.7. Immediately add appropriate vol. std dithizone soln, which depends on range to be covered (*see* table), and shake 1 min. Drain lower layers into series of tubes or vials and arrange in order. For lower ranges, i.e., <20 μg Pb, matching is best done by viewing longitudinally in small flat-bottom vials ca 75 mm long. For higher ranges, 20–50 μg and above, depth of column must be reduced, and matching is conveniently done by viewing transversely in Nessler tubes of matched diam., because even pure dithizone solns appear red by transmitted light if concn or depth of column is increased beyond certain point. If stds are kept covered when not in use, they should last ≥1 day.

For detn, place aliquot part, or entire amt, of the 50 mL 1% $HNO_3$ in which Pb has been isolated, **934.07D** or **E**, in separator, and if aliquot is taken, dil. to 50 mL with 1% $HNO_3$. Add 10 mL $NH_3$-cyanide mixt., (**f**), and mix. Immediately develop color by shaking 1 min with proper amt std dithizone soln. Drain lower layer into tube or vial similar to those used with stds and compare. If range is exceeded, repeat with smaller aliquot or re-ext with excess dithizone before draining from separator, isolate once more in 50 mL 1% $HNO_3$ reagent, and compare with stds covering higher range. Interpolation between steps of various ranges should be easily made.

If aliquot of the 50 mL 1% $HNO_3$ in which Pb has been isolated is taken, subtract only corresponding amt of total reagent blank from amt of Pb found.

(**b**) *Photometric methods.*—Absorption spectra of the 2 components in dithizone ext (Pb dithizone complex and free dithizone) show marked difference in ability to absorb 510 nm light, red Pb complex absorbing stongly and green dithizone transmitting freely. Thus, when absorption of light of this wavelength is detd photometrically, linear relationship is observed between amt of Pb and $A$. In making measurements, spectrophtr set at this wavelength or photometer equipped with blue-green filter centered at about this point can be used.

Stdze dithizone solns as follows: Using appropriate vols and concns of solns specified for various ranges (*see* above) in separators, prep. std colors as in visual color-matching procedure, satg std Pb and 1% $HNO_3$ solns with clear $CHCl_3$ before use, and thereby eliminating differences in vol. of ext between stds and unknowns. (It is unnecessary to prep. full 10 steps of the range, and number of stds may be limited to 5 or 6.) Develop colors by shaking separators 1 min, let stand few min, and filter exts thru specially prepd papers, (**m**). (Fitting 9 cm paper directly into mouth of 50 mL Pyrex beaker eliminates need of funnel in filtering operation.) Fill cell with filtered exts and det. $A$ for various steps of range.

Plot against amt of Pb to obtain std curve for particular lot of dithizone. Preferably calc. slope of line connecting std points and intercept of line on $A$ axis, making calcn by least squares method as in *Definitions of Terms and Explanatory Notes*.

Det. Pb content of unknown falling within the range of detg $A$, using std dithizone and same cell with which std readings were made, and calc. Pb from equation $X = (Y/b) - (a/b)$, using values of $a$ and $b$ detd previously. If protected from evapn and direct sunlight, std factors of dithizone solns should not change appreciably for ≥1 month (JAOAC **21**, 695(1938); **26**, 26(1943)).

For actual detn proceed as in (**a**), except to filter ext thru prepd papers before photometric measurement. Det. $A$, using stdzd dithizone with same cell used in making std curve, and read amt of Pb from this std curve or calc. from factor of dithizone soln. If range is exceeded, repeat with smaller aliquot, or re-ext and repeat with dithizone stdzd to cover higher range. If aliquot of the 50 mL 1% $HNO_3$ in which Pb has been isolated is taken, subtract only corresponding amt of total reagent blank from amt of Pb found.

Refs.: JAOAC **19**, 130(1936). Ind. Eng. Chem. Anal. Ed.**11**, 400(1939).

### G. Interferences

Interferences in colorimetric dithizone method are limited by use of KCN to $Sn^{+2}$, Bi, and Tl. Rarity of Tl makes its interference unlikely in ordinary work, and no method of removal is given (JAOAC **26**, 26(1943)). Dithizone itself is destroyed by strong oxidizing agents, such as free halogens and large amts of ferric Fe, under conditions of dithizone extn of Pb.

### H. Removal of Tin

(*Caution:* See safety notes on perchloric acid, bromine, and hydrogen sulfide.)

Sn becomes problem in analysis of canned foods; in amts >150 ppm it will usually appear in ash soln as milky suspension of $SnO_2$. It must be dissolved to facilitate filtration and to release occluded Pb. Quantities of Sn of this order may cause trouble by pptg under conditions of dithizone extn of Pb, **934.07D**.

Two methods for elimination of larger amts of Sn are given: (**a**) Volatilization as $SnBr_4$ from acid soln of ash, and (**b**) leaching mixed sulfides with warm Na polysulfide soln, when sulfide method of isolation, **934.07E**, has been applied. These methods may not eliminate Sn completely. Stannic Sn is not extd with dithizone, and as small amts of residual Sn will be in $Sn^{+4}$ form after application of either (**a**) or (**b**), final isolation of Pb by dithizone extn will eliminate Sn completely.

In general, amts <100 mg should not interfere in colorimetric dithizone methods of Pb detn provided Sn is in $Sn^{+4}$ form and preliminary isolation with dithizone is made; hence, this method of isolation should be applied wherever possible.

(**a**) *Volatilization as $SnBr_4$ from acid soln of ash.*— After almost C-free ash is obtained, **934.07B**, add 15–20 mL 40% redistd HBr. If nitrates were used as ash aids, cover casserole with watch glass and heat on steam bath until Br evolution diminishes; then rinse watch glass with $H_2O$ and bring to boil to complete expulsion of Br. (This process destroys undecomposed nitrates.) Add more HBr if necessary to dissolve ash, and examine solns for clearness. If there is insol. residue of $SnO_2$, add 50–100 mg pure Sn, (**g**), to simmering HBr soln of ash and let it dissolve. (Metallic Sn is best agent to bring ignited $SnO_2$ into soln. To be effective, ash soln must be in reduced state. $Fe_2O_3$ sometimes becomes "noble" during ashing and dissolves with difficulty, but treatment with metallic Sn also brings it into soln. Treatment with Sn is necessary only with contents of badly corroded cans.)

When soln of ash is free from milkiness due to $SnO_2$, add 20 mL 60% $HClO_4$ (double distd preferred), oxidize mixt. with few mL HBr-Br mixt., (**h**), and then add addnl 15 mL of the reagent portionwise, while soln is evapd to incipient fumes of $HClO_4$ (ca 150°) on hot plate. Repeat with addnl 10 mL portion HBr-Br mixt. if >100 mg Sn was used to dissolve ash. (Hot $HClO_4$ helps keep ash salts in soln and with Br holds Sn as volatile $SnBr_4$.) When HBr and Br are completely volatilized, cool, and take up with hot $H_2O$ (200 mL may be necessary if much $KClO_4$ is present). Filter off any small amts of dehydrated $SiO_2$, ext residue twice with 5 mL hot HCl-citric acid reagent, (**j**), and hot $H_2O$, treat dish if necessary with NaOH as in **934.07B**(*2*), and isolate Pb by dithizone extn as in **934.07D**, or by sulfide sepn, **934.07E**, finally detg Pb as in **934.07F**(**a**) or (**b**).

(**b**) *With sodium polysulfide.*—(Recommended for routine work on canned foods.)

Isolate Pb by sulfide pptn, **934.07E**, filter, and wash flask and filter with 3–6 portions of ca 5 mL each of warm Na polysulfide soln, (**i**). (Sn, As, and Sb sulfides are dissolved; CuS may be partially dissolved and repptd in filtrate.) Wash flask and residual sulfides several times with 3% $Na_2SO_4$ soln adjusted to pH 3.0–3.4 and satd with $H_2S$, and proceed as in **934.07E**, beginning "Dissolve sulfides, without previous washing, . . ." When ash contains much Sn, as when metallic

Sn has been added to dissolve insol. metallic oxides, sulfide ppt will be so bulky as to be difficult to handle, and it will be necessary to use volatilization method (**a**) before sulfiding. For colorimetric dithizone detn of Pb, ext $HNO_3$ soln of dissolved sulfides and proceed as in **934.07E** and **F**(**a**) or (**b**).

#### *I. Detection and Removal of Bismuth*

(*Caution:* See safety notes on nitric acid, bromine, cyanides, and arsenic trioxide.)

(**a**) *By dithizone at pH 2.0 after preliminary dithizone extraction at pH 8–11.*—(This method completely removes small amts of Bi.) (Ind. Eng. Chem. Anal. Ed. **7,** 285(1935)).

Ext metals from $CHCl_3$ dithizone ext with 50 mL 1% $HNO_3$ as in **934.07D**. Adjust acid ext to pH 2.0 (metacresol purple indicator) with 5% $NH_4OH$ soln and shake vigorously ca 1 min with 10 mL $CHCl_3$ soln of dithizone (200–250 mg/L). Let layers sep., and if $CHCl_3$ ext is orange red to red (Bi), drain off and ext with addnl 10 mL dithizone soln. If shades of green or purple are visible, indicating excess dithizone, drain $CHCl_3$ ext and ext aq. phase once more with 5 mL dithizone soln (shaking should be prolonged (3–5 min) to ensure complete extn of Bi). Continue extns until dithizone ext remains pure green. Adjust aq. soln to pH 8.5 with $NH_4OH$, add KCN, and ext with dithizone as in **934.07D**. Det. Pb colorimetrically as in **934.07F**(**a**) or (**b**).

(Method of Bambach and Burkey (Ind. Eng. Chem., Anal. Ed. **14,** 904(1942)) seps small amts of Bi from Pb by shaking out $CHCl_3$ soln of their mixed dithizonates with aq. soln buffered at pH 3.4; Bi remains as dithizonate in $CHCl_3$ phase, while Pb enters aq. phase and can be sepd Bi-free. Only *slight* excess of free dithizone should be present in $CHCl_3$ mixt. of dithizonates, otherwise Pb does not strip out completely. System of photometric detection and evaluation of Bi interference has also been outlined (JAOAC **26,** 26(1943)).

(**b**) *From acid soln of sulfides.*—(Intended for small amts of Bi, particularly when sulfide sepns may be necessary.) Dissolve mixed sulfides, **934.07E**, with hot $HNO_3$ and sep. Bi and Pb as in (**a**).

*Special conditions.*—(Intended for products contg large amts of Bi.) Dissolve inorg. Bi compds directly in HBr-Br, (**h**). Prep. org. Bi compds or Bi prepns mixed with org. matter contg little ash, as in **934.07B**, and dissolve residue in HBr-Br. If sample contains org. matter with appreciable ash material other than Bi compds, proceed as in **934.07B** or **K**, apply sulfide sepn, **934.07E**, and dissolve mixed sulfides in $HNO_3$. Evap. $HNO_3$ soln of sulfides to dryness in porcelain dish and treat with small portions HBr-Br mixt. Evap. contents of dish contg Bi dissolved in HBr-Br, after any of above methods of prepn, on steam bath to volatilize Sn and to convert other metals to bromides. Evap. to dryness, place in furnace with temp. control, and raise temp. gradually to 300°. ($AsBr_3$ and $SbBr_3$ volatilize first at 100° or above; $BiBr_3$ volatilizes as dense orange fumes at 300°.) After 5 min, or when fumes cease, remove dish, cool and treat again with small portions HBr-Br. Again evap. to dryness and heat addnl 5 min at 300–325° ($PbBr_2$ does not volatilize appreciably at <350°). Remove dish, cool, and dissolve residue in hot $HNO_3$. Proceed with removal of last traces of Bi at pH 2.0 and det. Pb as in (**a**).

### *Special Methods of Sample Preparation*

#### *J. Solution in Acids*

(Applicable to chemicals sol. in $H_2O$ or acid, e.g., phosphates, sulfates, etc., and org. products of type of tartrates and citrates.)

Dissolve 5–100 g sample, according to its nature and amt of Pb expected, in HCl in 400 mL beaker. With Ca phosphates, use 10–50 g. Dissolve in smallest practicable vol. of soln by warming and adding alternately small amts of hot $H_2O$ and HCl. Filter soln with suction (fritted glass preferred) into beaker or flask under bell jar and leach any residue with 10–25 mL hot HCl-citric acid, (**j**), followed by 10–25 mL hot 40% $NH_4OAc$ soln. Rinse beaker and filter with hot $H_2O$ and cool soln.

Proceed as in **934.07D**. If interfering ppt forms, again acidify and isolate Pb by sulfide pptn, **934.07E**. If it is difficult to obtain clear soln with Ca phosphates at pH 3.0–3.4 (sulfide ppt may be contaminated with excessive phosphates), redissolve ppt, add more citric acid soln, (**d**), readjust pH, and reppt sulfides; or make one sulfide pptn, dissolve sulfides in hot $HNO_3$, boil off $H_2S$, and ext Pb with dithizone, **934.07D**. Sometimes difficulty due to ppt formation in **934.07D** can be avoided by using smaller sample for extn and colorimetric detn. If Sn or Bi is suspected, remove by methods described in **934.07H** and **I**. Finally det. isolated Pb colorimetrically, **934.07F**.

#### *K. Complete Digestion*

(Applicable to most food or biological products; with difficulty to fats and oils, oily products, etc. *Caution:* See safety notes on distillation.)

Digest representative sample in Kjeldahl flask as in **963.21C**. Distil As, if desired, as $AsCl_3$, **963.21D**. If As is not to be distd, add 100 mL $H_2O$ and enough HCl to flask to dissolve any $CaSO_4$ in residue. Filter on fritted glass filter, pulverizing any insol. residue (anhyd. $SiO_2$ or $BaSO_4$) with flat-end stirring rod. Dissolve any $PbSO_4$ in flask and leach residue on filter with 10–20 mL hot HCl-citric acid soln, (**j**), followed by 10–20 mL hot 40% $NH_4OAc$. Finally rinse both flask and filter with hot $H_2O$. Isolate Pb by dithizone, **934.07D**, or sulfide pptn, **934.07E**, methods. (In general, sulfide method is preferable, especially when $BaSO_4$ or excessive $CaSO_4$ is present, as insol. sulfates readily occlude Pb.) If Bi and Sn are present, remove them as in **934.07H** or **I**. After isolation, det. Pb by colorimetric method, **934.07F**.

Refs.: JAOAC **17,** 108(1934); **18,** 315(1935); **19,** 130(1936).

CAS-7439-92-1 (lead)

## 930.34 Manganese in Food
**Final Action**

*See* **921.02** or **931.09★**.

## 971.21 Mercury in Food
**Flameless Atomic Absorption Spectrophotometric Method**
**First Action 1971**
**Final Action 1976**

(Rinse all glassware before use with $HNO_3$ (1 + 9). *Caution:* See safety notes on wet oxidation, nitric acid, perchloric acid, sulfuric acid, and mercury salts.)

#### *A. Apparatus*

(**a**) *Atomic absorption spectrophotometer.*—Instrumentation Laboratory, Inc., 113 Hartwell Ave, Lexington, MA 02173, Model 153 (or successors), or equiv. Equipped with Hg hollow cathode lamp and gas flow-thru cell (Fig. **971.21**), 25 (id)

× 115 mm with quartz windows cemented in place. *Operating conditions:* Wavelength 253.7 nm, slit width 160 μm, lamp current 3 ma, and sensitivity scale 2.5.

(**b**) *Diaphragm pump.*—Neptune Dyna-Pump, or equiv. Coat diaphragm and internal parts of pump with acrylic-type plastic spray. Use 16 gage Teflon tubing for all connections.

(**c**) *Water condenser.*—12–18 (id) × 400 mm borosilicate, 24/40 ₮ joint, modified to hold 6 mm Raschig rings. Fill condenser with Raschig rings to ht of 100 mm; then place 20 mm layer of 4 mm diam. glass beads on top of rings.

(**d**) *Gas inlet adapter.*—24/40 ₮ (Kontes Glass Co. No. K-181000).

(**e**) *Digestion flask.*—250 mL flat-bottom boiling flask with 24/40 ₮ joint.

### *B. Reagents*

(**a**) *Reducing soln.*—Mix 50 mL $H_2SO_4$ with ca 300 mL $H_2O$. Cool to room temp. and dissolve 15 g NaCl, 15 g hydroxylamine sulfate, and 25 g $SnCl_2$ in soln. Dil. to 500 mL.

(**b**) *Diluting soln.*—To 1 L vol. flask contg 300–500 mL $H_2O$, add 58 mL $HNO_3$ and 67 mL $H_2SO_4$. Dil. to vol. with $H_2O$.

(**c**) *Magnesium perchlorate.*—Drying agent placed in filter flask (Fig. **971.21**). Replace as needed. (*Caution:* $Mg(ClO_4)_2$ is explosive when in contact with org. substances.)

(**d**) *Mercury std solns.*—(*1*) *Stock soln.*—1000 μg/mL. Dissolve 0.1354 g $HgCl_2$ in 100.0 mL $H_2O$. (*2*) *Working soln.*—1 μg/mL. Dil. 1 mL stock soln to 1 L with 1*N* $H_2SO_4$. Prep. fresh daily.

### *C. Determination*

Weigh 5.0 g sample into digestion flask; add 25 mL 18*N* $H_2SO_4$, 20 mL 7*N* $HNO_3$, 1 mL *2% Na molybdate soln*, and 5–6 boiling chips. Connect condenser (with $H_2O$ circulating thru it) and apply gentle heat ca 1 hr. Remove heat and let stand 15 min. Add 20 mL $HNO_3$-$HClO_4$ (1 + 1) thru condenser. Turn off $H_2O$ circulating thru condenser and boil vigorously until white fumes appear in flask. Continue heating 10 min.

Cool. Cautiously add 10 mL $H_2O$ thru condenser while swirling liq. in flask. Again boil soln 10 min. Remove heat and wash condenser with three 15 mL portions $H_2O$.

Cool soln to room temp. Completely transfer digested sample with $H_2O$ to 100 mL vol. flask and dil. to vol. with $H_2O$. Transfer 25.0 mL aliquot from each sample to another digestion flask. Adjust vol. to ca 100 mL with dilg soln, (**b**).

Adjust output of pump to ca 2 L air/min by regulating speed of pump with variable transformer. Connect app. as in Fig. **971.21**, except for gas inlet adapter. With pump working and spectrophtr zeroed, add 20 mL reducing soln to dild aliquot. Immediately connect gas inlet adapter and aerate ca 3 min. (Adjust aeration time to obtain max. *A*.) Record *A*, disconnect pressure on "out" side of pump, and open vent on filter flask to flush system.

Prep. reagent blank and std curve by adding 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μg Hg to series of digestion flasks. To each flask add 100 mL dilg soln. Finally, add reducing soln and aerate stds as for sample.

Plot std curve from least squares linear regression of *A* against μg Hg. (*See* "Definitions of Terms and Explanatory Notes," or use calculator which performs linear regression.) Det. μg Hg in aliquot from curve. If μg Hg detd falls outside range of calibration, repeat detn with smaller aliquot of sample soln to bring μg Hg into region of std curve. From size of aliquot used, det. total μg Hg in original sample.

$$\text{ppm Hg} = \mu\text{g Hg/g sample}$$

Ref.: JAOAC **54,** 202(1971).

CAS-7439-97-6 (mercury)

## 977.15 Mercury in Fish

### Alternative Flameless Atomic Absorption Spectrophotometric Method

**First Action 1977**
**Final Action 1978**

(Rinse all glassware before use with $HNO_3$ (1 + 9). *Caution:* See safety notes on wet oxidation, nitric acid, sulfuric acid, and mercury salts.)

### *A. Apparatus*

*See* **971.21A(a)**, (**b**), (**e**), and in addn:

(**a**) *Boiling stones.*—6–8 mesh (Lurex Scientific, No. D-7325).

(**b**) *Gas inlet adapter.*—₮ 24/40 (Kontes Glass Co., No. K-181000). Cut off end of glass tube which extends downward from adapter and affix gas dispersion tube with fritted cylinder (Corning Glass Works, No. 39533, porosity 12C).

(**c**) *Trap.*—Construct from cut off bulb of 15 mL pipet and

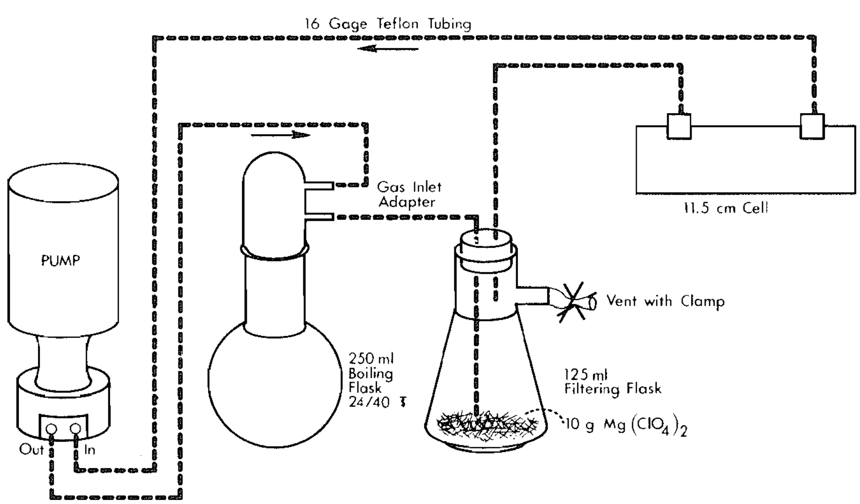


**FIG. 971.21—Apparatus for flameless atomic absorption analysis**

place between digestion flask and cell, replacing flask of Fig. **986.15B**, to trap overflow.

(**d**) *Water condenser*.—12–18 (id) × 300 mm Liebig condenser with ₮ 24/40 joint. Modify by making indentations in glass between lower std taper and $H_2O$ jacket with pointed C rod. Indent glass to hold 6 mm Raschig rings. Add 8–10 rings to condenser and cover with $^1/_8''$ (3.17 mm) id glass helices (Lurex Scientific, No. 181-2012) to ht of 90 mm.

### *B. Determination*

Weigh 5.0 g (wet wt) thoroly mixed fish sample into digestion flask, **971.21A(e)**. Rinse neck of flask with <5 mL $H_2O$, if necessary. Add ca 20 boiling stones, (**a**), 10–20 mg $V_2O_5$, and 20 mL $H_2SO_4$-$HNO_3$ (1 + 1). Quickly connect flask to condenser, (**d**), and swirl to mix. Circulate cold $H_2O$ thru condenser during digestion. Apply sufficient heat (luminous flame is suitable) to produce low initial boil (ca 6 min) and finish digestion with strong boil (ca 10 min). Swirl flask intermittently during digestion. No solid material should be apparent except for globules of fat after ca 4 min.

Remove flask from heat and wash condenser with 15 mL $H_2O$. Add 2 drops 30% $H_2O_2$ thru condenser and wash into flask with 15 mL $H_2O$. Cool digested fish soln to room temp. by placing flask, still connected to condenser, in beaker of $H_2O$. Disconnect flask, rinse ground joint with $H_2O$, and quant. transfer digest to 100 mL vol. flask. Ignore solidified fat; it does not interfere. Carefully rinse digestion flask with several portions $H_2O$ and dil. to vol. with rinse $H_2O$.

Pipet 25 mL soln into original digestion flask and add ca 75 mL dilg soln, **971.21B(b)**. Proceed as in **971.21C**, beginning "Adjust output of pump . . .", except aerate ca 1 min. 1 μg std should give $A \geq 0.400$.

Ref.: JAOAC **60,** 833(1977).

CAS-7439-97-6 (mercury)

## 974.14 Mercury in Fish
### Alternative Digestion Method
### First Action 1974
### Final Action 1976

(*Caution:* Do not change sample wt or acid vol. stated; otherwise excessive pressure during heating may result in explosion.)

### *A. Apparatus*

*Digestion vessel.—See* Fig. **974.14**. Stainless steel body supporting Teflon crucible and screw-on cap with Teflon liner to provide Teflon sealing surface. Teflon spout is snapped on outside rim to permit quant. transfer of contents without contact with metal parts. (Available from Uni-Seal Decomposition Vessels, PO Box 9463, Haifa, Israel.)

### *B. Digestion*

Accurately weigh 1 ± 0.1 g sample (*Caution:* Do not use >300 mg dry wt; for materials with high fat content do not use >200 mg dry wt.) into digestion vessel, add 5.0 mL $HNO_3$, and close vessel by tightening screw cap. Place vessel, without tilting, into preheated 150° oven 30–60 min or until clear. Remove vessel and let cool to room temp. Unscrew cap, snap on spout, and transfer with aid of 95 mL dilg soln, **971.21B(b)**, to 250 mL flask, **971.21A(e)**. Proceed as in **971.21C**, beginning "Adjust output of pump . . ."

Refs.: JAOAC **55,** 741(1972); **57,** 568(1974). Anal. Chem. **40,** 1682(1968).

CAS-7439-97-6 (mercury)

## 952.14 Mercury in Food
### Colorimetric Dithizone Method
### Final Action

### *A. Principle*

Sample is digested with $HNO_3$ and $H_2SO_4$ under reflux in special app., Hg is isolated by dithizone extn, Cu is removed, and Hg is estd by photometric measurement of Hg dithizonate.

### *B. Precautions*

Critical step is digestion of sample, which must be almost complete, otherwise residual org. matter may combine with Hg and prevent or hinder extn with dithizone. Oxidizing material in digest must also be destroyed or dithizone reagent is decomposed and Hg is not quant. extd. Because of volatility of Hg compds, careful heating of digest during sample prepn is required. Acidity of final sample soln (after partial neutzn with $NH_4OH$) before extn should be ca 1*N* and not >1.2*N*. Do not use silicone grease in stopcocks.

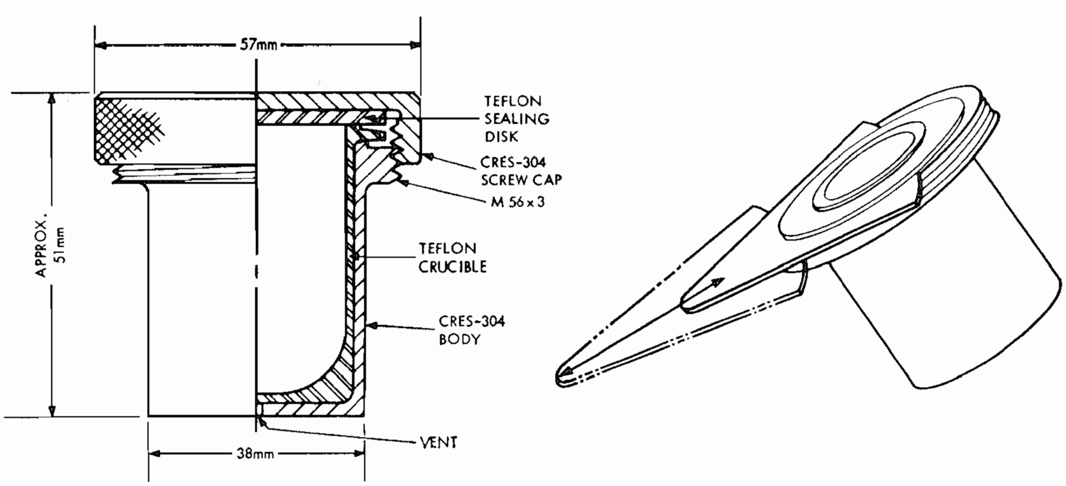


**FIG. 974.14—Digestion vessel**

### C. Apparatus

(As Hg compds tend to adsorb on glassware, app. and particularly separators should be rinsed with dil. $HNO_3$ and then with $H_2O$.)

*Special digestion apparatus.—See* Fig. **952.14**. App. is made from Pyrex with ₮ joints thruout. Unit *A* is modified Soxhlet extractor, 5 cm od, 200 mL capacity to overflow, without inner siphon tube but equipped with stopcock on tube leading to digestion flask, *D*. With stopcock open, app. is in reflux position; when closed, unit serves as trap for condensed $H_2O$ and acids. Top of *A* is attached to Friedrichs condenser, 35 cm long. Bottom of *A* is attached thru center neck of 2 neck ₮ 24/40 r-b 500 mL flask, *D*. Necks are 3 cm apart to provide clearance. Second neck is used for attaching 75 mL dropping funnel, *B*.

### D. Reagents

(**a**) *Mercury std solns.—(1) Stock soln.*—1 mg/mL. Prep. from dry, recrystd $HgCl_2$ (67.7 mg/50 mL). (2) *Working soln.*—2 μg/mL is convenient. Prep. from stock soln and store in Pyrex bottles. Add HCl in proportion of 8 mL/L to all stds before dilg to final vol.

(**b**) *Chloroform.—See* **945.58B(b)**.

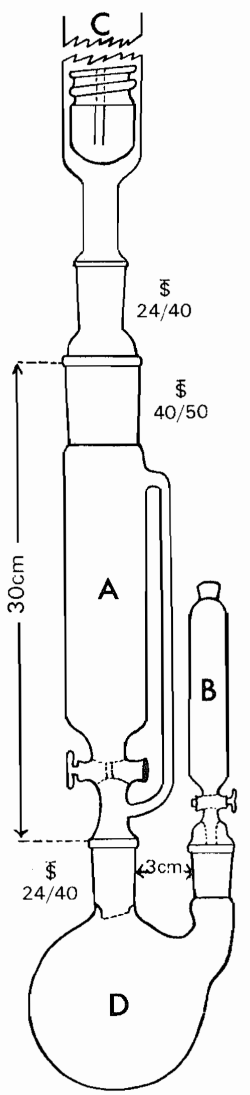


**FIG. 952.14—Special digestion apparatus for mercury residues**

(**c**) *Dithizone soln.—See* **934.07A(e)**. Reagent as now distributed needs no purification for this method. Prep. stock soln in redistd $CHCl_3$ (100 mg/L is convenient) and store in refrigerator. Prep. dilns as needed.

(**d**) *Sodium thiosulfate soln.*—1.5%. Prep. daily.

(**e**) *Sodium hypochlorite soln.*—Preferably 5% available Cl reagent. As distributed, reagent varies in available Cl content. Det. strength by **935.07B**. Store in refrigerator when not in use and det. titer monthly. (Certain prepns of hypochlorite intended for household use contain traces of Hg. If these prepns are used, det. blank. Reagent with >0.1 μg Hg/mL should not be used.)

(**f**) *Dilute acetic acid.*—30% by vol.

(**g**) *Hydroxylamine hydrochloride soln.*—20% w/v. Ext with dil. dithizone until $CHCl_3$ layer remains green, remove excess dithizone with $CHCl_3$, and filter.

### E. Preparation of Sample

(Conduct acid digestion in hood.)

In all detns use wt sample equiv. to ≤10 g dry wt.

(**a**) *Fresh fruits or vegetables and beverages.*—Place weighed sample in digestion flask with 6 glass beads, connect assembly, and add, thru dropping funnel, 20 mL $HNO_3$. Pass rapid stream of $H_2O$ thru condenser, adjust stopcock of Soxhlet unit to reflux position, and apply small flame to flask. Use asbestos board with 2–5 cm diam. hole between flask and flame. (Original reaction must not proceed violently or evolved $NO_2$ will carry vapors of digest mech. thru condenser and cause loss of Hg.) After initial reaction is complete, apply heat so that digest just refluxes. If mixt. darkens, add $HNO_3$ dropwise thru funnel as needed. Continue refluxing 0.5 hr, or until digest does not change consistency, and cool.

Slowly add 20 mL cold $HNO_3$-$H_2SO_4$ mixt. (1 + 1). (Use 10 mL acid mixt. for ≤5 g (dry wt) of sample.) Heat with small flame, subsequently adding $HNO_3$ dropwise as needed to dispel darkening of digest. Continue heating until fibrous material (fruit skin, cellulose, etc.) is apparently digested. Turn stopcock of Soxhlet unit to trap $H_2O$ and acids, and continue heating. Let digest become dark brown (not black) before adding further increments of $HNO_3$. (Fats and waxes cannot be totally digested by the hot acids under reflux. Therefore no attempt should be made to effect complete digestion in this step.) When all except fat and wax is in soln, let digest cool, and cautiously drain $H_2O$ and acids into main digest. Cool, and pour two 25 mL portions $H_2O$ thru condenser and intermediate unit. Remove reaction flask, chill under cold $H_2O$ or by surrounding with ice to solidify fats and waxes, and filter off insol. matter on small pledget of glass wool. Rinse reaction flask and filter pad successively with two 10 mL portions $H_2O$. Remove Soxhlet unit, and wash it and flask with hot $H_2O$ to remove insol. material. Pour hot $H_2O$ thru condenser to remove volatile fats and oils. Discard all washings.

Connect flask contg filtered sample soln to assembled app., heat, and collect $H_2O$ and acids in trap. Complete digestion, using small addns of $HNO_3$ as needed. In final stage of digestion, adjust flame until digest reaches incipient boiling (soln simmers) and acid vapors do not rise beyond lower half of condenser. Continue heating 15 min after last addn of $HNO_3$. Digest should now be colorless or pale yellow. Let digest cool, drain trapped liqs carefully into reaction flask, and add two 50 mL portions $H_2O$ thru condenser. Reflux soln until all $NO_2$ is expelled from app. Add 5 mL *40% w/v urea soln* and reflux 15 min. (Digest should be colorless or pale yellow.)

(**b**) *Dried fruit, cereal, seeds, and grains.*—Dil. sample with 50 mL $H_2O$ before adding $HNO_3$, and proceed with sample prepn as in (**a**).

(**c**) *Meats, fish, and biological material.*—Because of high fat and protein content of these materials, conduct initial digestion carefully to avoid foaming of digest into condenser. Add 20 mL $HNO_3$ to sample, swirl flask, and let stand 0.5 hr in digestion assembly before heating. Add 25 mL $H_2O$ and heat cautiously with small rotating flame until initial vigorous reaction is over and foaming ceases. Proceed as in (**a**).

### F. Isolation of Mercury

Titr. 1 mL prepd sample soln, **952.14E**, with std alkali. Add calcd amt of concd $NH_4OH$ to reduce acidity to 1.0*N*: swirl flask during addn of the $NH_4OH$ to avoid local excess. (Soln should never be ammoniacal to avoid formation of Hg complexes.)

Transfer sample soln to 500 mL separator. Add 10 mL 4 mg/L dithizone and shake vigorously 1 min. (If characteristic green of dithizone is visible in $CHCl_3$ layer, indicating excess of dithizone, amt of Hg is within 0–5 μg.) Let layers sep., and drain $CHCl_3$ layer quickly to second separator contg 25 mL 0.1*N* HCl and 5 mL $H_2NOH.HCl$ soln. (Small amt of oxidizing material may still be present. On long contact with dithizone soln, oxidizing substances may destroy dithizone reagent and prevent extn of Hg.)

Repeat extn of sample soln with two 5 mL portions dithizone soln, transferring $CHCl_3$ layer successively to second separator. If first extn indicates >5 μg Hg, add stronger concns of dithizone, as indicated by table, **952.14H**, until, after 1 min vigorous shaking, $CHCl_3$ layer contains dithizone in marked excess. Drain $CHCl_3$ layer into second separator contg 0.1*N* HCl and again ext sample soln with two 10 mL portions 4 mg/L dithizone soln, draining each successive ext into second separator.

Shake contents of second separator vigorously 1 min, and drain $CHCl_3$ layer into third separator contg 50 mL 0.1*N* HCl. (Shaking dithizone ext with dil. acid in second separator removes entrained org. matter. With biological materials or those of high protein content, aq. layer is usually light yellow because of nitrated org. compds. Small amts are carried into third separator where they are destroyed by Cl.) Ext soln in second separator with 1–2 mL $CHCl_3$ and transfer org. layer to third separator.

To contents of third separator add 2 mL $Na_2S_2O_3$ soln, shake vigorously 1 min, let layers sep., drain off $CHCl_3$ as completely as possible, and discard. (Cu if present is removed as dithizonate.) Ext again with 1–2 mL $CHCl_3$, drain carefully, and discard. Add 3.5 mL NaOCl reagent (or enough soln of different titer to furnish 175 mg available Cl) to decompose Hg thiosulfate complex and to oxidize excess thiosulfate, and shake vigorously 1 min. Add 5 mL $H_2NOH.HCl$ reagent from pipet, taking care to wet both stopper and neck of separator. Shake vigorously 1 min. Hold mouth of separator in front of air vent and blow out any remaining gaseous Cl. Stopper separator and shake vigorously 1 min. (It is imperative that all hypochlorite be reduced. Trace amts remaining would oxidize dithizone, subsequently added, to yellow oxidized form which would be measured in photometer as Hg.) Ext soln with 2–3 mL $CHCl_3$, drain off org. layer carefully, and discard. Final aq. soln should now be colorless.

### G. Determination

To third separator add 3 mL 30% HOAc and appropriate vol. and concn of dithizone soln as indicated by table, **952.14H**, and proceed with colorimetric detn of Hg as in **952.14H**, converting *A*, measured at 490 nm, to μg Hg from working curve.

### H. Preparation of Standard Curve

Following table is useful in prepg std curve and for establishing approx. Hg range in sample soln when 1 cm cells are used:

| Hg Range, μg | Dithizone Concn, mg/L | Volume Dithizone, mL |
|---|---|---|
| 0–10 | 6 | 5 |
| 0–50 | 10 | 25 |
| 0–100 | 10 | 40 |

Prep. working curve of required range, starting with blank and extending to final std of range, with 4 intermediate increments. Add appropriate amts of Hg to 50 mL 0.1*N* HCl in separator. Add 5 mL $H_2NOH.HCl$ reagent and 5 mL $CHCl_3$, and shake vigorously 1 min. Let layers sep., drain off $CHCl_3$, and discard, being careful to remove as completely as possible all droplets of $CHCl_3$. Add 3 mL 30% HOAc and appropriate vol. dithizone soln, shake vigorously 1 min, and let layers sep. (HOAc aids in stabilizing mercuric dithizonate.) Insert cotton pledget into stem of separator and collect dithizone ext (discarding first mL) in test tube for transfer to appropriate cell. Make photometer readings at 490 nm. (Since both dil. dithizone and mercuric dithizonate are somewhat unstable, read immediately.) Plot *A* against μg Hg.

Ref.: JAOAC **35**, 537(1952).

CAS-7439-97-6 (mercury)

## 983.20 Mercury (Methyl) in Fish and Shellfish

### Gas Chromatographic Method

**First Action 1983**
**Final Action 1988**

(*Caution:* See safety notes on benzene and acetone.)

### A. Principle

Org. interferences are removed from homogenized sample by acetone wash followed by benzene wash. Protein-bound Me Hg is released by addn of HCl and extd into benzene. Benzene ext is concd and analyzed for $CH_3HgCl$ by GC.

### B. Reagents

(**a**) *Solvents.*—Acetone, benzene, and isopropanol are all distd in glass (Burdick & Jackson Laboratories, Inc.; EM Science OmniSolv reagents). *Note:* Benzene is a possible carcinogen.

(**b**) *Hydrochloric acid soln (1 + 1).*—Add concd HCl to equal vol. of distd or deionized $H_2O$ and mix. Ext HCl soln 5 times with $^1/_4$ its vol. of benzene by shaking vigorously 15 sec in separator. Discard benzene exts. Soln may be mixed in advance but must be extd immediately before use.

(**c**) *Carrier gas.*—GC quality Ar-$CH_4$ (95 + 5).

(**d**) *Sodium sulfate.*—Heat overnight in 600° furnace, cool, and store in capped brown bottle. Line cap with Al foil to prevent contamination from cap.

(**e**) *Methyl mercuric chloride std solns.*—Keep tightly stoppered. (*1*) *Stock std soln.*—1000 μg Hg/mL. Weigh 0.1252 g $CH_3HgCl$ into 100 mL vol. flask. Dil. to vol. with benzene. (*2*) *High intermediate std soln.*—40 μg Hg/mL. Dil. 10.0 mL stock soln to 250.0 mL with benzene. (*3*) *Low intermediate std soln.*—2.0 μg Hg/mL. Dil. 10.0 mL high intermediate std soln to 200.0 mL with benzene. (*4*) *Working std solns.*—0.010–0.30 μg Hg/mL. Prep. monthly by dilg with benzene in vol. flasks as follows: Dil. 15 mL of 2.0 μg Hg/mL std to 100.0 mL, 10.0 mL to 100.0 mL, and 10.0 mL to 200.0 mL for 0.30, 0.20, and 0.10 μg Hg/mL, resp. Dil. 20 mL of 0.10 μg Hg/mL std to 25.0 mL, 10.0 mL to 25.0 mL, 10.0 mL to 50.0 mL, and 10.0 mL to 100.0 mL for 0.080, 0.040, 0.020, and 0.010 μg Hg/mL, resp.

(**f**) *Mercuric chloride column treatment soln.*—1000 ppm $HgCl_2$. Dissolve 0.1 g $HgCl_2$ in 100 mL benzene.

### C. Apparatus

Wash all glassware with detergent (Micro Laboratory Cleaner, International Products, PO Box 118, Trenton, NJ 08601-0118, or equiv.) and rinse thoroly with hot tap $H_2O$ followed by distd or deionized $H_2O$.

(**a**) *Centrifuge*.—Model UV (replacement Model PR-7000) (International Equipment Co.), or equiv.

(**b**) *Centrifuge tubes*.—50 mL capacity with ground glass or Teflon-lined stoppers.

(**c**) *Kuderna-Danish (K-D) concentrators*.—250 mL flask (No. K570001, Kontes Glass Co.) and 10 mL graduated concentrator tube (No. K570050, size 1025, Kontes Glass Co.).

(**d**) *Snyder distilling column*.—Use No. K-503100, size 0003 (Kontes Glass Co.) as is or modify Kontes No. K503000, size 121, in either of 2 ways: (*i*) Shorten 3-section, 3-ball column to 2-section, 2-ball column by cutting off top at uppermost constriction. (*ii*) Insulate 3-section, 3-ball column by wrapping glass wool around top section and holding it in place with Al foil. Glass wool and foil must surround only top section above top ball.

(**e**) *Carborundum boiling chips*.—20 mesh, HCl-washed.

(**f**) *Graduated cylinders*.—Class A, 25 mL capacity, with ground-glass stopper (Kimble 20036, or equiv.).

(**g**) *Transfer pipets*.—Disposable glass, Pasteur-type $5^3/_4$ in. long (No. 13-678-6A, Fisher Scientific Co., or equiv.).

(**h**) *Dropping pipets*.—5 mL capacity (No. 13-710B, Fisher Scientific Co., or equiv.).

(**i**) *Gas chromatograph*.—Hewlett-Packard Model 5710A or equiv., equipped with linear $^{63}Ni$ electron capture detector and 6 ft × 2 mm id silanized glass column packed with 5% DEGS-PS on 100–120 mesh Supelcoport (Supelco, Inc., No. 1-1870). Pack column *no closer* than 2.0 cm from injection and detector port nuts and hold packing in place with 2 cm high quality, silanized glass wool at both ends. Install oxygen scrubber and molecular sieve dryer (No. HGC-145, Analabs, Inc., or equiv.) between carrier gas supply and column. Condition column according to manufacturer's instructions as follows: Flush column 0.5 h with carrier gas flowing at 30 mL/min at room temp. Then heat 1 h at 100°. Next, heat column to 200° at programmed heating rate of 4°/min and hold at 200° overnight. Do not connect column to detector during this conditioning process. Maintain 30 mL/min carrier gas flow at all times during conditioning, treatment, and use. Operating conditions: column 155°; injector 200°; detector, 300°; carrier gas flow 30 mL/min; and recorder chart speed 0.5–1.0 cm/min. Under these conditions and with $HgCl_2$ column treatment procedure described below, $CH_3HgCl$ peak will appear 2–3 min after sample injection.

### D. Mercuric Chloride Column Treatment

5% DEGS-PS conditioned according to manufacturer's instructions can be used to det. $CH_3HgCl$ only after treatment by $HgCl_2$ soln, (**f**). Treat column any time column has been heated to 200°. Because column performance degrades with time, also treat column periodically during use. Perform appropriate $HgCl_2$ treatment procedures described below. Procedure (**b**) produces most stable baseline and is recommended over procedure (**c**) for routine use.

(**a**) *Following 200° column conditioning*.—If column has just been conditioned overnight at 200°, use this procedure. Adjust column temp. to 160° and connect detector. When baseline is steady, treat column by injecting 20 μL $HgCl_2$ treatment soln 5 times at 5–10 min intervals. (Change in column performance may be monitored by injecting 5 μL 0.010 μg Hg/mL std soln before and between $HgCl_2$ treatment soln injections.) During treatment procedure, large broad peaks will elute. ($CH_3HgCl$ peak retention time will decrease and peak ht will increase.) Approximately $1^1/_2$–$1^3/_4$ h after last $HgCl_2$ treatment soln injection, a final large peak will elute. ($CH_3HgCl$ peak ht and retention time will be stable.) This broad peak and $CH_3HgCl$ peak ht stability signal completion of treatment process. Adjust column temp. to 155° and wait for steady baseline; then column is ready for use.

(**b**) *On day preceding sample extract analysis*.—If column has been treated by procedure (**a**) or used at 155° to analyze sample exts, column may be treated at end of working day for next day's use as follows: Lower column temp. to 115° and inject 20 μL $HgCl_2$ treatment soln *one* time. Broad peaks will elute between 11 and 15 h after $HgCl_2$ injection. Next working day, increase column temp. to operating temp. When baseline is steady (ca 15–30 min), column is ready for use.

(**c**) *During sample extract analysis at 155°*.—If column has been used at 155° for ext analysis and column performance has degraded enough to require $HgCl_2$ treatment, increase column temp. to 160°, inject *one* 20 μL aliquot of $HgCl_2$ treatment soln, and monitor baseline. Large, broad peaks will elute $1$–$1^1/_2$ h after $HgCl_2$ injection, signaling completion of treatment process. Decrease column temp. to 155° and wait for steady baseline; then column is ready for use.

### E. Extraction of Methyl Mercury Chloride

*Perform all operations, except weighing, in laboratory hood.* Accurately weigh 2 g homogenized sample into 50 mL centrf. tube. Add 25 mL acetone, stopper, and shake vigorously 15 s. Remove stopper, cover with foil, and centrf. 2–5 min at 2000 rpm. Carefully decant and discard acetone. (Use dropping pipet to remove acetone, if necessary.) Repeat 25 mL acetone wash step twice more. Break up tissue with glass stirring rod before shaking, if necessary. Add 20 mL benzene, stopper, and shake vigorously 30 s. Remove stopper, cover with foil, and centrf. 2–5 min at 2000 rpm. Carefully decant (or draw off with dropping pipet) and discard benzene. Extraneous peaks in final GC anal. chromatograms indicate that more vigorous shaking with acetone and benzene is required.

Add 10 mL HCl soln to centrf. tube contg acetone and benzene-washed sample. Break up tissue with glass stirring rod, and ext sample by adding 20 mL benzene and shaking gently but thoroly 2 min. Remove stopper, cover with foil, and centrf. 5 min at 2000 rpm. If emulsion forms, add 2 mL isopropanol and gently stir benzene layer to break emulsion, taking care not to disturb aq. phase, and recentrf. Carefully transfer benzene layer to K-D concentrator, using 5 mL dropping pipet. Rinse centrf. tube walls with 3–4 mL benzene and transfer rinse to K-D concentrator. Repeat extn step twice more, adding 20 mL benzene and shaking *1* min each time. Combine all 3 benzene exts in K-D concentrator.

Place 4–6 boiling chips in K-D concentrator, connect Snyder column, wet Snyder column bubble chambers with 3–4 drops of benzene, and immediately place tube in steam bath or vigorously boiling $H_2O$ bath. Evap. so that 8 mL remains when cooled to room temp. Cool. Disconnect concentrator tube and quant. transfer soln to 25 mL g-s graduate using Pasteur-type transfer pipet. Dil. to 20.0 mL with benzene and mix. Add 4 g $Na_2SO_4$ and mix again. $Na_2SO_4$ must be added to 20 mL concd sample ext within 10 h of first acetone wash. Tightly stoppered exts may be held overnight at this point. Analyze by GC.

### F. Chromatography

Verify that system is operating properly by injecting 5 μL vols of 0.01 μg Hg/mL working std soln into chromatograph. Difference between $CH_3HgCl$ peak hts for 2 injections should be ≤4%. Check detector linearity by chromatographing all 0.01–0.30 μg Hg/mL working std solns.

Inject duplicate 5 μL vols. (equiv. to 0.5 mg sample) of ext. Difference between $CH_3HgCl$ peak hts for 2 injections should be ≤4%. Next, inject duplicate 5 μL vols. of std soln with $CH_3HgCl$ concn approx. equal to or slightly greater than ext $CH_3HgCl$ concn. Because column performance and peak ht slowly decrease with time, calc. each sample concn by comparison to std soln injected immediately after sample.

Calc. Me Hg content of homogenate in μg Hg/g (ppm Hg) by comparing av. $CH_3HgCl$ peak ht of duplicate sample injections with av. $CH_3HgCl$ peak ht of duplicate std injections.

$$\text{ppm Hg} = (R/R') \times (C'/C) \times 20$$

where $R$ = av. peak ht of duplicate sample injections; $R'$ = av. peak ht of duplicate std injections; $C$ = g sample; $C'$ = concn of Hg in $CH_3HgCl$ std soln (μg Hg/mL).

Ref.: JAOAC **66**, 1121(1983).

CAS-7439-97-6 (mercury)

## 988.11 Mercury (Methyl) in Fish and Shellfish

### Rapid Gas Chromatographic Method

### First Action 1988

#### *A. Principle*

Org. interferences are removed from homogenized seafood by acetone wash followed by toluene wash. Protein-bound Me Hg is released by addn of HCl and extd into toluene. Toluene ext is analyzed for $CH_2HgCl$ by electron capture GC.

#### *B. Reagents*

Equiv. reagents may be used.

(**a**) *Solvents.*—Acetone, toluene, and isopropanol, all distd in glass (Burdick and Jackson Laboratories, Inc., or EM Science OmniSolv® reagents). *Caution:* Toluene is harmful if inhaled and is flammable; conduct all operations with toluene in laboratory hood.

(**b**) *Hydrochloric acid soln (1 + 1).*—Add concd HCl to equal vol. distd or deionized $H_2O$ and mix. Use 2 vols toluene to ext potential interferences from 1 vol. HCl soln by vigorously shaking mixt. 15 s in separator. Discard toluene ext. Repeat extn step 4 times. Soln may be mixed in advance. However, extn must be performed immediately before HCl soln is used to avoid formation of electron-capturing compds which produce extraneous peaks in chromatograms.

Before beginning analysis, check quality of reagents by chromatographing blank taken thru method. Do not use HCl and solvs which produce extraneous peaks at retention time of Me Hg.

(**c**) *Carrier gas.*—GC quality Ar-$CH_4$ (95 + 5).

(**d**) *Sodium sulfate.*—Anhyd. reagent grade. Heat overnight in 600° furnace, let cool, and store in capped bottle. Line cap with acetone-washed Al foil to prevent contamination from cap. Peaks appearing at 14–15 min may be eliminated by refiring $Na_2SO_4$ (600° overnight).

(**e**) *Methyl mercuric chloride std solns.*—Keep tightly stoppered. Seal stopper with Teflon tape. (*1*) *Stock std soln.*—1000 μg Hg/mL. Weigh 0.1252 g $CH_3HgCl$ (ICN-K&K Laboratories, Inc., PO Box 28050, Cleveland, OH 44128-0250) into 100 mL vol. flask. Dil to vol. with toluene. (*2*) *High level intermediate std soln.*—40 μg Hg/mL. Dil. 10.0 mL stock std soln to 250.0 mL with toluene. (*3*) *Low level intermediate std soln.*—2.0 μg Hg/mL. Dil. 10.0 mL high level intermediate std soln to 200.0 mL with toluene. (*4*) *Working std solns.*—0.005–0.10 μg Hg/mL. Prep. monthly by dilg with toluene in vol. flasks as follows: Dil. 10.0 mL of 2.0 μg Hg/mL soln to 200.0 mL for 0.10 μg Hg/mL. Dil. 20.0 mL of 0.10 μg Hg/mL soln to 25.0 mL, 15.0 mL to 25.0 mL, 10.0 mL to 25.0 mL, 10.0 mL to 50.0 mL, 10.0 mL to 100.0 mL, and 10.0 mL to 200.0 mL for 0.080, 0.060, 0.040, 0.020, 0.010, and 0.005 μg Hg/mL, resp.

(**f**) *Mercuric chloride column treatment soln.*—1000 ppm $HgCl_2$. Dissolve 0.1 g $HgCl_2$ in 100 mL toluene.

(**g**) *Fortification solns.*—(*1*) *Stock soln.*—1000 μg Hg/mL. Weigh 0.1252 g $CH_3HgCl$ into 100 mL vol. flask. Dil to vol. with $H_2O$. (*2*) *Working fortification soln.*—15 μg Hg/mL. Dil. 1500 μL stock fortification soln to 100.0 mL with $H_2O$.

#### *C. Apparatus*

Wash all glassware with detergent (Micro Laboratory Cleaner, International Products, PO Box 118, Trenton, NJ 08601-0118) and rinse thoroly with hot tap $H_2O$ followed by distd or deionized $H_2O$. Then rinse 3 times with acetone and 3 times with toluene. Dry in hood.

Equiv. app. may be used except use packed column specified.

(**a**) *Centrifuge.*—Model IEC CRU-5000 or CR6000 (International Equipment Co.).

(**b**) *Centrifuge tubes.*—Glass, 50 mL capacity with Teflon-lined screw caps (Cat. No. 9212-K78, Thomas Scientific).

(**c**) *Graduated cylinders.*—Glass, class A, 50 mL capacity, with ground-glass stoppers (Kimble 20036).

(**d**) *Transfer pipets.*—Disposable glass, Pasteur-type.

(**e**) *Dropping pipets.*—Glass, 5 mL capacity (No. 13-710B, Fisher Scientific Co.).

(**f**) *Mechanical shaker.*—Model S-500 shaker-in-the-round, with timer (Glas-Col Apparatus Co., 711 Hulman St, PO Box 2128, Terre Haute, IN 47802.)

(**g**) *Gas chromatograph.*—Hewlett-Packard Model 5710A equipped with linear $^{63}Ni$ electron capture detector, Model 7131A recorder, and 6 ft × 2 mm id silanized glass column packed with 5% DEGS-PS on 100–120 mesh Supelcoport (Supelco, Inc.). Pack column *no closer* than 2.0 cm from injection and detector port nuts and hold packing in place with 2 cm high quality, silanized glass wool at both ends. Install oxygen scrubber and molecular sieve dryer (No. HGC-145, Analabs, Inc.) between carrier gas supply and column. Condition column according to manufacturer's instructions as follows: Flush column 0.5 h with carrier gas flowing at 30 mL/min at room temp. Then heat 1 h at 50°. Next, heat column to 200° at 4°/min and hold at 200° overnight. Do not connect column to detector during this conditioning process. Maintain 30 mL/min carrier gas flow at all times during conditioning, treatment, and use. Operating conditions: column 155°, injector 200°, detector 300°; carrier gas flow 30 mL/min; recorder chart speed 0.5–1.0 cm/min. Under these conditions and with $HgCl_2$ column treatment procedure described below, $CH_3HgCl$ peak appears 2–3 min after injection of ext.

#### *D. Mercuric Chloride Column Treatment*

Column of 5% DEGS-PS, conditioned according to manufacturer's instructions, can be used to det. $CH_3HgCl$ only after treatment by $HgCl_2$ soln, (**f**). Because column performance degrades with time, also treat column periodically during use. Perform appropriate $HgCl_2$ treatment procedures described below.

(**a**) *Following 200° column conditioning and after every 2–3 days of analyses.*—If column has just been conditioned according to manufacturer's instructions or has been used 2–3 days to analyze exts, proceed as follows: Adjust column temp. to 200° and inject 20 μL $HgCl_2$ treatment soln 5 times at 5–10 min intervals. Maintain 200° temp. overnight. Chromatogram will contain large, broad peaks. Adjust column temp. to 155° next morning and inject 20 μL $HgCl_2$ treatment soln 2 more times. Large, broad chromatgc peaks appearing at ca 1–

2 h signal completion of treatment process and that column is ready for use.

(**b**) *On day preceding analyses.*—If column has been treated by procedure (**a**) or used 1 day at 155° to analyze exts, column may be treated at end of working day for next day's use as follows: Lower column temp. to 115° and inject 20 μL $HgCl_2$ treatment soln 1 time. After large, broad peaks appear in chromatogram (11–20 h), treatment process is complete. Next working day, increase column temp. to 155° operating temp. When baseline is steady, column is ready for use.

(**c**) *During extract analyses at 155°.*—If column has been used at 155° to analyze exts or if column performance and peak ht have degraded enough to require $HgCl_2$ treatment, inject two 20 μL aliquots of $HgCl_2$ treatment soln. Large, broad peaks will appear in chromatogram 1–2 h after $HgCl_2$ injection, signaling completion of treatment process. Wait for steady baseline; then column is ready for use.

#### *E. Extraction of Methyl Mercuric Chloride*

Perform all operations except weighing in laboratory hood. Take empty centrf. tube thru all steps for method blank detn. Accurately weigh 1 g homogenized test sample into 50 mL centrf. tube. Add 25 mL acetone; tightly cap and *vigorously* shake tube by hand 15 s. Loosen cap and centrf. 5 min at 2000 rpm. Carefully decant and discard acetone. (Use dropping pipet to remove acetone, if necessary.) Repeat 25 mL acetone wash step 2 more times. Break up tissue with glass stirring rod before shaking tube, if necessary. Add 20 mL toluene; tightly cap and vigorously shake tube by hand 30 s. Loosen cap and centrf. 5 min at 2000 rpm. Carefully decant (or draw off with dropping pipet) and discard toluene. Extraneous peaks in final GC chromatogram may indicate that more vigorous shaking with acetone and toluene is required. In products for which Me Hg recoveries are to be detd, fortify tissue at this point by adding working fortification soln, (**g**), to centrf. tubes.

Add 2.5 mL HCl soln, (**b**), to centrf. tube contg acetone- and toluene-washed sample. Break up tissue with glass stirring rod, if necessary. Ext $CH_3HgCl$ by adding 20 mL toluene and shaking tube *gently* but *thoroly* 5 min on mech. shaker at setting 5 (2 min by hand). Loosen cap and centrf. 5 min at 2000 rpm. If emulsion is present after centrifugation, add 1 mL isopropanol and gently stir into toluene layer with glass stirring rod to reduce emulsion. Do not mix isopropanol with aqueous phase. Add equal amts of isopropanol to blank and test solns. If emulsion is not present, do not add isopropanol to blank or test solns. Vigorous mixing of isopropanol with HCl may produce interfering peaks in chromatograms. Recentrifuge. With dropping pipet, carefully transfer toluene to graduated cylinder. Rinse walls of centrf. tube with 1–2 mL toluene and transfer rinse to graduated cylinder. Repeat extn step 1 more time. Combine both exts in graduated cylinder, dil. to 50 mL with toluene, stopper, and mix well. Add 10 g $Na_2SO_4$ and mix again. Tightly stoppered exts (sealed with Teflon tape) may be refrigerated and held overnight at this point. Analyze by GC.

#### *F. Gas Chromatography*

Verify that system is operating properly by injecting 5 μL std soln contg 0.005 μg Hg/mL into GC system. Diff. between $CH_3HgCl$ peak hts for 2 injections should be ≤4%. Check detector linearity by chromatographing all working std solns.

Inject 5 μL std soln with concn approx. equal to or slightly greater than concn of ext. Immediately after $CH_3HgCl$ peak appears, inject another 5 μL ext. Immediately after $CH_3HgCl$ and background peaks for ext appear, inject another 5 μL aliquot of std soln. Because column performance and peak ht slowly decrease with time, calc. Hg concn in each test sample by comparing peak ht for each test ext to average peak ht for std solns injected immediately after test ext.

Correct ht of $CH_3HgCl$ peak for test ext by subtracting ht of peak for method blank obtained at same attenuation and recorder sensitivity. Calc. Me-bound Hg content of test sample expressed as μg Hg/g (ppm Hg) by comparing ht of peak from injection of test ext to av. ht of peak from dup. injections of std soln as follows:

$$\mu g\ Hg/g\ fish = (R/R') \times (C'/C) \times 50$$

where $R$ = corrected ht of $CH_3HgCl$ peak from injection of test ext, $R'$ = av. ht of $CH_3HgCl$ peak from dup. injections of std soln, $C$ = wt (g) of test portion, $C'$ = concn (μg/mL) of Hg in std soln, and 50 = final vol. (mL).

Ref.: JAOAC **70**, 24(1987).

CAS-7439-97-6 (mercury)

## 975.34 Nickel in Tea

### Atomic Absorption Spectrophotometric Method

**Final Action**

*See* **971.20**.

## 974.15 Selenium in Food

### Fluorometric Method

**First Action 1974**
**Final Action 1976**

#### *A. Apparatus*

(**a**) *Fluorometer.*—Filter fluorometer or spectrophotofluorometer capable of excitation at 366 nm and detection of fluorescence at 525 nm. (*Caution:* See safety notes on photofluorometers.)

(**b**) *Cuvets or tubes.*—Pyrex culture tubes, 12 × 75 mm, selected by matching, are suitable for fluorometer.

(**c**) *Wrist-action shaker.*—Model BB (Burrell Corp.), or equiv., set at max. speed.

(**d**) *Separators.*—Glass, 250 and 125 mL, with Teflon stopcocks.

#### *B. Reagents*

(Use anal. grade reagents and glass-distd $H_2O$ thruout except as noted.)

(**a**) *Nitric acid.*—Distil from glass, discarding first and final 10%.

(**b**) *Dilute sulfuric acid.*—5*N*. Dil. 140 mL $H_2SO_4$ to 1 L with $H_2O$.

(**c**) *Ammonium hydroxide soln.*—Approx. 6*N*. Dil. 400 mL $NH_4OH$ to 1 L with $H_2O$.

(**d**) *Disodium EDTA soln.*—0.02*M*. Dissolve 7.445 g $Na_2H_2EDTA.2H_2O$ and dil. to 1 L with $H_2O$.

(**e**) *2,3-Diaminonaphthalene* (*DAN*) *soln.*—1 mg/mL. Pulverize DAN (purest grade available; product from Aldrich Chemical Co. has been found satisfactory) in clean mortar to fine powder. Insert glass wool plug in stem of 250 mL separator and add 150 mL 5*N* $H_2SO_4$. Transfer 0.150 g DAN to separator and place on shaker 15 min to dissolve. Add 50 mL cyclohexane and shake 5 min. Let phases sep. 5 min, drain lower phase into another separator, and discard cyclohexane (upper) phase. Repeat cyclohexane extn twice more; after third extn, drain lower phase into low-actinic g-s flask, add 1 cm layer hexane, and store in cold. Soln is stable several weeks.

(**f**) *Selenium std soln.*—(*1*) *Stock soln.*—100 μg/mL. Dissolve 0.1000 g black Se (purity ≥99.9%) in ca 5 mL $HNO_3$, (**a**), and warm to dissolve. Dil. with $H_2O$ and 20 mL 5*N* $H_2SO_4$ to 1 L. (*2*) *Working soln.*—Dil. stock soln with $H_2O$ and 5*N* $H_2SO_4$ to give Se concns in 0.1*N* $H_2SO_4$ appropriate for level of Se expected in sample. Store all solns in all-glass containers. Solns are stable indefinitely.

### C. Preparation of Standard Curve and Fluorometric Blank

Conduct appropriate vols of Se std solns (≤10 mL contg ≤800 ng Se) and 10 mL $H_2O$ each thru entire detn, including digestion, along with samples. Zero fluorometer against blank soln and read fluorescence at 525 nm or subtract blank fluorescence from that of stds. Plot reading against ng Se/6 mL cyclohexane soln. Prepare new std curve daily.

### D. Determination

(To ensure adequate cleanliness for fluorometry, acid-wash all glassware except cells. In particular, clean Kjeldahl flasks and erlenmeyers, separators, centrf. tubes, and glass beads before each detn. Rinse glassware with hot $H_2O$, dry in oven, and wash with hot $HNO_3$-$H_2SO_4$ (1 + 1). Rinse with hot tap $H_2O$ followed by distd $H_2O$ and dry in oven or let air dry. Rinse cells with alcohol followed by acetone. Do not use plastic ware other than that mentioned. *Caution:* See safety notes on wet oxidation; nitric acid, perchloric acid, sulfuric acid.)

Place accurately weighed sample contg ≤1.0 g dry matter and ≤0.8 μg Se with 3 glass beads into 100 mL Kjeldahl flask contg 10 mL $H_2O$, and swirl to wet sample. Add 10 mL $HNO_3$, (**a**). (Alternatively, omit the 10 mL $H_2O$, add 10 mL $HNO_3$, or more if all $HNO_3$ is absorbed by sample, and let digest overnight at room temp.) Heat cautiously to reduce vol. to ca 5 mL, taking care to prevent severe foaming or bumping, and cool. Add 6.0 mL 70% $HClO_4$ and 5.0 mL $H_2SO_4$, return to cool heater, and heat until soln first turns yellow and then becomes colorless. Avoid charring of sample during digestion which may result in loss of Se. If charring occurs, repeat analysis with new sample, using higher $HNO_3$-$HClO_4$/sample wt ratio. If this fails, add small amts of $HNO_3$ at first signs of darkening.

Remove flask from heat, swirl to wet entire bulb area and lower neck of flask, replace flask on heater, and continue heating until soln becomes colorless and white fumes appear.

Remove flask from heat, swirl, add 1.0 mL 30% $H_2O_2$, rinsing walls of flask, and swirl until fuming ceases. Resume heating until contents boil briskly and white fumes are again evolved. Repeat addn of $H_2O_2$ and heating twice more, and continue final heating 5 min after appearance of white fumes. Let flask cool, add 30 mL $H_2O$, rinsing walls of flask, and mix thoroly. Transfer quant. to 250 mL g-s erlenmeyer, using two 10 mL and one 5 mL $H_2O$ rinses. Add, successively with mixing, 10.0 mL EDTA soln, 25.0 mL 6*N* $NH_4OH$, and 5.0 mL DAN soln. Bring quickly to brisk boil and boil exactly 2 min.

Let reaction mixt. stand at room temp. for definite interval between 1 and 2 hr. Use *same* interval for all samples, stds, and blank in set. Accurately add 6.0 mL cyclohexane, stopper flask, and place on shaker 5 min. Transfer to 125 mL separator, and let phases sep. ca 5 min. Discard lower aq. phase and drain cyclohexane soln into 15 mL centrf. tube. Centrf. 5 min to further sep. $H_2O$ and transfer ca 5 mL to fluorometer cell.

Zero fluorometer against reagent blank and read fluorescence of sample at 525 nm. Alternatively, subtract fluorescence of blank from that of sample. Det. Se content from std curve. Altho fluorescence readings for both samples and blanks increase with time, net readings (sample − blank) remain constant with 1–2 hr complexing period.

Ref.: JAOAC **57**, 368, 373(1974).

CAS-7782-49-2 (selenium)

## 939.09★ Selenium in Food

**Titrimetric Method**

**Final Action**
**Surplus 1975**

*See* **25.121–25.126**, 12th ed.

## 915.02★ Tin in Food

**Gravimetric Method**

**Final Action 1976**
**Surplus 1980**

*See* **25.131–25.133**, 13th ed.

## 912.02★ Tin in Food

**Volumetric Method**

**Final Action**
**Surplus 1980**

*See* **25.134–25.135**, 13th ed.

## 980.19★ Tin in Food

**Atomic Absorption Spectrophotometric Method**

**First Action 1980**
**Surplus 1986**

*See* **25.161–25.163**, 14th ed.

## 985.16 Tin in Canned Foods

**Atomic Absorption Spectrophotometric Method**

**First Action 1985**
**Final Action 1988**

### A. Principle

Samples are digested with $HNO_3$ and then HCl and are dild. Aq. KCl is added to samples and stds to reduce pos. instrument interference. Sn is detd by AAS at 235.5 nm with oxidizing $N_2O$-$C_2H_2$ flame.

### B. Reagents and Apparatus

(**a**) *Atomic absorption spectrophotometer.*—With simultaneous background correction and $N_2O$-$C_2H_2$ burner.

(**b**) *Tin std solns.*—(*1*) *Stock soln.*—1 mg Sn/mL. Dissolve 1.000 g Sn (reagent grade) in ca 200 mL concd HCl, add ca 200 mL $H_2O$, cool to ambient temp., and dil. to 1 L with $H_2O$. (*2*) *Working solns.*—0, 50, 100, 150, and 200 μg Sn/mL. Into each of five 100 mL vol. flasks, pipet 10 mL concd HCl, 1.0 mL KCl soln, (**c**), and 0, 5, 10, 15, or 20 mL Sn stock soln. Dil. to vol. with $H_2O$.

(**c**) *Potassium chloride soln.*—10 mg K/mL. Dissolve 1.91 g KCl and dil. to 100 mL with $H_2O$.

(**d**) *Nitric acid.*—Concd. Test purity of lot by dilg portion 1:4 v/v with $H_2O$ and aspirating into AA spectrophtr. Absence of Sn signal indicates suitability for analysis.

### C. Preparation of Sample

Accurately (±0.01 g) weigh sample into 250 mL erlenmeyer: 30–40 g juices or drinks, 20 g foods contg 50–75% $H_2O$, and 5–10 g solids or semisolids. Limit fat or oil content to 2–4 g and total organics to ca 5 g. Dry in oven at 120°.

Do not add $HNO_3$ to samples unless there is time to complete this stage of digestion in the same day. Add 30 mL concd $HNO_3$ to flask and, within 15 min, heat gently in hood to initiate digestion, avoiding excessive frothing. Gently boil until 3–6 mL digest remains or until sample just begins to dry on bottom. Do not let sample char. Remove flask from heat. Without delay, continue as follows, including 2 empty flasks for reagent blanks: Add 25 mL concd HCl, and heat gently ca 15 min until sample bumping from evolution of $Cl_2$ stops. Increase heat, and boil until 10–15 mL vol. remains, using similar flask with 15 mL $H_2O$ to est. vol. Add ca 40 mL $H_2O$, swirl, and pour into 100 mL vol. flask, rinsing once with ca 10 mL $H_2O$. When HCl is present in digest, samples may stand overnight or longer.

Pipet 1.0 mL KCl soln into each vol. flask. Cool to ambient temp. and dil. to vol. with $H_2O$, adding addnl $H_2O$ to approx. compensate for vol. of fat in flask. Mix well and filter ca 30–50 mL thru dry, medium porosity paper into dry, polypropylene or polyethylene screw-cap bottle. Do not filter blanks. Cap bottles until analysis. Solns are stable several months.

### D. Determination

(*Caution:* Due to explosive nature of gases, take care when igniting and using flame. Warming tape on $N_2O$ regulator may be needed to maintain steady gas flow.) Using 200 μg/mL std and 235.5 nm Sn line, optimize spectrophtr, burner, and flame according to manufacturer's instructions. Then increase $N_2O$ flow or decrease $C_2H_2$ flow to give oxidizing flame; red part should be ca 4 mm above burner slot. This reduces sensitivity but improves precision to 0 ± 0.0004 $A$ for blank and 0.201 ± 0.001 $A$ for 100 μg/mL std. Periodically monitor sensitivity of a std; if sensitivity decreases >20%, turn off flame and carefully clean burner slot.

Zero spectrophtr while aspirating $H_2O$ but do not adjust zero until after detns; autozero reduces precision. Aspirate $H_2O$ before and after each sample, std, and blank soln. Take three 5 s readings for each soln, average, and ref. all $A$ measurements to $A$ of $H_2O$.

Record $A$ for stds, draw calibration curve, and visually check for inaccurate stds. Two times blank-corrected $A$ for 50 μg/mL std should not differ by more than 3% from blank-corrected $A$ for 100 μg/mL std.

Block std blank with 50 μg/mL std, and using ratio of $A$, calc. concn of std blank:

$$\text{Std blank } (\mu g/mL) = [A_o/(A' - A_o)] \times 50$$

where $A_o$ and $A'$ refer to blank and mean of readings for 50 μg/mL blocking std, resp.

Add std blank concn to nominal std concns to obtain true std concns.

Measure $A$ of sample blanks as for std blank and calc:

$$\text{Sample blank } (\mu g/mL) = (A_o/A') \times \text{true concn of 50 } \mu g/mL \text{ std}$$

where $A_o$ and $A'$ refer to blank and 50 μg/mL std, resp. Calc. mean concn of sample blanks, $B$.

Det. sample soln concns by one of 2 ways: (*1*) Measure $A$ of sample solns (max. 3 samples) and 50 μg/mL std (or 100 μg/mL std, depending on sample concn level), blocking samples with stds. Calc. blank-corrected sample soln concns:

$$\text{Sample concn } (\mu g/mL) = (A/A' \times \text{true std concn}) - B$$

where $A$ and $A'$ refer to sample and std, resp.

When high accuracy is not required or when calibration curvature is extensive, use procedure (*2*) after confirmation that sensitivity changes and baseline drift are absent during analytical run. (*2*) Calibrate using blank and 50, 100, and 150 μg/mL stds. Run sample blanks and samples, and calc. soln concns using either instrument microprocessor or calibration curve. Calc. mean of sample blank concns, $B$. Calc. blank-corrected soln concns (μg/mL) by subtracting $B$ from soln concns.

For both (*1*) and (*2*), calc. sample concns:

$$\text{Sample } (\mu g/g) = [\text{blank-corrected soln concn/sample wt (g)}] \times 100$$

Ref.: JAOAC **68**, 209(1985).

CAS-7440-31-5 (tin)

## 973.36 Titanium in Cheese

### Spectrophotometric Method

**First Action 1973**
**Final Action 1976**

(*Caution:* See safety notes on sulfuric acid.)

### A. Standard Solution

*Titanium dioxide std soln.*—0.1 mg/mL. Accurately weigh 50 mg $TiO_2$ and transfer to 250 mL beaker; add 15 g anhyd. $Na_2SO_4$ and 50 mL $H_2SO_4$. Add boiling chips, cover with watch glass, and heat to bp on hot plate to dissolve. Cool, and cautiously add 100 mL $H_2O$ with stirring. (Warm on steam bath if soln becomes cloudy.) Cool, transfer soln to 500 mL vol. flask contg 200 mL $H_2O$, and dil. to vol. with $H_2O$.

### B. Preparation of Sample

Weigh, to nearest 0.1 g, 10 g prepd sample, **955.30**, into 100 mL Pt dish and char under IR lamp. Place in cold furnace and ignite at 850° to white ash.

Cool, add ca 1.5 g anhyd. $Na_2SO_4$ and 10 mL $H_2SO_4$, cover with watch glass, and bring to bp on hot plate to dissolve. Turn heat off and let cool on hot plate. Cautiously rinse cover, carefully add ca 30 mL $H_2O$, and mix with stirring rod to disperse any insol. salts. Heat on steam bath if insol. material forms cake on bottom of dish.

Transfer quant. to 100 mL vol. flask with aid of ca 40 mL $H_2O$. If soln is cloudy, heat on steam bath or in boiling $H_2O$ bath to clarify. Cool, and dil. to vol. with $H_2O$.

### C. Preparation of Standard Curve

Transfer 0, 1, 2, 3, 4, and 5 mL $TiO_2$ std soln to sep. 5 mL g-s graduates (or vol. flasks) and dil. to vol. with $H_2SO_4$ (1 + 9). Add 0.2 mL 30% $H_2O_2$, mix, and det. $A$ on recording spectrophtr in 1.0 cm cells from 650 to 325 nm against 0.2 mL 30% $H_2O_2$ in 5.0 mL $H_2SO_4$ (1 + 9). Det. $A$ at max., ca 408 nm, and prep. std curve.

### D. Determination

Transfer 3.0 mL sample soln to 5 mL g-s graduate (or vol. flask), dil. to vol. with $H_2SO_4$ (1 + 9), and continue as in **973.36C**, beginning "Add 0.2 mL 30% $H_2O_2$, . . ."

Det. mg $TiO_2$ in sample from std curve, and calc. as % $TiO_2$.

Ref.: JAOAC **56**, 535(1973).

CAS-7440-32-6 (titanium)

## 944.09 Zinc in Food
### Colorimetric Method
### Final Action 1976

#### *A. Principle*

Method involves wet oxidn of sample; elimination of Pb, Cu, Cd, Bi, Sb, Sn, Hg, and Ag as sulfides with added Cu as scavenger agent; simultaneous elimination of Co and Ni by extg metal complexes of α-nitroso-β-naphthol and dimethylglyoxime, resp., with $CHCl_3$; extn of Zn dithizonate with $CCl_4$; transfer of Zn to dil. HCl; and final extn of Zn dithizonate for color measurement.

#### *B. Reagents*

(All $H_2O$ must be redistd from glass. Pyrex glassware should be used exclusively and must be scrupulously cleaned with hot $HNO_3$. Purify $HNO_3$ (usually unnecessary) and $NH_4OH$ by distn in Pyrex if appreciably contaminated. Test $H_2SO_4$ if Zn contamination is suspected.)

(**a**) *Copper sulfate soln*.—2 mg Cu/mL. Dissolve 8 g $CuSO_4.5H_2O$ in $H_2O$ and dil. to 1 L.

(**b**) *Ammonium citrate soln*.—Dissolve 225 g $(NH_4)_2HC_6H_5O_7$ in $H_2O$, make alk. to phenol red with $NH_4OH$ (pH 7.4, first distinct color change), and add 75 mL in excess. Dil. to 2 L. Ext this soln immediately before use as follows: Add slight excess of dithizone and ext with $CCl_4$ until solv. layer is clear bright green. Remove excess dithizone by repeated extn with $CHCl_3$, and finally ext once more with $CCl_4$. (It is essential that excess dithizone be entirely removed, otherwise Zn will be lost during elimination of Co and Ni.)

(**c**) *Dimethylglyoxime soln*.—Dissolve 2 g reagent in 10 mL $NH_4OH$ and 200–300 mL $H_2O$, filter, and dil. to 1 L.

(**d**) *Alpha-nitroso-beta-naphthol soln*.—Dissolve 0.25 g in $CHCl_3$ and dil. to 500 mL.

(**e**) *Chloroform*.—Redistd.

(**f**) *Diphenylthiocarbazone (dithizone) soln*.—Dissolve 0.05 g dithizone in 2 mL $NH_4OH$ and 100 mL $H_2O$, and ext repeatedly with $CCl_4$ until solv. layer is clear bright green. Discard solv. layer and filter aq. portion thru washed ashless paper. (This soln is best prepd as needed, since it is only moderately stable, even when kept in dark and under refrigeration.)

(**g**) *Carbon tetrachloride*.—Redistd.

(**h**) *Dilute hydrochloric acid*.—0.04*N*. Dil. required amt of HCl with $H_2O$ (redistd acid may be used altho usually unnecessary).

(**i**) *Zinc std solns*.—(*1*) *Stock soln*.—500 μg/mL. Dissolve 0.500 g pure granulated Zn in slight excess of dil. HCl and dil. to 1 L. (2) *Working soln*.—5 μg/mL. Dil. 10 mL stock soln to 1 L with 0.04*N* HCl.

#### *C. Preparation of Sample*

(*Caution:* See safety notes on wet oxidation, nitric acid, perchloric acid, and sulfuric acid.)

Weigh, into suitable size erlenmeyer, representative sample ≤25 g, estd to contain 25–100 μg Zn. If sample is liq., evap. to small vol. Add $HNO_3$ and heat cautiously until first vigorous reaction subsides somewhat; then add 2–5 mL $H_2SO_4$. Continue heating, adding more $HNO_3$ in small portions as needed to prevent charring, until fumes of $SO_3$ evolve and soln remains clear and almost colorless. Add 0.5 mL $HClO_4$ and continue heating until it is almost completely removed. Cool, and dil. to ca 40 mL. (Wet digestion and subsequent sulfide sepn may also be advantageously performed in small Kjeldahl flask.)

#### *D. Separation of Sulfide Group*

(*Caution:* See safety notes on bromine and hydrogen sulfide.)

To $H_2SO_4$ soln add 2 drops Me red and 1 mL $CuSO_4$ soln, and neutze with $NH_4OH$. Add enough HCl to make soln ca 0.15*N* with respect to this acid (ca 0.5 mL excess in 50 mL soln is satisfactory); pH of soln as measured with glass electrode is 1.9–2.1. Pass stream of $H_2S$ into soln until pptn is complete. Filter thru fine paper (Whatman No. 42, or equiv., previously fitted to funnel and washed with HCl (1 + 6), then with redistd $H_2O$). Receive filtrate in 250 mL beaker, and wash flask and filter with 3 or 4 small portions $H_2O$. Gently boil filtrate until odor of $H_2S$ can no longer be detected; then add 5 mL satd Br-$H_2O$ and continue boiling until Br-free. Cool, neutze to phenol red with $NH_4OH$, and make slightly acid with HCl (excess of 0.2 mL 1 + 1 HCl). Dil. resultant soln to definite vol. For optimum conditions of measurement, soln should contain 0.2–1.0 μg Zn/mL.

#### *E. Elimination of Nickel and Cobalt*

Transfer 20 mL aliquot of prepd soln to 125 mL separator; add 5 mL $NH_4$ citrate soln, 2 mL dimethylglyoxime soln, and 10 mL α-nitroso-β-naphthol soln; and shake 2 min. Discard solv. layer and ext with 10 mL $CHCl_3$ to remove residual α-nitroso-β-naphthol. Discard solv. layer.

#### *F. Isolation and Estimation of Zinc*

To aq. phase following removal of Ni and Co, which at this point has pH of 8.0–8.2, add 2.0 mL dithizone soln and 10 mL $CCl_4$, and shake 2 min. Let phases sep. and remove aq. layer as completely as possible, withdrawing liq. with pipet attached to vac. line. Wash down sides of separator with ca 25 mL $H_2O$ and without shaking again draw off aq. layer. Add 25 mL 0.04*N* HCl and shake 1 min to transfer Zn to acid-aq. layer. Drain and discard solv., being careful to dislodge and remove drop that usually floats on surface. To acid soln add 5.0 mL $NH_4$ citrate soln and 10.0 mL $CCl_4$ (pH of soln at this point is 8.8–9.0).

Det. vol. dithizone to be added as follows: To separator contg 4.0 mL working Zn std (20 μg), dild to 25 mL with 0.04*N* HCl, 5.0 mL citrate buffer, and 10.0 mL $CCl_4$, add dithizone reagent in 0.1 mL increments, shaking briefly after each addn until faint yellow in aq. phase indicates bare excess of reagent. Multiply vol. dithizone soln required by 1.5 and add this vol. (to nearest 0.05 mL) to all samples. Shake 2 min. Pipet exactly 5.0 mL solv. layer into clean, dry test tube, dil. with 10.0 mL $CCl_4$, mix, and det. *T* (or *A*) at 540 nm.

#### *G. Preparation of Standard Curves*

Prep. series of separators contg 0, 5, 10, 15, and 20 μg Zn dild to 25 mL with 0.04*N* HCl; add 5.0 mL citrate buffer, and proceed as with final extn of Zn, **944.09F**.

Plot *T* in logarithmic scale (or *A* on linear scale) against concn and draw smooth curve thru points. (Intercept of this curve may vary slightly from day to day, depending on actual concn of dithizone used in final extn, but slope should remain essentially same.)

Refs.: JAOAC **27**, 325(1944); **28**, 271(1945).

CAS-7440-66-6 (zinc)

## 969.32 Zinc in Food
### Atomic Absorption Spectrophotometric Method
### First Action 1969
### Final Action 1971

(*Caution:* See safety notes on AAS, wet oxidation, nitric acid, and sulfuric acid.)

### A. Principle

Representative sample is dry or wet ashed. Residue is taken up in acid and dild to optimum working range. *A* of this soln as detd by AA spectrophotometry at 213.8 nm is converted to Zn concn thru calibration curve.

### B. Reagents

(Use Pyrex glassware exclusively; clean thoroly before use with hot $HNO_3$. If glass beads are used to prevent bumping, clean first with strong alkali followed by hot $HNO_3$. Since Pt used in laboratory may contain significant traces of metals, clean Pt dishes by $KHSO_4$ fusion followed by 10% HCl leach.)

(**a**) *Zinc std solns.*—(*1*) *Stock soln.*—500 μg/mL. Dissolve 0.500 g pure Zn metal in 5–10 mL HCl. Evap. almost to dryness and dil. to 1 L with $H_2O$. Soln is stable indefinitely. (*2*) *Working soln.*—Dil. aliquots of stock soln with $H_2SO_4$ (1 + 49) or 0.1*N* HCl (depending on method of ashing) to obtain ≥5 solns within range of instrument. Prep. stds in 0–10 μg/mL range daily. (Do not use <2 mL pipets or <25 mL vol. flasks.)

(**b**) *Acids.*—Reagent grade $HNO_3$, HCl, and $H_2SO_4$. Test acids for freedom from Zn by AA measurement of appropriately dild sample. If contaminated, purify $HNO_3$ and HCl by distn. Further test purity of reagents and efficiency of cleaning by conducting blank detns by appropriate ashing method.

### C. Preparation of Sample Solution

Prep. representative sample by mixing, blending, or grinding.

(**a**) *Wet ashing.*—Accurately weigh, into 300 or 500 mL Kjeldahl flask, representative sample ≤10 g, estd to contain 25–100 μg Zn. (If sample is liq., evap. to small vol.) Add ca 5 mL $HNO_3$ and cautiously heat until first vigorous reaction subsides. Add 2.0 mL $H_2SO_4$ and continue heating, maintaining oxidizing conditions by adding $HNO_3$ in *small* increments (large amts may introduce Zn) until soln is colorless. Continue heating until dense fumes of $H_2SO_4$ are evolved and all $HNO_3$ has been removed. Cool, dil. with ca 20 mL $H_2O$, filter thru fast paper (pre-washed) into 100 mL vol. flask, and dil. to vol. with $H_2O$. Dil. further, if necessary, with $H_2SO_4$ (1 + 49) to attain working range of spectrophtr.

(**b**) *Dry ashing.*—Accurately weigh, into clean Pt dish, representative sample estd to contain 25–100 μg Zn. Char under IR lamp and ash at temp. ≤525° until C-free. (Raise temp. of furnace slowly to 525° to avoid ignition.) Dissolve ash under watch glass in min. vol. HCl (1 + 1). Add ca 20 mL $H_2O$ and evap. to near dryness on steam bath. Add 20 mL 0.1*N* HCl and continue heating ca 5 min. Filter thru fast paper into 100 mL vol. flask. Wash dish and filter with several 5–10 mL portions of 0.1*N* HCl, cool, and dil. to vol. with 0.1*N* HCl. Dil. further, if necessary, with 0.1*N* HCl to attain working range of instrument.

### D. Determination

Set instrument to previously established optimum conditions or according to manufacturer's instructions. Det. *A* of ashed soln or diln, and ≥5 stds within optimum working range, taking ≥2 readings (before and after sample readings). Flush burner with $H_2O$ and check 0 point between readings. Det. Zn content from std curve obtained by plotting *A* against μg Zn/mL:

$$\text{ppm Zn} = [(\mu g\ \text{Zn/mL from curve}) \times (\text{diln factor, mL})]/\text{g sample}$$

Refs.: JAOAC **51,** 1042(1968).

CAS-7440-66-6 (zinc)

# 10. Pesticide and Industrial Chemical Residues


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## MULTIRESIDUES GENERAL CONSIDERATIONS

### 970.52 Organochlorine and Organophosphorus Pesticide Residues
#### General Multiresidue Methods

**A. Principle**

Thoroly mixed sample is extd with $CH_3CN$ (high-$H_2O$ foods) or aq. $CH_3CN$ (low-$H_2O$ or high sugar foods). Fat is extd from fatty foods and partitioned between pet ether and $CH_3CN$. Aliquot (nonfatty samples) or entire soln (fatty samples) of $CH_3CN$ is dild with $H_2O$ and residues are extd into pet ether. Residues are purified by chromatgy on Florisil column, eluting with mixt. of pet and Et ethers. Residues in concd eluates are measured by GC and identified by combinations of gas, thin layer, or paper chromatgy.

Analyst competence in applying method for trace residues should be assured before analysis. Recoveries of added compds thru method should be ≥80%.

Absence of interferences arising from laboratory and reagent contamination should also be assured by regular performance of reagent blanks. Solvs in particular, because of their concn during methods, can contribute significant interference if not sufficiently purified. Solvs of adequate purity are com. available from several manufacturers, but each batch must be tested under conditions of method in which it will be used.

Other reagents and app. (rubber, plastics, glass wool, etc.) are also potential source of interferences. *See* references for recoveries obtained during collaborative and validation studies and Table **970.52A** for commodities approved. *See* **970.52H**, introductory par., and **970.52H(c)** for GC performance requirements: sensitivity, sepn capability, and linearity. Behavior of >200 pesticides and industrial chems in method is given in JAOAC **61**, 640(1978).

**B. General Reagents**

Solvs must be purified and final distn conducted in all-glass app. (*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, acetonitrile, diethyl ether, hexane, and petroleum ether.) *See* **970.52A**.

*Solvent purity test.*—Electron capture GC requires absence of substances causing detector response as indicated by following test: Place 300 mL solv. in Kuderna-Danish concentrator fitted with 3-ball Snyder column and calibrated collection vessel, and evap. to 5 mL. Inject 5 μL conc. from 10 μL syringe into gas chromatograph, using conditions described in **970.52H(c)**. Conc. must not cause recorder deflection >1 mm from baseline for 2–60 min after injection.

(**a**) *Acetonitrile.—See solv. purity test.* Purify tech. $CH_3CN$ as follows: To 4 L $CH_3CN$ add 1 mL $H_3PO_4$, 30 g $P_2O_5$, and boiling chips, and distil in all-glass app. at 81–82°. Do not exceed 82°.

Some lots of reagent grade $CH_3CN$ are impure and require distn. Generally vapors from such lots will turn moistened red litmus paper blue when held over mouth of storage container. Pronounced amine odor is detectable.

(**b**) *Acetonitrile saturated with petroleum ether.*— Sat. $CH_3CN$, (**a**), with redistd pet ether, (**m**).

(**c**) *Alcohol.*—USP, reagent grade, or MeOH, ACS.

(**d**) *Alcoholic alkali soln.*—2%. Dissolve 2 g KOH in alcohol, and dil. to 100 mL.

(**e**) *Eluting solvent, 6%.*—Dil. 60 mL Et ether, (**h**), to 1 L with redistd pet ether, (**m**).

(**f**) *Eluting solvent, 15%.*—Prep. as in (**e**), using 150 mL Et ether.

(**g**) *Eluting solvent, 50%.*—Prep. as in (**e**), using 500 mL Et ether.

(**h**) *Ethyl ether.—See solv. purity test.* Redistd at 34–35°, and stored under N. Add 2% alcohol. Must be peroxide-free by test in *Definitions of Terms and Explanatory Notes*.

(**i**) *Florisil.*—60/100 PR grade, activated at 675°C (1250°F), available from Floridin Co., 3 Pennsylvania Center, Pittsburgh, PA 15235. When 675°C activated Florisil is obtained in bulk, transfer immediately after opening to ca 500 mL (1 pt) glass jars, or bottles, with g-s or foil-lined, screw-top lids, and store in dark. Heat ≥5 hr at 130° before use. Store at 130° in g-s bottles or in air-tight desiccator at room temp. and reheat at 130° after 2 days.

Prep. mixed pesticide std soln in hexane contg 1, 4, 1, 2, 1, 2, and 4 μg/mL, resp., of ronnel, ethion, heptachlor epoxide, parathion, dieldrin, endrin, and malathion.

Test each batch of activated Florisil by placing 1 mL mixed pesticide std on prepd column and eluting as in *Cleanup*, **970.52O**. Conc. eluates from Florisil column to 10 mL. Inject aliquot (*see* **970.52H**) of each eluate into gas chromatograph and det. quant. recovery of each compd as in **970.52R**. Florisil that quant. elutes heptachlor epoxide, ronnel, and ethion in 6% eluate; dieldrin, endrin, and parathion in 15% eluate; and malathion in 50% eluate, is satisfactory.

Adsorptivity of lots of Florisil may be tested with lauric acid and size of column adjusted to compensate for variation in adsorptivity (JAOAC **51**, 29(1968)). Test adjusted column before use by performing elution test above.

**Table 970.52A Compounds and Commodities to Which General Method Applies**

| Compound | Official Final Action |
|---|---|
| Dieldrin (CAS-60-57-1)<br>Heptachlor epoxide (CAS-1024-57-3) | Group I nonfatty foods, dairy products, fish, vegetable oils, whole eggs |
| BHC (CAS-608-73-1)<br>DDE (CAS-72-55-9)<br>DDT (CAS-8017-34-3)<br>TDE (CAS-72-54-8) | Group I nonfatty foods, dairy products, fish, whole eggs |
| Lindane (CAS-58-89-9)<br>Methoxychlor (CAS-72-43-5)<br>Ethylan (CAS-72-56-0) | Group I nonfatty foods, dairy products, whole eggs |
| Aldrin (CAS-309-00-2)<br>Endrin (CAS-72-20-8)<br>Heptachlor (CAS-76-44-8)<br>Mirex (CAS-2385-85-5) | Group I nonfatty foods, whole eggs |
| *o,p'*-DDT (CAS-8017-34-3)0 | Group I nonfatty foods, dairy products |
| Diazinon (CAS-333-41-5)<br>Ethion (CAS-563-12-2)<br>Malathion (CAS-121-75-5)<br>Me parathion (CAS-298-00-0)<br>Parathion (CAS-56-38-2)<br>Ronnel (CAS-299-84-2) | Group II nonfatty foods |
| PCBs (CAS-12767-79-2) | Poultry fat, fish, dairy products |

*Group I nonfatty foods:* apples*, apricots, barley*, beets, bell peppers, broccoli*, cabbage*, cantaloupes, cauliflower*, celery, collard greens, corn meal and silage, cucumbers*, eggplant, endive, grapes*, green beans, hay, kale*, mustard greens*, oats*, peaches, pears, peas, plums, popcorn, potatoes*, radishes, radish tops, spinach, squash*, strawberries, sugar beets, sweet potatoes, tomatoes*, turnips*, turnip greens*, wheat*
*Group II nonfatty foods:* Group I nonfatty foods marked with asterisk (*) plus carrots, green peppers, and lettuce

(**j**) *Hexane.—See solv. purity test.* Reagent grade, redistd in all-glass app.

(**k**) *Magnesium oxide.*—Adsorptive magnesia (Fisher Scientific Co. No. S-120). Treat as follows: Slurry ca 500 g with $H_2O$, heat on steam bath ca 30 min, and filter with suction. Dry overnight at 105–130° and pulverize to pass No. 60 sieve. Store in closed jar.

(**l**) *Magnesia-Celite mixture.*—Mix treated MgO, (**k**), with Celite 545, 1 + 1 by wt. Pet ether ext of Celite should be free of electron-capturing substances.

(**m**) *Petroleum ether.—See solv. purity test.* Reagent grade, redistd in all-glass app. at 30–60°.

(**n**) *Sodium sulfate.*—Anhyd., granular.

### C. Reagents for Thin Layer Chromatography

(**a**) *Aluminum oxide.*—Neutral $Al_2O_3$ "G" (Type E, EM Science No. 1090), or equiv., for TLC.

(**b**) *Developing solvents for organochlorine pesticides.*—(*1*) *n*-Heptane, com. grade. (*2*) *n*-Heptane contg 2% reagent grade acetone.

(**c**) *Chromogenic agent for organochlorine pesticides.*—Dissolve 0.100 g $AgNO_3$ in 1 mL $H_2O$, add 20 mL 2-phenoxyethanol (Practical, Eastman Kodak Co.), dil. to 200 mL with acetone, add very small drop 30% $H_2O_2$, and mix. Store in dark overnight and decant into spray bottle. Discard after 4 days.

(**d**) *Developing solvents for organophosphorus pesticides.*—(*1*) *Immobile.*—15 or 20% *N,N*-dimethylformamide (DMF) in ether. Dil. 75 or 100 mL DMF to 500 mL with ether and mix. (*2*) *Mobile.*—Methylcyclohexane.

(**e**) *Chromogenic agents for organophosphorus pesticides.*—(*1*) *Stock dye soln.*—Dissolve 1 g tetrabromophenolphthalein Et ester (Eastman No. 6810) in 100 mL acetone. (*2*) *Dye soln.*—Dil. 10 mL stock dye soln (*1*) to 50 mL with acetone. (*3*) *Silver nitrate soln.*—Dissolve 0.5 g $AgNO_3$ in 25 mL $H_2O$ and dil. to 100 mL with acetone. (*4*) *Citric acid soln.*—Dissolve 5 g granular citric acid in 50 mL $H_2O$ and dil. to 100 mL with acetone.

### D. Reagents for Paper Chromatography★ —Surplus 1980

*See* **29.004**, 13th ed.

### E. General Apparatus

(**a**) *High-speed blender.*—Waring Blendor, or equiv.

(**b**) *Chromatographic tubes.*—With Teflon stopcocks and coarse fritted plate or glass wool plug; 22 mm id × 300 mm.

(**c**) *Chromatographic tubes without stopcocks.*—22 mm id × 300 or 400 mm.

(**d**) *Filter tubes.*—Approx. 22 mm id × 200 mm with short delivery tube and coarse fritted plate or glass wool plug.

(**e**) *Kuderna-Danish concentrators.*—500 and 1000 mL with Snyder distilling column and 5 or 10 mL plain, vol., and graduated receiving flasks (Kontes Glass Co. No. K-570000, K-621400, and K-570050, or equiv.).

(**f**) *Separators.*—1000 and 125 mL with Teflon stopcocks.

(**g**) *Micro-Snyder column.*—2-ball (Kontes Glass Co. No. K-569001, or equiv.).

(**h**) *Micro-Vigreaux column.*—Kontes Glass Co. No. K-569251, or equiv.

### F. Apparatus for Thin Layer Chromatography

(**a**) *Desaga/Brinkmann standard model applicator, or equiv.*

(**b**) *Desaga/Brinkmann standard mounting board, or equiv.*

(**c**) *Desaga/Brinkmann drying rack, or equiv.*—Accommodates ten 8 × 8″ plates.

(**d**) *Desaga/Brinkmann model 51 stainless steel desiccating cabinet, or equiv.*

(**e**) *Window glass.*—8 × 8″, double strength window glass plates of uniform width and thickness; smooth off corners and edges with file or other tool.

(**f**) *Chromatographic tank and accessories.*—With metal instead of glass troughs.

(**g**) *Dipping tank and accessories.*—Stainless steel, $8^1/_2$ × $8^1/_2$ × $^1/_4$–$^3/_{16}$″ inside width with metal supports and close-fitting U-shaped cover ca 9 × $^1/_2$″. Capacity ca 300 mL.

(**h**) *Spotting pipets.*—1 μL.

(**i**) *Spray bottle.*—8 oz (Thomas Scientific No. 2753-J10 or Lurex Scientific No. 131-0514, 250 mL).

(**j**) *Chromatography spray flask.*—250 mL (Microchemical Specialties Co., 1825 Eastshore Hwy, Berkeley, CA 94710, No. S-4530-D).

(**k**) *Tank liner.*—Cut 2 pieces, $12^1/_4$ × $8^3/_4$″, from desk blotter, white or colored, and bend into L-shape to fit tank.

(**l**) *Strong ultraviolet light source.*—Such as germicidal lamps (General Electric Co., Nela Park, Cleveland, OH 44112), either (*1*) two 30 watt, 36″ tubes, No. G30T8, mounted in std 30 watt reflector fixture ca 20 cm above papers; or (*2*) two 15 watt, 18″ tubes, No. G15T8, mounted in std 15 watt desk lamp fixture placed ca 10 cm above papers. Shield to protect eyes and skin at all times.

### G. Apparatus for Paper Chromatography★ —Surplus 1980

*See* **29.007**, 13th ed.

### H. Apparatus for Gas Chromatography

(*See also* JAOAC **47,** 326–342(1964); **49,** 8–21(1966).)

Gas chromatgc system when operated with column, (**b**), and approx. conditions described in *Gas Chromatography,* **970.52R**, should be capable of producing ca $^1/_2$ scale deflection for 1 ng heptachlor epoxide by electron capture detection and for 2 ng parathion by KCl-thermionic detection, and should resolve mixt. of heptachlor, aldrin, heptachlor epoxide, ethion, and carbophenothion into sep. peaks. Retention time for aldrin should be ca 4.5 min. Compds of interest must not be degraded by any part of GC system.

**(a)** *Gas chromatograph.*—Instrument consisting of on-column injection system, all-glass column in oven controlled to ±0.1°, electron capture and thermionic detectors, each with independent power supply, electrometer, and appropriate mv recorder.

**(b)** *Column.*—Glass, 1.85 m (6′) × 4 mm id packed with 10% DC-200 (w/w) on solid support: *(1)* 80–100 mesh Chromosorb W HP (Manville Filtration and Minerals, manufacturer, but available thru many GC distributors); *(2)* 80–100 mesh Gas-Chrom Q (Applied Science Laboratories, Inc.); *(3)* 80–90 mesh Anakrom ABS (Analabs, Inc.). DC-200 may be replaced by OV-101 (available from many GC distributors).

Weigh 2 g Dow Corning 200 silicone fluid (12,500 centistokes) or OV-101 into beaker. Dissolve in $CHCl_3$ and transfer to 300 mL Morton-type flask, using total of ca 100 mL $CHCl_3$. Add 18 g solid support, *(1)*, *(2)* or *(3)*, to flask. Swirl, and let stand ca 10 min. Place flask on rotary evaporator and remove solv. slowly with intermittent rotation, using 50° $H_2O$ bath and slight vac. (Foaming may occur initially.) When solids appear damp, increase vac. Remove last traces of $CHCl_3$ without rotation or by air drying. Use only free-flowing material to fill column. Use care at all stages of column prepn to prevent fracturing solid support. Condition column at 250–260° with N flow of ca 100 mL/min ≥48 hr or until endrin exhibits single peak.

**(c)** *Electron capture detector (ECD).*—Concentric design, for use with dc voltage supply and $^3H$ source (ca 150 mCi $^3H$, U.S. Nuclear Regulatory Commission license is required.)

Det. detector operating characteristics as follows: Apply dc voltage to detector. After system becomes stable (overnight), det. current-voltage relationship at various voltages between 200 and 0 v. (Current measurements at voltages of 200, 150, 100, 75, 50, 40, 30, 25, 20, 15, 10, 8, 6, 4, 2, and 1 provide points for smooth curve.) Slightly lower, stable, standing current may be obtained after detector has been at operating temp. several hr. This is probably due to loss of some easily removed radioactive material. Det. and plot response-voltage relationship at $1 \times 10^{-9}$ amp full scale sensitivity for 1 ng injections of heptachlor epoxide at same voltages used in obtaining current-voltage curve. Select as operating voltage that voltage at which heptachlor epoxide causes ca 40–50% full scale recorder deflection. Check linearity of system from 0.2 to 2.0 ng heptachlor epoxide.

Other electron capture detectors may be substituted for dc voltage concentric design $^3H$ detector, which is no longer marketed. Const current, variable frequency $^{63}Ni$ electron capture detectors are acceptable substitutes when operated at conditions to produce stable, reproducible, linear responses. Optimum conditions may produce more sensitive response than from $^3H$ detector. To maintain same method limit of quantitation of $^3H$ detector, inject proportionately smaller equiv. sample wt into $^{63}Ni$ detector system. The $^{63}Ni$ electron capture detector may provide different relative responses for pesticides than those obtained with $^3H$ electron capture detector. Use of Ar-$CH_4$ carrier gas, as recommended for most $^{63}Ni$ detectors, precludes use of KClTD dual detection system, **(d)**, **(h)**–**(j)**.

**(d)** *Potassium chloride thermionic detector (KClTD).*—Flame ionization detector modified to incorporate coil with KCl coating prepd as in *(1)* or *(2)*. Detector voltage is 300 v dc. Use in dual arrangement with electron capture detector.

All dual detector systems described are capable of comparable performance. In-series, **(h)**, arrangement is preferred because of simplicity and ease of operation.

*(1) Coil with potassium chloride for in-series dual detector.—See* Fig. **970.52A** (may be used with all detector arrangements). Wind Pt-Ir wire (B&S gage 26) on 7 mm diam. rod into 2 turn helix so that turns are touching. Approx. 5 mm below helix, continue to wind wire on 3 mm rod, or rod with same outside diam. as flame jet, making 3-turn spiral. Cut wire so that 7 mm helix is supported 4 mm above flame jet when 3 mm spiral is slipped over jet. Fill 30 mL tall-form Pt crucible ca $^1/_4$ full with KCl (ACS). Heat with Meker burner until all salt melts. Continue heating until bottom of crucible glows red, imparting pink glow to melt. Remove heat and begin dipping the 2-turn helix of coil into melt at 5 sec intervals as melt cools. (Make sure only 2-turn helix touches melt and do not raise coil above top of crucible.) When melt is at proper temp., salt clings to coil. Remove coil from melt. Place probe in center of coil while salt is molten. This causes crystn around probe tip. Remove center of coil. Remove any rough edges on coil coating by holding coil in burner flame 1 sec; id of properly coated coil is 5 mm. Position coil over flame jet.

*(2) Coil with potassium chloride for parallel and in-series split dual detectors.—See* Fig. **970.52B**. Wind Pt-Ir wire (B&S gage 26) on 5 mm diam. rod into 5-turn helix so that turns are close together or touching. Continue to wind wire on 3 mm rod, or rod having same outside diam. as flame jet, making 3-turn spiral. Cut wire so that 5 mm helix is supported 2 mm above flame jet when 3 mm spiral is slipped over jet. Grasp formed wire by end opposite 5 mm helix with forceps. Dip 5 mm helix into satd KCl (recrystd twice from $H_2O$) soln, or apply KCl soln with dropper. Fuse in flame. (*Caution:* Use safety glasses; spattering occurs.) Repeat application of KCl soln 3–4 times until helix is coated with fused KCl. Coating should appear almost crystal clear. Position coil over flame jet.

**(e)** *Hydrogen.*—From generator or cylinder of compressed H gas (cylinder preferred). Equip cylinder with pressure drop of stainless steel capillary tubing (0.020″ id) to restrict H flow to ca 30 mL/min at 20 lb delivery pressure. Place H source close to detector and use gas lines with min. dead vol. to reduce outgassing time in lines. (For fine precise control of H flow, insert Nupro Fine Metering Valve, "S" series (Swagelok Co., 31400 Aurora Rd, Solon, OH 44138; Part Number B-1S) between exit end of capillary tubing pressure drop and inlet of detector H line. *Caution:* Do not use Nupro valve as shut-off valve. Repeated tightening damages needle.) Use Swagelok fittings for all connections.

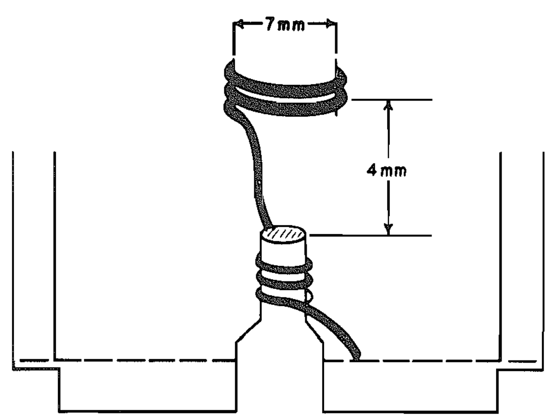


**FIG. 970.52A—KCl thermionic detector coil for in-series dual detection system**

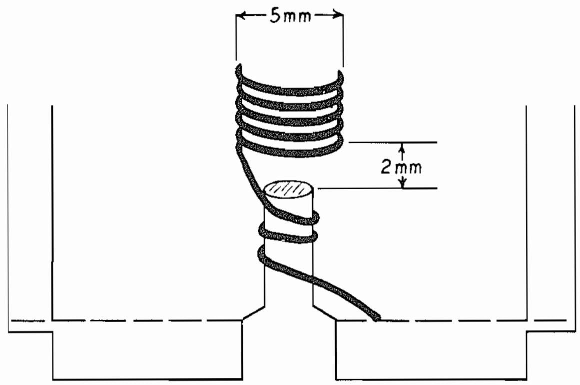


**FIG. 970.52B—KCl thermionic detector coil for parallel and in-series split dual detection systems**

(**f**) *Air.*—Min. air requirement for thermionic detector is 300 mL/min. Cylinder of compressed air or aquarium air pump is recommended.

(**g**) *Capillary T-tube.*—(*See* Figs. **970.52C** and **970.52D**.) Prep. 1:1 stream splitter (*B*) for parallel and in-series split dual detection systems. Fit two 4.5 cm lengths of stainless steel capillary tubing, 0.010″ id, $^1/_{16}$″ od, into 1 cm length of std wall, $^1/_8$″ stainless steel tubing. Fit 1″ length of No. 16 hypodermic tubing at right angles in hole drilled into the piece of $^1/_8$″ tubing. Silver braze all connections. Prep. capillary T-tube (*E*) for introducing purge gas to parallel system. Fit two 2.5 cm lengths of No. 16 hypodermic tubing into 1 cm length of std wall, $^1/_8$″ stainless steel tubing. Fit 1 cm length of No. 16 hypodermic tubing at right angles in hole drilled into piece of $^1/_8$″ tubing. Silver braze as above.

(**h**) *Assembly of in-series dual detection system.*— Assemble as in Fig. **970.52E**. Introduce column effluent (*A*) of 120 mL/min directly to ECD inlet. Connect ECD outlet directly to KClTD inlet, using No. 16 std wall Teflon tubing.

*Note:* For in-series, (**h**), and in-series split, (**i**), operation, thoroly check ECD for gas leaks, particularly at Teflon insulator.

(**i**) *Assembly of in-series split dual detection system.*—Assemble as in Fig. **970.52C**. Introduce column effluent (*A*) of 120 mL/min directly to ECD inlet. Connect 1:1 stream splitter (*B*) between ECD outlet and KClTD inlet so that only 60 mL N/min enters KClTD and remaining 60 mL N/min exits to atm. Use No. 16 std wall Teflon tubing for all connections. *See Note* in (**h**).

(**j**) *Assembly of parallel dual detection system.*— Assemble as in Fig. **970.52D**. Split column effluent (*A*) of 120 mL/min by passing thru 1:1 stream splitter (*B*) so that each detector receives 60 mL effluent/min. Increase flow to ECD by introducing 60 mL N/min from second N source (*C*) thru capillary T-tube (*E*). Preheat N from *C* by passing thru stainless steel capillary tube (*D*) (0.040″ id) which extends 120 cm into column bath and returns to detector bath where addnl 35 cm of tubing is coiled into small helix. Connect capillary tubes and splitters to detectors with No. 16 std wall Teflon tubing. Measure flow at each end of splitter (*B*) to ensure exact 1:1 split.

(**k**) *Potassium chloride thermionic detector operation.*—Zero recorder with zero control before detector flame is ignited (no signal). Turn on H (ca 30 mL/min) and ignite flame. Adjust H with flame burning to give baseline current (BLC) of 0.2–0.8 × $10^{-8}$ amp. (Sensitivity to P compds is directly related to KCl temp., which depends on H concn in flame.) Select operational electrometer setting and adjust H concn to obtain 40–50% full scale recorder deflection for 2 ng parathion entering detector. When baseline has stabilized, measure BLC precisely, at electrometer setting of 1 × $10^{-8}$ amp full scale. Return to operational electrometer setting and zero recorder pen, using current balance control to "buck out" current generated by detector. Check linearity of system from 0.4 to 4.0 ng parathion. Monitor BLC frequently during operation. If drift occurs, readjust H concn to maintain same BLC. *For accurate quantitation, BLC must be identical during chromatgy of sample and std.*

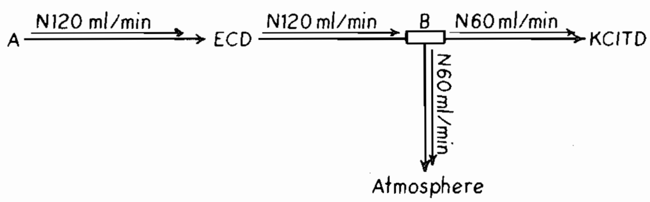


**FIG. 970.52C—In-series split dual detection system**

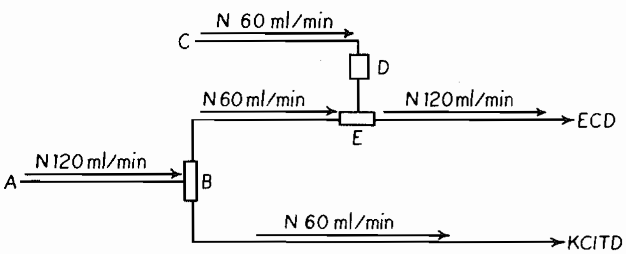


**FIG. 970.52D—Parallel dual detection system**

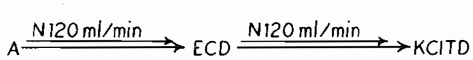


**FIG. 970.52E—In-series dual detection system**

### Concentration Technics

#### *I. Purified Extracts*

(Never evap. purified exts to dryness.)

(**a**) *To approximately 5 mL or more.*—Evap. on steam bath in Kuderna-Danish concentrator fitted with 3-ball Snyder column and vol. flask or graduated collection tube; 20-mesh boiling chip is necessary.

(**b**) *To less than 5 mL.*—Evap. to ca 5 mL as in (**a**). Remove collection tube from concentrator and fit tube with 2-ball micro-Snyder or micro-Vigreux column. Evap. to slightly less than desired vol., permit condensate to drain into tube, and remove column. Min. attainable vol. is 0.2–0.4 mL.

#### *J. Extracts Containing Fats, Oils, or Plant Extractives*

(**a**) *Kuderna-Danish concentrator.*—Fitted with 3-ball Snyder column and vol. flask or graduated collection tube. Use on steam bath.

(**b**) *Flash evaporator.*—Keep flask in $H_2O$ bath at room temp.

(**c**) *Beaker.*—Evap. in beaker in $H_2O$ bath at 35–40° under stream of clean, dry air. Remove from heat and air stream as soon as last of solv. evaps. Let residual $H_2O$ evap. spontaneously. Solvs may be evapd from fats on steam bath for short periods.

### Preparation of Sample and Extraction

#### K. Nonfatty Foods

(*Caution:* See safety notes on blenders, distillation, flammable solvents, toxic solvents, acetonitrile, and petroleum ether.)

Pit *soft fruits*, if necessary. Chop or blend representative sample of *leafy or cole-type vegetables, pitted soft fruits, firm fruits, and roots*. Mix thoroly to obtain homogeneous sample before taking portions for analysis. Grind *dry or low moisture products*, e.g., hays, to pass No. 20 sieve and mix thoroly. Proceed as in **(a)**, **(b)**, **(c)**, or **(d)**.

**(a)** *High moisture (more than 75% $H_2O$) products containing less than 5% sugar.*—*(1) Products other than eggs.*—Weigh 100 g chopped or blended sample into high-speed blender jar, add 200 mL $CH_3CN$ and ca 10 g Celite, and blend 2 min at high speed. Filter with suction thru 12 cm buchner fitted with sharkskin paper into 500 mL suction flask. Transfer filtrate to 250 mL graduate and record vol. ($F$). Transfer measured filtrate to 1 L separator, and proceed as in **(e)**. *(2) Whole eggs.*—Discard shells and blend combined yolks and whites at *low* speed $\geq$5 min or until sample is homogeneous. Low-speed blending will minimize foaming or "whipping" of sample. Weigh $\leq$25 g thoroly mixed yolks and whites into high-speed blender jar, and proceed with addn of $CH_3CN$ as in *(1)*.

**(b)** *High moisture (more than 75% $H_2O$) products containing 5–15% sugar.*—Add 200 mL $CH_3CN$ and 50 mL $H_2O$ to 100 g sample in blender and proceed as in **(a)**. Transfer $\leq$250 mL filtered ext (record vol. ($F$)) to 1 L separator, and proceed as in **(e)**.

**(c)** *High moisture (more than 75% $H_2O$) products containing 15–30% sugar, e.g., grapes.*—Heat mixt. of 200 mL $CH_3CN$ and 50 mL $H_2O$ to 75°, add to 100 g sample in blender, and immediately proceed as in **(a)**. Before filtered ext cools, transfer $\leq$250 mL (record vol. ($F$)) to 1 L separator. Let cool to room temp. and proceed as in **(e)**.

**(d)** *Dry or low-moisture products, e.g., hays.*—Add 350 mL 35% $H_2O$-$CH_3CN$ (350 mL $H_2O$ dild to 1 L with $CH_3CN$) to 20–50 g ground sample in blender (if larger sample is required, add enough addnl extn mixt. to wet sample and permit thoro blending). Blend 5 min at high speed, and proceed as in **(a)**, beginning "Filter with suction . . ." Transfer $\leq$250 mL filtered ext (record vol. ($F$)) to 1 L separator, and proceed as in **(e)**.

**(e)** *Transfer of residues to petroleum ether.*—Carefully measure 100 mL pet ether and pour into separator contg filtrate. Shake vigorously 1–2 min and add 10 mL satd NaCl soln and 600 mL $H_2O$. Hold separator in horizontal position and mix vigorously 30–45 sec. Let sep., discard aq. layer, and gently wash solv. layer with two 100 mL portions $H_2O$. Discard washings, transfer solv. layer to 100 mL g-s cylinder, and record vol. ($P$). Add ca 15 g anhyd. $Na_2SO_4$ and shake vigorously. Do not let ext remain with $Na_2SO_4$ >1 hr or losses of organochlorine pesticides by adsorption may result. Transfer soln directly to Florisil column, **970.52O**, or conc. to 5–10 mL in Kuderna-Danish concentrator for transfer.

**(f)** *Calculation for fruits and vegetables.*—Calc. g sample as $S \times (F/T) \times (P/100)$; where $S$ = g sample taken; $F$ = vol. filtrate; $T$ = total vol. (mL $H_2O$ in sample + mL $CH_3CN$ added − correction in mL for vol. contraction); $P$ = mL pet ether ext; and 100 = mL pet ether into which residues were partitioned. When 50 mL $H_2O$ is added to $CH_3CN$ for extn of high sugar products, total vol., $T$, is increased by 45, *i.e.*, $T$ = 325 instead of 280 for samples contg 85% $H_2O$.

*Example:* 100 g sample contains 85 g $H_2O$; 200 mL $CH_3CN$ is added; vol. contraction is 5 mL. Total vol., $T$, is 280 mL. If vol. filtrate is 235 mL, vol. pet ether ext is 85 mL, and residue is transferred to 100 mL pet ether, then $100 \times (235/280) \times (85/100) = 71$ g sample.

Consult refs on food composition for av. $H_2O$ content. Water content of most fresh fruits and vegetables may be assumed to be 85%.

For 25 g whole eggs and 200 mL $CH_3CN$, use 215 as $T$.

**(g)** *Calculation for dry or low moisture products, e.g., hays.*—Calc. g sample as in fruits and vegetables, **(f)**, except $T$ = total vol. (mL $H_2O$ in sample + mL 35% $H_2O$-$CH_3CN$ added − correction in mL for vol. contraction). If $H_2O$ content of sample is $\leq$10%, disregard and use vol. of extg mixt. as $T$.

#### L. Fat-Containing Foods

(After isolation of fat, proceed with $CH_3CN$ partitioning, **970.52H**.)

**(a)** *Animal and vegetable fats and oils.*—If solid, warm until liq. and filter thru dry filter.

**(b)** *Butter.*—Warm at ca 50° until fat seps and decant fat thru dry filter.

**(c)** *Milk.*—(*Caution:* See safety notes on distillation, flammable solvents, diethyl ether, and petroleum ether.) To 100 mL fluid milk (dil. evapd milk 1 + 1 with $H_2O$) in 500 mL centrf. bottle, add 100 mL alcohol or MeOH and ca 1 g Na or K oxalate, and mix. Add 50 mL ether and shake vigorously 1 min; then add 50 mL pet ether and shake vigorously 1 min. Centrf. ca 5 min at ca 1500 rpm. Blow off solv. layer with wash bottle device, *Notes*, into 1 L separator contg 500– 600 mL $H_2O$ and 30 mL satd NaCl soln. Re-ext aq. residue twice, shaking vigorously with 50 mL portions ether-pet ether (1 + 1); centrf. and blow off solv. layer into separator after each extn. Mix combined exts and $H_2O$ cautiously. Drain and discard $H_2O$. Rewash solv. layer twice with 100 mL portions $H_2O$, discarding $H_2O$ each time. (If emulsions form, add ca 5 mL satd NaCl soln to solv. layer or include with $H_2O$ wash.) Pass ether soln thru column of anhyd. $Na_2SO_4$, 50 × 25 mm od, and collect eluate in 400 mL beaker. Wash column with small portions pet ether and evap. solv. from combined exts at steam bath temp. under air current to obtain fat.

**(d)** *Cheese.*—Place 25–100 g (to provide 3 g fat) diced sample, ca 2 g Na or K oxalate, and 100 mL alcohol or MeOH in high-speed blender and blend 2–3 min. (If experience with product indicates emulsions will not be broken by centrfg, add 1 mL $H_2O$/2 g sample before blending.) Pour into 500 mL centrf. bottle, add 50 mL ether, and shake vigorously 1 min; then add 50 mL pet ether and shake vigorously 1 min (or divide between two 250 mL bottles and ext each by shaking vigorously 1 min with 25 mL each ether). Proceed as in **(c)**, beginning "Centrf. ca 5 min at ca 1500 rpm."

**(e)** *Fish.*—(*Caution:* See safety notes on blenders, distillation, flammable solvents, and petroleum ether.)

Weigh 25–50 g thoroly ground and mixed sample into high-speed blender. (If fat content is known or can be estd, adjust sample size so that max. of ca 3 g fat will be extd.) Add 100 g anhyd. $Na_2SO_4$ to combine with $H_2O$ present and disintegrate sample. Alternately blend and mix with spatula until sample and $Na_2SO_4$ are well mixed. Scrape down sides of blender jar and break up caked material with spatula. Add 150 mL pet ether and blend at high speed 2 min. Decant supernate pet ether thru 12 cm buchner, fitted with 2 sharkskin papers, into 500 mL suction flask. Scrape down sides of blender jar and break up caked material with spatula. Re-ext residue in blender jar with two 100 mL portions pet ether and blend 2 min each time. (After blending 1 min, stop blender, scrape down sides of blender jar, and break up caked material with spatula; continue blending 1 min.) Scrape down sides of blender jar and break up caked material between extns. Decant supernate pet ether from repeat blendings thru buchner and combine with first ext. After last blending, transfer residue from blender jar to buchner, and rinse blender jar and material in buchner with three 25–50 mL portions pet ether. Immediately after last rinse, press residue in buchner with bottom of beaker to force out remaining pet ether. Pour combined exts thru 40 × 25 mm od column of anhyd. $Na_2SO_4$ and collect eluate in 500 or 1000 mL Kuderna-Danish concentrator with plain tube. Wash flask and column with small portions pet ether and evap. most of pet ether from combined exts and rinses in Kuderna-Danish concentrator. Transfer fat soln to tared beaker, using small amts pet ether. Evap. pet ether at steam bath temp. under current of dry air to obtain fat. When pet ether is completely removed, weigh and record wt of fat extd.

Record wt of fat taken for cleanup. ((Wt fat for cleanup/wt fat extd) × wt original sample = wt sample analyzed.) If it is known that ≤3 g fat will be extd from particular sample, do not isolate and weigh fat before $CH_3CN$ partitioning. Detn is then on basis of wt of original sample.

*Notes:* To siphon off ether, use tube similar to delivery tube of ordinary wash bottle but with intake end bent up into U shape in opposite direction to outlet end, with opening 6–12 mm higher than bottom of U, cut off horizontally. (Avoid excessive constriction when bending.) Set delivery tube loosely enough in stopper that it can be raised or lowered. In operating, adjust opening of U bend to ca 3 mm above surface of aq. layer and blow ether layer off by gently blowing thru mouthpiece tube inserted in adjacent hole in stopper.

### M. Soil

(Official final action (1976) for aldrin, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT, dieldrin, endrin, heptachlor, heptachlor epoxide, lindane, and *p,p'*-TDE (DDD))

Weigh 10.0 g undried soil, sieved thru 2 mm sieve and mixed thoroly, into 250 mL erlenmeyer. Add 7 mL *0.2M $NH_4Cl$ soln* (10.7 g/L) and let stand 15 min. Add 100 mL hexane-acetone (1 + 1), stopper tightly, and shake overnight (≥12 hr) on reciprocal or wrist-action shaker at 180 rpm.

Carefully pour supernate, avoiding aq.-clay phase, thru 2–3 cm column (22 mm id) of Florisil, **970.52B(i)**, and collect eluate in 1 L separator. Rinse flask and soil with two 25 mL portions hexane-acetone and decant thru column. Rinse column with 10 mL hexane-acetone.

Add 200 mL $H_2O$ to separator and shake gently ca 30 sec. Drain aq. phase into second separator and ext with 50 mL hexane. Combine hexane layers in first separator and wash with 100 mL $H_2O$. Drain and discard $H_2O$. Pour hexane thru 2 cm column (22 mm id) $Na_2SO_4$, conc. to 100 mL, and make preliminary injection of 5–10 μL into gas chromatograph. If peaks are present at retention times of DDE or dieldrin, conc. to 10 mL in Kuderna-Danish concentrator, **970.52I(a)**, and sep. DDE or dieldrin as in **970.52O**. (This cleanup may also be necessary with exts from high org. matter soils.) Proceed as in **970.52Q**, using ECD, **(b)**. To calc. to dry basis, dry sep. sample of 10 g ca 16 hr at 105° to obtain % solids.

Refs.: JAOAC **51**, 403, 472(1968); **56**, 728(1973); **57**, 604(1974).

## Cleanup Technics

### N. Acetonitrile Partitioning

(*Caution:* See safety notes on distillation, flammable solvents, and petroleum ether. Different fats and oils may show varying tendencies to emulsion formation.)

Weigh ≤3 g fat into 125 mL separator, and add pet ether so that total vol. of fat and pet ether is 15 mL. Add 30 mL $CH_3CN$ satd with pet ether, **970.52B(b)**, shake vigorously 1 min, let layers sep., and drain $CH_3CN$ into 1 L separator contg 650 mL $H_2O$, 40 mL satd NaCl soln, and 100 mL pet ether. Ext pet ether soln in 125 mL separator with 3 addnl 30 mL portions $CH_3CN$ satd with pet ether, shaking vigorously 1 min each time. Combine all exts in the 1 L separator.

(If experience with particular sample (e.g., fish) indicates that cleanup may not be sufficient, perform partitioning as follows: Drain $CH_3CN$ phase from first partitioning into second 125 mL separator contg 15 mL pet ether, shake vigorously 1 min, let layers sep., and drain $CH_3CN$ into 1 L separator contg 650 mL $H_2O$, 40 mL satd NaCl soln, and 100 mL pet ether. Pass $CH_3CN$ phase from each of 3 addnl partitionings thru same 15 mL pet ether in 125 mL separator. Shake vigorously each time and combine $CH_3CN$ exts in the 1 L separator.)

Hold separator in horizontal position and mix thoroly 30–45 sec. Let layers sep. and drain aq. layer into second 1 L separator. Add 100 mL pet ether to second separator, shake vigorously 15 sec, and let layers sep. Discard aq. layer, combine pet ether with that in original separator, and wash with two 100 mL portions $H_2O$. Discard washings and draw off pet ether layer thru 50 × 25 mm od column of anhyd. $Na_2SO_4$ into 500 mL Kuderna-Danish concentrator. Rinse separator and then column with three ca 10 mL portions pet ether. Evap. combined ext and rinses to ca 10 mL in Kuderna-Danish concentrator for transfer to Florisil column.

### O. Florisil Cleanup

(*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, diethyl ether, and petroleum ether.)

Prep. 22 mm id Florisil column, **970.52E(b)**, contg 10 cm, after settling (or amt detd by lauric acid test, **970.52B(i)**), of activated Florisil topped with ca 1 cm anhyd. $Na_2SO_4$. Prewet column with 40–50 mL pet ether. Place Kuderna-Danish concentrator with vol. flask or graduated collection flask under column to receive eluate. Transfer pet ether ext or conc. to column, letting it pass thru at ≤5 mL/min. Rinse containers and $Na_2SO_4$, if present, with two ca 5 mL portions pet ether, pour rinsings onto column, rinse walls of tube with addnl small portions pet ether, and elute at ca 5 mL/min with 200 mL 6% eluting solv., **970.52B(e)**. Change receivers and elute with 200 mL 15% eluting solv., **970.52B(f)**, at ca 5 mL/min. Change receivers and elute with 200 mL 50% eluting solv., **970.52B(g)**, at ca 5 mL/min.

Conc. each eluate to suitable definite vol. in Kuderna-Danish concentrator. When vol. <5 mL is needed, use 2-ball micro-Snyder or micro-Vigreux column.

First eluate (6%) contains *organochlorine pesticides* (aldrin, BHC, DDE, DDD (TDE), *o,p'*- and *p,p'*-DDT, heptachlor, heptachlor epoxide, lindane, methoxychlor, mirex, and ethylan), *industrial chems* (polychlorinated biphenyls (PCB)), and *organophosphorus pesticides* (ethion and ronnel) and is usually suitable for GC directly. If further cleanup is necessary, repeat Florisil cleanup, using new column. Second eluate (15%) contains *organochlorine pesticides* (dieldrin and endrin) and *organophosphorus pesticides* (diazinon, Me parathion, and parathion). If further cleanup is necessary, det. organophosphorus pesticides by GC and TLC; then proceed with *Magnesia Cleanup*, **970.52P**, and/or *Saponification*, **970.52Q**, which are applicable only to organochlorine pesticides in 15% eluate (organophosphorus pesticides are degraded). Third eluate (50%) contains *organophosphorus pesticide* malathion.

### P. Magnesia Cleanup

(Applicable only to organochlorine pesticides in 15% eluate when addnl cleanup is necessary)

Transfer ca 10 g MgO-Celite mixt., **970.52B(l)**, to chromatgc tube without stopcock, **970.52E(c)**, using vac. to pack. Prewash with ca 40 mL pet ether, discard prewash, and place Kuderna-Danish concentrator under column. Transfer 15% Florisil eluate, concd to ca 5 mL, to column, rinsing with small portions pet ether. Force pet ether into column with slight vac. or pressure. Then elute with 100 mL pet ether. Conc. eluate to suitable vol. Proceed with detn, or saponification, if required.

### *Q. Saponification–First Action*

(Applicable only to those chems stable to hot alkali treatment. Use as supplemental cleanup if 15% eluate or MgO–Celite eluate is not substantially free from oily materials.)

Conc. pet. ether–ether (85 + 15) fraction under current of air to 2 mL, add 1 mL 2% alc. KOH, attach micro-Snyder column, and carefully reduce to ≤1 mL on steam bath. Reflux sample 15 min, remove, and cool. Add 2 mL alcohol–$H_2O$ (1 + 1) and 5 mL hexane, and shake 1 min. Centrf. to sep. layers. Transfer as much hexane layer as possible to second tube, using disposable Pasteur pipet, and repeat extn with 5 mL hexane. Conc. combined hexane to appropriate vol. for GC analysis.

## *Detection Methods*

### *R. Gas Chromatography—Tentative Identification and Quantitative Measurement*

(Applicable to organochlorine pesticides, organophosphorus pesticides, and polychlorinated biphenyls (PCB). Method is applicable to PCB residues when present alone in sample. If pesticidal or other compds are detected in chromatogram of the PCB residue, other chemical or physical operations must be applied to eliminate or minimize their interference before PCB quantitation.)

Inject suitable aliquot (3–8 μL) of concd eluate from Florisil or MgO-Celite column contg amt of compd within linear range into gas chromatograph, **970.52H**, using 10 μL syringe. Tentatively identify residue peaks on basis of retention times. Measure area or ht of residue peak(s) and det. residue amt by comparison to peak area or ht obtained from known amt of appropriate ref. material(s). To ensure valid measurement of residue amt, size of peaks from residue and ref. std should be within ±25%. Chromatograph ref. material(s) immediately after sample.

Measure PCB residues by comparing total area or ht of residue peaks to total area or ht of peaks from appropriate Aroclor(s) (Analabs, Inc.) ref. materials. Measure total area or ht response from common baseline under all peaks. Use only those peaks from sample that can be attributed to chlorobiphenyls. These peaks must also be present in chromatogram of ref. material. Mixt. of Aroclors may be required to provide best match of GC patterns of sample and ref.

Alternatively, det. PCB residues by individual peak area comparisons using Aroclor ref. material wt factors in Table **970.52B**. Calc. each PCB peak against appropriate individual ref. peak with exactly same absolute retention. Sum individual peak values to obtain total ppm PCB. (This method is recommended for PCB residues with chromatgc patterns which are altered extensively from that of any Aroclor ref.)

(**a**) *Recommended operating conditions for 10% DC-200 or OV-101 column.*—Glass column, 1.8 m (6′) × 4 mm id. Temps (°): injector, 225; column 200; $^3$H electron capture detector, 210 max.; carrier gas flow, 120 mL N/min.

(**b**) *Electron capture detection (ECD).*—(Use for detn of organochlorine pesticides in fruits, vegetables, and food contg fats and for detn of PCB in foods and paperboard.) Select for $^3$H electron capture detector operating voltage that voltage (ca 50 v dc) at which 1 ng heptachlor epoxide produces 40–50% full scale recorder deflection at 1 or 3 × $10^{-9}$ amp full scale sensitivity.

Operate $^{63}$Ni electron capture detector to produce stable, reproducible, linear response, and adjust amt of injected sample to accommodate differences in instrument sensitivity.

(**c**) *Potassium chloride thermionic and electron capture dual detection.*—(Use one of the 3 dual detection systems specified in **970.52H(h)**, (**i**), (**j**), for detn of organophosphorus and organochlorine pesticides and PCB. In-series system, (**h**), is preferred because of simplicity and ease of operation.) (*1*) *In-series dual detection.*—Operate ECD as in (**b**). For KCITD, adjust H flow producing 0.2–0.8 × $10^{-8}$ amp baseline current and select electrometer setting at which 2 ng parathion produces 40– 50% full scale recorder deflection. (*2*) *In-series split dual detection.*—Same as (*3*), *Parallel*, except ECD receives entire injection and KCITD receives ½ amt injected into column. (*3*) *Parallel dual detection.*— Same as (*1*), *In-series dual*, except column effluent is split; therefore, inject twice as much sample to obtain desired limit of quantitation.

**Table 970.52B Weight % Factors for Individual Gas Chromatographic Peaks in Aroclor Reference Materials**
**(Peaks are identified by their retention time relative to *p,p′*-DDE=100 at conditions consistent with 970.52R(a) and (b))**

| | AROCLOR | | | | |
|---|---|---|---|---|---|
| $R_{DDE}$(100×) | 1016 (77-029)[a] | 1242 (71-696)[a] | 1248 (71-697)[a] | 1254 (71-698)[a] | 1260 (71-699)[a] |
| 11 | 0.2 | | | | |
| 16 | 3.8 | 3.4 | 0.3 | | |
| 21 | 8.1 | 10.3 | 1.1 | | |
| 24 | 1.2 | 1.1 | 0.2 | | |
| 28 | 16.8 | 15.8 | 6.0 | | |
| 32 | 7.6 | 7.3 | 2.6 | | |
| 37 | 18.5 | 17.0 | 8.7 | | |
| 40 | 14.6 | 13.0 | 7.4 | | |
| 47 | 11.6 | 9.9 | 15.7 | 7.1 | |
| 54 | 7.7 | 7.1 | 9.3 | 2.7 | |
| 58 | 6.4 | 4.4 | 8.3 | 1.2 | |
| 70 | 3.4 | 8.7 | 18.2 | 14.7 | 2.4 |
| 78 | | 1.9 | 6.4 | | |
| 84 | | | 4.6 | 18.6 | 3.6 |
| 98 | | | 3.4 | 8.3 | |
| 104 | | | 3.3 | 14.1 | 2.8 |
| 112 | | | 1.0 | | |
| 117 | | | | | 4.4 |
| 125 | | | 2.3 | 15.6 | 11.0 |
| 146 | | | 1.2 | 9.0 | 13.3 |
| 160 | | | | | 5.5 |
| 174 | | | | 7.4 | 10.0 |
| 203 | | | | 1.3 | 10.9 |
| 232-244 | | | | | 11.2 |
| 280 | | | | | 12.5 |
| 332 | | | | | 4.2 |
| 360-372 | | | | | 5.4 |
| 448 | | | | | 0.8 |
| 528 | | | | | 2.0 |

[a] Food and Drug Administration Lot Nos. (Wt factors are valid only for these FDA Lot Nos.) Aroclor ref. materials are available from Food and Drug Administration, Division of Contaminants Chemistry, HFF-420, 200 C St SW, Washington, DC 20204.

## *Thin Layer Chromatography—Confirmation of Identity*

### *Method I*

(Applicable to organochlorine and organophosphorus pesticides except where indicated)

### S. Preparation of Adsorbent Layer

Before coating, wash plates in hot soapy water and thoroly rinse with distilled $H_2O$. Press plates snugly into position on mounting board that has retaining ledge on one side and one end. Plastic board is mounted so that long side with raised ledge faces operator while short side with ledge is to right of operator. Before coating, wipe plates with few mL alcohol. Position applicator, trough open, with left edge 6 mm in from edge of first plate to be coated.

To coat 5 plates, weigh 30 g $Al_2O_3$ G, **970.52C(a)**, into 250 mL ₮ erlenmeyer. Add 50 mL $H_2O$, stopper, and shake moderately 45 sec. Violent shaking produces bubbles, resulting in "pock-marked" layer.

*Suspensions that contain adsorbents with binders set rapidly, and entire operation from prepn of slurry to final coating must be completed within 2 min.*

After shaking, immediately pour slurry into applicator chamber. Rotate chamber by turning large lever handle thru 180°. After few sec, slurry begins to flow out of exit slit. Grasp applicator with both hands and pull it manually with steady motion across series of plates. Approx. 5 sec is required for actual coating operation. Immediately after application, tap edge of mounting board or shake entire board gently to smooth out slight ripples or imperfections in wet coating.

Let coated plates dry in position on mounting board 15 min. Then dry plates in forced-draft oven 30 min at 80°. Remove plates and cool.

Examine plates carefully in transmitted and reflected light for imperfections or irregularities in coating. Discard any plates showing extensive rippling or mottling of layer.

Prep. 5 more plates while first set is drying. Be sure applicator is thoroly cleaned and dried before reusing. The 10 coated and dried plates may be prewashed immediately.

### T. Prewashing of Adsorbent Layer

Scrape 1 cm of adsorbent off edge of plate with razor blade. Pour 15 mL 50% aq. acetone into metal trough inside chromatgc tank. Cut out 2 × 20 cm strip of Whatman No. 1 filter paper, wet with solv., and place over scraped off portion with 6 mm overlapping adsorbent layer. Place plate in chromatgc tank, seal tank with masking tape, and develop with 50% aq. acetone to within 4 cm from top of plate (75–90 min). Remove plate from tank, remove filter paper wick, invert plate, and dry in hood 5 min. Dry plate 45 min at 80°. Remove plate from oven, cool, and store in desiccator. Use prepd plates within 1 week after prepn.

### U. Sample Spotting

Make pencil mark 4 cm from bottom of plate at both sides. Imaginary line between the two points indicates sample spotting or origin "line." Draw line (which removes coating) completely across plate 14 cm from bottom edge; this line represents solv. front after development. On lower edge of adsorbent starting 2 cm in from left edge of plate, make 18 marks with pencil at 1 cm intervals. (Fewer marks with longer intervals may be used, if desired. Marks serve as horizontal guides to sample application. Identity of samples and stds may be etched directly into adsorbent layer above these marks above solv. front line.)

Imaginary spotting "line" is actually shadow line cast by strong light source from wooden ruler supported 2 cm above plate. Align ruler shadow on the two 4 cm marks on either edge of plate. Shadow line and 18 marks, resp., serve as vertical and horizontal guides for sample application.

For optimum semiquant. detn, spot aliquot of sample as follows:

(**a**) *Organochlorine pesticides.*—Adjust aliquot to give residue spot within range 0.005–0.1 μg. Spot stds and std mixts at 0.002, 0.005, 0.01, 0.02, 0.05, 0.1, and 0.2 μg. Sample spots >0.2 μg are difficult to det. quant. and <0.005 μg may be difficult to distinguish. Spot all 6% Florisil eluates on one plate and 15% Florisil eluates on another plate.

(**b**) *Organophosphorus pesticides.*—Adjust aliquots of sample and stds to give spot within range 0.1–0.5 μg. Spot 6, 15, and 50% Florisil eluates on same plate. Ronnel and ethion are not resolved; spot each std sep. Spot diazinon, Me parathion, and malathion sep. or as mixt.

Vol. of sample ext spotted should be ≤10 μL, if possible, and spotting should be done repeatedly with 1, 2, or 3 μL Kontes spotting pipet. Spot std and sample solns with same pipet. For best results, keep size of spotted samples as small as possible.

### V. Development

(**a**) *Organochlorine pesticides.*—Place liners and metal trough in tank, **970.52F(f)**. Presat. liner by pouring 75 mL developing solv., **970.52C(b)**, into bottom of tank ≥30 min before developing plate. Presatn decreases development time and improves uniformity of $R_f$ values.

For plates spotted with 6% Florisil eluates, pour 50 mL *n*-heptane into trough. Place lower edge of plate in metal trough with top of plate leaning against side of tank. Place glass cover plate on tank and seal with masking tape.

For plates spotted with 15% Florisil eluates, use acetone-*n*-heptane (2 + 98) as developing solv.

(**b**) *Organophosphorus pesticides.*—Prep. chromatgc tank, **970.52F(f)**, after samples and stds have been spotted on plate. Place liners and metal trough in tank. Pour 50 mL methylcyclohexane, **970.52C(d)**(*2*), into trough, and 75 mL into bottom of tank. Quickly fill dipping tank, **970.52F(g)**, to within 4–5 cm from top with immobile solv., **970.52C(d)**(*1*). Invert plate and dip with uncoated side touching back wall of tank to prevent front wall from scraping the adsorbent layer during dipping operation. Dip plate *just* to spotting line, remove, and immediately place in metal trough, with top portion of plate leaning against side of tank. Place glass cover plate on tank and seal with masking tape.

When solv. front in (**a**) or (**b**) *just* reaches pencil line 10 cm above spotting "line," remove plate and dry in hood 5 min.

### W. Spraying

(*Caution:* See safety notes on spraying chromatograms.)

(**a**) *Organochlorine pesticides.*—Support plate on one side and spray fairly heavily with chromogenic agent, **970.52C(c)**, using lateral motions of spray bottle perpendicular to direction of solv. flow. Spray until plate appears translucent or soaked with reagent. Underspraying will result in poor sensitivity. After spraying, dry plate in hood 15 min; then immediately place under UV light source and proceed as in **970.52X**.

(**b**) *Organophosphorus pesticides.*—Immediately spray plate moderately heavily and uniformly with dye soln, **970.52C(e)**(*2*), using lateral motions of spray flask, **970.52H(j)**, perpendicular to direction of solv. flow. Plate should be vivid blue after spraying. Using spray bottle, **970.52H(i)**, overspray plate lightly and uniformly with $AgNO_3$ soln, **970.52C(e)**(*3*) (at this point plate should be bluish purple and spots should be discernible).

After 2 min, overspray plate moderately and uniformly with citric acid soln, **970.52C(e)**(*4*), using spray bottle, **970.52H(i)**. After spraying, thiophosphate pesticides should immediately appear as vivid blue or purple spots against yellow background. Color of spots reaches max. intensity ca 5–10 min after citric acid spraying. After ca 10 min, background begins to change from yellow to greenish blue, masking spots. At this point, respraying plate with citric acid soln changes background back to yellow and makes spots stand out as well as or better than originally. Evaluate chromatogram ≤10 min after respraying. Blue spots fade completely and irreversibly after 30–40 min from time of original citric acid spraying.

### X. Exposure

(*Caution:* See safety notes on hazardous radiations.)

Expose plate to UV light until spot for std of lowest concn appears; 5 ng of most organochlorine pesticides should be visible after 15–20 min exposure with equipment described under **970.52H(1)**. Exposure times >30 min will not harm plates. For best results, place plates 8 cm from bottom edge of lamps.

## Method II

(Applicable only to organochlorine pesticides)

### Y. Preparation of Adsorbent Layer

Weigh 40 g $Al_2O_3$ G, **970.52C(a)**, into 500 mL centrf. bottle. Add 80 mL 0.2% $HNO_3$, shake well, and centrf. at ca 1200 rpm 1–2 min. Decant supernate into 100 mL graduate, and record vol. (35–40 mL should be recovered). Add 80 mL $H_2O$, breaking up material on bottom of centrf. bottle with glass rod, if necessary. Shake well and centrf. as before. Decant and record vol. supernate recovered (60–70 mL). Add 2 addnl 80 mL portions $H_2O$, shake well, centrf., and decant.

Weigh the $Al_2O_3$ and $H_2O$ that has been retained. (Wt should be ca 100 g.) Add 10 mL 1% $AgNO_3$ soln and enough $H_2O$ to make total wt 120–130 g. Shake well, place in applicator, and prep. plates as in **970.52S**. Let plates air dry in position on mounting board 15 min. Place in metal drying rack, in vertical position, 30 min at 100°.

### Z. Sample Spotting

Spot as in **970.52U**. Draw line across plate 4 cm from top (which removes coating). Next, scrape 6 mm of coating from each side of plate. (Irregularities in thickness of coating on these outer edges cause uneven flow of mobile solv.) Make pencil mark at each side of layer 2.5 cm from bottom of plate; imaginary line between these 2 points indicates sample spotting line. Spot samples and stds at 1 cm intervals.

### AA. Development and Exposure of Plates

(*Caution:* See safety notes on hazardous radiations.)

Develop plates as in **970.52V**, except use only 25–30 mL mobile solv. in trough, since spotting line has been lowered to 2.5 cm. Use *n*-heptane to develop 6% Florisil eluates, and acetone-*n*-heptane (2 + 98) for 15% Florisil eluates.

Plates may be exposed to UV light after short drying period (ca 5 min) after removal from tank. Spots of aldrin, DDE, and isomers of DDT will appear within 5–10 min after exposure; lindane, endrin, dieldrin, and all others will require more time. Plates may be exposed 1.5–2 hr without appreciable darkening of background.

### BB. Paper Chromatography*

*See* **29.028**, 13th ed.

Refs.: JAOAC **42,** 734(1959); **44,** 171(1961); **46,** 186(1963); **48,** 668(1965); **49,** 460, 463, 468(1966); **50,** 430, 623, 1205(1967); **51,** 311, 666, 892(1968); **52,** 1280(1969); **53,** 152, 355, 1300(1970); **54,** 325, 525(1971); **55,** 284(1972); **56,** 721, 1015(1973); **59,** 169(1976); **61,** 282(1978); **63,** 277(1980).

## 985.22 Organochlorine and Organophosphorus Pesticide Residues

### Gas Chromatographic Method

**First Action 1985**
**Final Action 1986**

(Applicable to residues of acephate, α-BHC, chlorpyrifos, dieldrin, monocrotophos, and omethoate in lettuce, strawberries, and tomatoes)

### A. Principle

Nonfatty sample is blended with acetone and filtered; pesticides are transferred from aq. filtrate to org. phase by shaking with pet ether and $CH_2Cl_2$; after drying, org. phase is concd in presence of pet ether and then acetone to remove $CH_2Cl_2$; aliquot of concd org. phase is injected into various GC systems for detn of wide variety of pesticide residues.

Absence of cleanup steps permits examination for residues of many chem. types, including many that would not be recovered thru methods requiring Florisil or charcoal column chromatgc step.

### B. Reagents and Apparatus

(**a**) *Solvents.*—Acetone, $CH_2Cl_2$, pet. ether, distd in glass (Burdick & Jackson Laboratories), or equiv.

(**b**) *Sodium sulfate.*—Anhyd., granular.

(**c**) *Glass wool.*—Rinse with acetone and alcohol several times and dry. Washed glass wool will be somewhat brittle.

(**d**) *High-speed blender.*—Waring Blendor, or equiv.

(**e**) *Kuderna-Danish concentrator.*—500 mL with Snyder column and fitted with vol. flask or graduated receiving tube. Calibrate receiving tube with acetone delivered from a buret. Use buret-corrected vol. for sample wt calcn.

(**f**) *Separatory funnels.*—1 L, with Teflon stopcocks.

(**g**) *Gas chromatograph.*—(*1*) *For organochlorine residues.*—Instrument contg any suitable methyl silicone column, such as 2% OV-101, on 80–100 mesh Chromosorb W (HP), 6 ft × 2 mm id glass, and Hall 700A HECD halogen-specific detector. Column, 200°; He carrier cas, 60 mL/min; detector 900°, H reaction gas 60–100 mL/min; *n*-propanol solv. 0.35 mL/min; electrometer range 10 in OPR/FLT mode; attenuation 5. (*2*) *For organophosphorus residues.*—Instrument with column contg 2% stabilized DEGS on 80–100 mesh Chromosorb W (HP), 4 ft × 2 mm id silanized glass, and P-specific flame photometric detector (526 nm filter). Column 180°; detector 200°; He carrier gas, 60 mL/min. Condition column (disconnect detector) by passing carrier gas thru column 0.5 h at ≤80°. Program temp. at 1–2°/min to 230° and hold overnight. Establish stable flame at electrometer setting that will produce 50% full scale deflection for 1.5 ng chlorpyrifos and 6 ng monocrotofos. If necessary, increase air/O until ≥50% response. Baseline noise should be <2%.

(**h**) *Reference std materials.*—Acephate, BHC, chlorpyrifos, *p,p'*-DDT, dieldrin, methamidophos, monocrotophos, and omethoate (U.S. Environmental Protection Agency, Pesticides and Industrial Chemical Respository (MD-8), Research Triangle Park, NC 27709). Prep. all stds in acetone. *Mixed stds.*—For Hall system, std soln should contain at least chlorpyrifos, dieldrin, and *p,p'*-DDT. For flame photometric detector, std soln should contain at least methamidophos and chlorpyrifos. Do not use mixed std solns for quant. of unknowns.

(**i**) *Std solns.*—Prep. all stock solns and dilns in glass-distd acetone. Prep. GC std solns so 4 μL injection causes 30–70% full scale deflection in properly functioning system. Suggested concns are given below. Check responses before beginning analysis. Store all std solns in tightly stoppered containers in refrigerator. Let equilibrate 1 h at room temp. before using.

| Compd | ng/μL |
|---|---|
| acephate | 0.5 |
| α-BHC | 0.1 |
| chlorpyrifos | 0.5 |
| dieldrin | 0.2 |
| *p,p'*-DDT | 0.5 |
| methamidophos | 0.2 |
| monocrotophos | 2.0 |
| omethoate | 2.0 |

### *C. Preparation of Sample*

Chop or blend fruits and vegetables and mix thoroly. Weigh 100 g chopped or blended sample into high-speed blender jar, add 200 mL acetone, and blend 2 min at high speed. *Do not* add Celite. Filter with suction thru 12 cm Buchner funnel fitted with sharkskin paper. (*Note*: Rinse filter paper with acetone before filtration of sample to remove artifacts that can interfere with analysis.) Collect ext in 500 mL suction flask. Filtration is normally complete in <1 min. Continuation of vac. for excessive period can reduce vol. of ext and cause error in calcn.

Place 80 mL sample ext in 1 L sep. funnel, and add 100 mL pet ether and 100 mL $CH_2Cl_2$. Shake vigorously 1 min. Transfer lower aq. layer to second 1 L sep. funnel. Dry upper org. layer in first sep. funnel by passing thru ca $1^1/_2$ in. $Na_2SO_4$ supported on washed glass wool in 4 in. funnel, collecting in 500 mL Kuderna-Danish concentrator fitted with vol. flask or calibrated receiving tube. To sep. funnel with aq. phase, add 7 g NaCl and shake vigorously 30 s until most NaCl is dissolved. Add 100 mL $CH_2Cl_2$, shake 1 min, and dry lower org. phase thru same $Na_2SO_4$. Ext aq. phase with addnl 100 mL $CH_2Cl_2$, and dry as above. Rinse $Na_2SO_4$ with ca 50 mL $CH_2Cl_2$. Attach Snyder column on Kuderna-Danish concentrator (boiling chips may be added) and start evapn slowly by placing only receiver tube into steam. After 100–150 mL has evapd, concentrator may be exposed to more steam. When liq. level in hot concentrator tube is ca 2 mL, add 100 mL pet ether thru Snyder column and reconc. to ca 2 mL. Add 50 mL pet ether and repeat concn step. Add 20 mL acetone and reconc. to ca 2 mL. Do not let soln go to dryness during any concn step. Adjust vol. of ext to suitable definite vol. with acetone.

*Calculation of equivalent sample weight*.—Calc. equiv. sample wt in final soln as follows:

$$\frac{\text{mg sample equiv.}}{\mu\text{L final ext}} = 100 \times \frac{80}{200 + W - 10} \times \frac{1}{\text{mL final vol.}}$$

where 200 = mL acetone blended with 100 g sample; $W$ = amt (mL) $H_2O$ present in sample; and 10 = adjustment for water-acetone vol. contraction. Thus, when sample contains 85% $H_2O$ (85 mL/100 g) and final ext vol. is 7 mL, each μL contains:

$$100 \times \frac{80}{200 + 85 - 10} \times \frac{1}{7}$$

$$= 4.15 \text{ mg sample equiv.}/\mu\text{L final ext}$$

### *D. Determination*

Check that both GC systems are working properly by injecting mixed std soln into each. Inject ca 12 mg sample equiv. into each system. Tentatively identify any GC responses on basis of retention times. Quantitate residue peak(s) by area comparison with that obtained from known amt of ref. material(s). To ensure valid measurement of residue amt, area of peaks from residue and ref. std should be within ±25%. *Caution:* Repeated injection of sample exts which have had min. cleanup can be detrimental to GC columns. Replace packing material at front of GC columns as needed to maintain chromatgc quality and prolong column life.

Refs.: JAOAC **68,** 64(1985); **70,** 329(1987).

CAS-30560-19-1 (acephate)
CAS-319-84-6(α-BHC)
CAS-2921-88-2 (chlorpyrifos)
CAS-60-57-1 (dieldrin)
CAS-50-29-3 (DDT)
CAS-1113-02-6 (omethoate)
CAS-10265-92-6 (methamidophos)
CAS-6923-22-4 (monocrotophos)

## ORGANOCHLORINE RESIDUES

## 983.21 Organochlorine Pesticide and Polychlorinated Biphenyl Residues in Fish

**Gas Chromatographic Method**

**First Action 1983**
**Final Action 1985**

### *A. Principle*

Chlorinated pesticides and polychlorinated biphenyls (PCBs) are extd from prepd fish sample with pet. ether, cleaned up on Florisil column, and detd by GC against ref. stds.

See **970.52B, E, H–J** for general app., reagents, and technics.

### *B. Apparatus*

(**a**) *Gas chromatograph*.—With on-column injection system, 6 ft glass column (4 mm id), packed with 10% DC-200 on 80–100 mesh Chromosorb WHP, and electron capture detector. Other liq. phases such as 5% OV-101 on suitable supports may be substituted if known to give adequate resolution for compds present in samples.

Linearized $^{63}$Ni detector capable of producing $^1/_2$ scale deflection for 1 ng heptachlor epoxide is suggested; however, other equiv. electron capture detectors may be used. Operate GC in accordance with manuf. directions, adjusting to provide necessary response and resolution.

(**b**) *Chromatographic tube*.—10 mm id × 300 mm column with Teflon stopcock, coarse fritted disk, ₮ 24/40 top joint (Kontes Glass Co. K-420550, or equiv.).

(**c**) *Kuderna-Danish concentrators*.—Snyder distg column (Kontes K-503000-0121); 125 mL K-D flask (Kontes K-570001-9010) (special item) ₮ 19/22 lower joint; 10 mL concentrator tube (Kontes K-570050-1025).

(**d**) *Micro Snyder column*.—Kontes K-569251, ₮ 19/22.

### *C. Reagents*

(**a**) *Florisil*.—PR grade, 60–80 mesh (Floridin Co.). Must meet **970.52B(i)** specifications.

(**b**) *Solvents*.—Pet. ether, ethyl ether, hexane, and alcohol, known to be suitable for pesticide residue detn.

(**c**) *Glass wool* (*Pyrex*).—Must be free of interference with electron capture detection.

(**d**) *Sodium sulfate*.—Anhyd., granular, reagent grade, free of interference with electron capture detection.

### *D. Extraction*

Weigh 20 g thoroly ground and mixed sample into metal blender cup. Moisten 40 g granular $Na_2SO_4$ with pet. ether and add to sample. Mix sample, using stirring rod, let stand 20 min, and mix again. Add 100 mL pet. ether to sample and blend 1–2 min. (Lourdes blender in series with rheostat set at 40–60%, or equiv., may be used.) Centrf. balanced sample cup 1–2 min at ca 2000 rpm to obtain clear pet. ether ext. Place glass wool plug in funnel, overlay with 20 g granular $Na_2SO_4$, and place funnel in 250 mL vol. flask. Decant pet.

ether ext thru $Na_2SO_4$ into vol. flask. Mix sample again with stirring rod, add 100 mL pet. ether, and ext as before. Repeat using 70 mL pet. ether. Dil. to vol. with pet. ether.

Transfer 25 mL aliquot to tared 100 mL flat bottom extn flask. Place flask on steam bath to evap. solv., leave addnl 30 min on steam bath, remove, and cool. Weigh flask and det. % fat in fish.

For fish contg <10% fat, transfer 25 mL aliquot to 125 mL K-D concentrator. For fish contg >10% fat, take aliquot contg not >200 mg fat. Add several granules of 20–30 mesh carborundum and conc. to ca 3 mL on steam bath. Let cool and remove Snyder column. Rinse concentrator with two 1 mL portions of pet. ether and, using only current of air, conc. sample to 3 mL for transfer to Florisil column.

### E. Florisil Cleanup

Use 4 g Florisil adjusted for lauric acid value (JAOAC **51,** 29(1968)). Add Florisil to 300 × 10 mm id chromatgc tube and add $Na_2SO_4$ to ht 2 cm above Florisil. Completely open stopcock, tap tube to settle adsorbent, and mark tube 1 cm above $Na_2SO_4$ layer.

Add 20–25 mL pet. ether wash to Florisil column; as solv. level reaches mark, place 125 mL K-D flask under column. Using disposable Pasteur pipet, transfer 3 mL sample to column, and wash tube with 1 mL pet. ether and add wash to column. Solv. level must not go below mark. Temporarily close stopcock if necessary. Add 35 mL pet. ether–ether mixt. (94 + 6) and elute PCBs and DDT and its analogs. When solv. level reaches mark, change K-D flask, and add 35 mL pet. ether–ether (85 + 15) to elute compds such as dieldrin and endrin. Add several granules of carborundum to first concentrator, attach Snyder column, and carefully conc. on steam bath. Let concentrator cool, remove Snyder column and evap. solv. under air to appropriate vol. for GC detn. Fractions contg mixt. of PCBs and chlorinated compounds such as DDE may require addnl sepn technics.

### F. Additional Cleanup

Often addnl cleanup is required for second fraction (85 + 15) to prevent deterioration of GC column. Use **970.52Q**.

### G. Gas Chromatography

*See* **970.52R**.

Ref.: JAOAC **66,** 969(1983).

## 984.21 Organochlorine Pesticide Residues in Animal Fats

### Gel Permeation Chromatographic Method

**First Action 1984**
**Final Action 1985**

(Applicable to beef, poultry, and swine fats)

### A. Principle

Liq. fats are dissolved in $CH_2Cl_2$–cyclohexane (1 + 1). Residues are sepd from lipid by gel permeation chromatgy (GPC), and identified and measured in concd eluates by GC-EC detection.

### B. Reagents and Apparatus

**(a)** *Solvents.*—$CH_2Cl_2$, cyclohexane ($C_6H_{12}$), isooctane. Must meet criteria in **970.52B**.

**(b)** *Gel permeation chromatographic system (GPC).*—AutoPrep gel permeation chromatograph (Model 1002B, Analytical Bio-Chemistry Laboratories, Inc., PO Box 1097, Columbia, MO 65205) or equiv. with 60 g BioBeads SX-3 resin, 200–400 mesh, in 60 × 2.5 cm id chromatgc tube, ca 48 cm bed length, elution solv. $CH_2Cl_2$-$C_6H_{12}$ (1 + 1). Flow rate calibrated to 5.0 mL/min, operating pressure 7–10 psig.

**(c)** *Flash evaporator.*—Rotary evapn system with 30° $H_2O$ bath.

**(d)** *Gas chromatograph.*—EC detector ($^{63}$Ni) operated as in **970.52H**. 1.85 m × 4 mm id column packed with 1.5% SP2250/1.95% S-2401 on 100–120 mesh Supelcoport (Supelco, Inc.). Operating conditions: injector 250°, column 200°, detector 350°, N flow 60–80 mL/min. Condition column 2 days at 250°.

### C. GPC Calibration Procedure

Chromatographic system will fractionate effluent from column into 23 equal fractions for elution calibration. (It is necessary to det. correct "dump" and "collect" times for desired residues, as function of pump flow rate.) Before fractionating, check flow rate with SX-3 gel column connected and adjust flow to 5.0 ± 0.2 mL/min (start pump ≥5 min before measuring flow to let flow equilibrate and improve accuracy). Fractionate vol. of eluant from 150 to 320 mL to ensure residue collection. Evap. fractions, resuspend in isooctane, and analyze to det. collection vol. for samples (fractionation procedure is described in instrument manual). Check calibration for quant. recovery with 2.0 g corn oil fortified with relevant compds.

### D. Preparation of Sample

Place ca 40 g representative fat sample in glass funnel (8.0 cm) with glass wool plug. Place funnel in flint glass bottle or 250 mL beaker on hot plate at ≤110° until fat ceases to drip. Mix thoroly.

### E. Cleanup

Weigh 2.0 g liq. fat into 10 mL vol. flask. (Fortifications of corn oil to check recoveries may be made here with stds dild in $CH_2Cl_2$-$C_6H_{12}$ (1 + 1).) Dil. to 10 mL with $CH_2Cl_2$-$C_6H_{12}$ (1 + 1) and mix thoroly. Centrf. or filter if particulate matter is visible. Use ca 7 mL sample to load sample loops on precalibrated GPC (5 mL aliquot (1.0 g equiv. of sample) is accepted into sample loop). Process thru GPC system using dump/collect times from fractionation procedure and collect eluate in 250 mL boiling flask. Rotary-evap. to just dry at ≤30°. Transfer quant. with 10 mL isooctane or equiv. GC-EC compatible solvent into a precalibrated culture tube. Adjust vol. under gentle, dry N stream to 5.0 mL.

### F. Gas Chromatography

Inject 3–6 μL aliquots into a gas chromatograph operated as in **970.52H** with $^{63}$Ni ECD. Measure peaks (ht or area). If necessary, dil. sample to give residue concn approx. that of std soln. Inject aliquot of pesticide std soln (in same solv. as sample) and again measure peaks.

Each residue, ppm = concn std (μg/mL) × (peak size sample/peak size std) × (μL std/μL sample) × (diln vol/1.0 g sample).

(*Note:* Since only 5 mL of original 10 mL vol. contg 2.0 g fat is injected into GPC sample loop, only 1.0 g fat is analyzed.)

Refs.: JAOAC **67,** 284(1984); **68,** 267(1985).

## 976.23 Endosulfan, Endosulfan Sulfate, Tetradifon, and Tetrasul Pesticide Residues

### Gas Chromatographic Method

**First Action 1976**
**Final Action 1977**

(Applicable to apples and cucumbers)

### A. Principle

Pesticides are extd with $CH_3CN$, partitioned with pet ether, eluted thru Florisil column with mixts of $CH_2Cl_2$, hexane, and $CH_3CN$, and detd by gas chromatgy. Method is variation of **970.52A–R**, as it applies to nonfatty foods. Pesticides are eluted from Florisil column with different eluants to improve cleanup for these compds.

### B. Apparatus

*See* **970.52E(a)–(h)** and **970.52H(a)–(c)**.

### C. Reagents

(**a**) *Florisil.—See* **970.52B(i)**.

(**b**) *Solvents.*—Hexane, $CH_2Cl_2$, and $CH_3CN$, all distd in glass and free from electron capturing substances (*see* **970.52B**).

(**c**) *Eluant mixtures.*—(*1*) *Eluant I.*—20% $CH_2Cl_2$-hexane. Dil. 200 mL $CH_2Cl_2$ with hexane. Let mixt. reach room temp. and adjust vol. to 1 L with hexane. (*2*) *Eluant II.*—50% $CH_2Cl_2$-0.35% $CH_3CN$-49.65% hexane. Pipet 3.5 mL $CH_3CN$ into 500 mL $CH_2Cl_2$, and dil. with hexane. Let mixt. reach room temp.; dil. to 1 L with hexane.

### D. Preparation of Sample and Extraction

*See* **970.52K(a)**, **(b)**, **(e)–(g)**.

### E. Column Chromatography

(*Caution:* See safety notes on distillation, acetonitrile, and hexane.)

Add wt activated Florisil detd from lauric acid absorption value, **970.52B(i)**, to 22 mm id chromatgc tube, **970.52E(b)**. Gently tap chromatgc column to settle Florisil. Top column with ca 12 mm anhyd. granular $Na_2SO_4$. Wet column with 40–50 mL hexane. Use Kuderna-Danish concentrator with volumetric or graduated tube to collect eluate. Transfer pet ether or hexane soln of sample ext to column, and let it elute at ca 5 mL/min. Rinse container (and $Na_2SO_4$, if present) with 2 ca 5 mL portions hexane, transfer rinsings to column, and rinse walls of chromatgc tube with addnl small portions of hexane. Elute tetrasul at ca 5 mL/min with 200 mL eluant I. Change receivers and elute endosulfan I and II, endosulfan sulfate, and tetradifon at ca 5 mL/min with 200 mL eluant II. Conc. each eluate to suitable definite vol. in Kuderna-Danish concentrator. For evapn <5 mL, use 2-ball micro Snyder or Vigreux column.

### F. Determination

*See* **970.52H(a)–(c)**.

Using the 10% DC-200 column, retention times relative to aldrin are ca 1.6 for endosulfan I, 2.2 for endosulfan II, 2.5 for tetrasul, 2.7 for endosulfan sulfate, and 5.4 for tetradifon.

Ref.: JAOAC **59,** 209(1976).

CAS-115-29-7 (endosulfan)
CAS-1031-07-8 (endosulfan sulfate)
CAS-116-29-0 (tetradifon)
CAS-2227-13-6 (tetrasul)

## 974.21 Polychlorinated Biphenyls in Paper and Paperboard

### Gas Chromatographic Method

**First Action 1974**
**Final Action 1982**

### A. Apparatus

(**a**) *Gas chromatograph.*—Equipped with electron capture detector and 1.85 m (6′) × 4 mm id glass column contg either (*1*) 10% DC-200 or (*2*) 1 + 1 mixt. of 15% QF-1 + 10% DC-200 on 80–100 mesh Chromosorb W(HP). Operating conditions: temps (°)—column and detector 200, injector 225; flow rate, 120 mL N/min; concentric design electron capture detector operated at dc voltage to cause 1/2 full scale recorder deflection for 1 ng heptachlor epoxide when full scale deflection is $1 \times 10^{-9}$ amp (*see* **970.52H(c)**).

(**b**) *Chromatographic tubes.—See* **970.52E(b)**.

(**c**) *Filter tube.—See* **970.52E(d)**.

(**d**) *Kuderna-Danish concentrator.—See* **970.52E(e)**, and (**g**).

(**e**) *West condenser.*—400 mm jacket length with ₮ inner drip joint to fit 250 and 500 mL erlenmeyers.

### B. Reagents

(**a**) *Florisil.—See* **970.52B(i)**.

(**b**) *Alcoholic potassium hydroxide soln.*—2% KOH in alcohol or redistd MeOH.

(**c**) *Petroleum ether.—See* **970.52B(m)**.

(**d**) *Polychlorinated biphenyls.*—Com. mixts (Aroclors) for ref. in GC detn (Analabs, Inc.).

### C. Extraction

(*Caution:* See safety notes on flammable solvents, and petroleum ether.)

Cut paper sample representative of lot into pieces ca 6 × 6 mm and mix thoroly.

Weigh 10 g sample into 250 mL erlenmeyer. Do not pack tightly. (*See* note below if vol. of 10 g sample is >50 mL.) Add 60 mL 2% alc. KOH, and fit flask with West condenser cooled with circulating cold tap $H_2O$. Reflux gently on steam bath 30 min. Rinse inside of condenser with small amt of alcohol. Transfer soln thru glass wool plug in small funnel, to 250 mL separator, avoiding transfer of any paper material. Rinse paper and flask with three 40 mL portions pet ether, combining rinses in separator. Add 60 mL $H_2O$ to separator and shake vigorously 30 sec. Drain lower aq. layer into second 250 mL separator. Add 60 mL pet ether to second separator and shake vigorously 30 sec. Discard aq. layer and combine pet ether layers in first separator. Rinse second separator with several small portions pet ether, collecting rinses in first separator. Wash pet ether with three 40 mL portions $H_2O$, discarding each wash. Dry pet ether thru 50 mm column, (**c**), of anhyd. $Na_2SO_4$, collecting eluate in Kuderna-Danish concentrator. Rinse separator and then column with 3 ca 20 mL portions pet ether, collecting rinses. Conc. combined pet ether ext and rinses on steam bath to ca 5 mL. Ext is ready for cleanup on Florisil column, **974.21D**. If experience with particular sample types indicates that Florisil column cleanup is not required, proceed to GC detn, **970.52R**.

*Note:* Adequate extn of low density paper such as newspaper or tissue paper will require adjustment of either amt of sample to <10 g or vol. of reflux soln to >60 mL. Preferably, reduce sample to wt that is completely covered and wetted by 60 mL KOH soln. Increase in vol. of reflux soln >60 mL must be accompanied by proportional increases in vols of pet ether rinses of sample, $H_2O$ diluent added to alc. reagent in separator, and size of erlenmeyers and separators.

Refs.: JAOAC **56,** 957(1973); **57,** 518(1974).

### D. Florisil Cleanup

Proceed as in **970.52O**, pars 1 and 2, except prep. 10 g column, pre-wet column with 20 mL pet ether, and elute at ca 5 mL/min with 150 mL pet ether. Concd eluate is suitable for analysis by GC with electron capture detection, **970.52R**.

*Note:* Waxes, if present in ext, can be removed before Florisil chromatgy by partitioning between pet ether and $CH_3CN$, **970.52N**.

## ORGANOPHOSPHORUS RESIDUES

### 974.22 Organophosphorus Pesticide Residues

**Carbon Column Cleanup Method**

**First Action 1974**
**Final Action 1976**

($CH_3CN$ extn and charcoal cleanup column using KCl thermionic or flame photometric detector for residues of parathion, paraoxon, carbophenothion and its O analog, and EPN on apples and green beans)

#### A. Reagents

(**a**) *Solvents*.—Redistd from glass (*see* **970.52A**): EtOAc, $CH_2Cl_2$, benzene, hexane, $CH_3CN$, and isopropanol.

(**b**) *Acid-treated charcoal*.—Slurry 200 g Norit SG Extra (no longer marketed) or 100 g Nuchar C-190N (no longer marketed) with 500 mL HCl, cover with watch glass, and stir mag. while boiling 1 hr. Add 500 mL $H_2O$, stir, and boil addnl 30 min. Collect charcoal in buchner and wash with $H_2O$ until washings are neut. to universal indicator paper. Dry at 130° in forced-draft oven.

(**c**) *Magnesium oxide*.—*See* **970.52B(k)**.

(**d**) *Adsorbent mixture*.—Mix 1 part acid-treated charcoal, 2 parts hydrated MgO, and 4 parts Celite 545, acid washed. Keep sealed.

(**e**) *Pesticide std solns*.—Prep. solns contg 1 μg/mL EtOAc of each of following: parathion, paraoxon, carbophenothion, carbophenothion O analog, and EPN.

(**f**) *Eluting soln*.—$CH_3CN$-benzene (1 + 1).

*Purity test*.—Reagents must be free of substances causing KCl thermionic or flame photometric detector response, as indicated by following test: Carry reagents thru entire method, and inject 5 μL from final conc. into gas chromatograph, using conditions described in **968.24F**. Conc. must not cause recorder deflection >1 mm from baseline for 2–60 min after injection.

#### B. Apparatus

*See also* **970.52E** and **968.24B(a)**.

(**a**) *Vacuum adapter*.—Kontes Glass Co., No. K-954002, or equiv.

(**b**) *Gas chromatograph*.—With potassium chloride thermionic detector (See **968.24B(i)** and (**k**).) or flame photometric detector (See **974.22B(e)** and (**i**).).

(**c**) *Column*.—*See* **968.24B(j)**.

(**d**) *Potassium chloride thermionic detector* (KClTD).—*See* **970.52H(d)**(*1*) or (*2*), (**e**), (**f**), and (**k**). Also check linearity of GC system to paraoxon and carbophenothion O analog.

(**e**) *Flame photometric detector* (*FPD*).—With P selective optical filter for 526 nm wavelength (Tracor Instruments, Inc.). Equiv. to KClTD for detn of organophosphorus pesticides in fruits and vegetables. (*Note:* Older commercial models of FPD may give rise to adsorption and/or degradation of O analogs of organophosphorus pesticides within detector's gas mixing chamber. Design changes of detectors manufactured after mid-1973 have generally corrected this problem. Flameout in FPD, on injection of sample, can be avoided by letting H enter detector (lower part) so that H and GC column effluent mix before burner area. Air-O enters detector thru upper part. This arrangement reverses that recommended by manufacturer. Specifications for physical modification of FPD to correct above problems are available from Division of Chemistry and Physics, Food and Drug Administration, Washington, DC 20204.) Use highly stabilized 0–750 v dc variable power supply capable of 10 ma output (Keithley Instruments, Inc., 28775 Aurora Rd, Cleveland, OH 44139, or equiv.), 6.3 v ac ignitor power supply, electrometer with bucking capability of $1 \times 10^{-6}$ amp (Tracor Instruments, or equiv.), and variable transformer capable of delivering 150 watts to control temp. of flame housing. Strip chart recorder should be compatible with electrometer.

(**f**) *Hydrogen*.—From cylinder of compressed H gas. Equip cylinder with regulator, delivery line, and variable flow controller capable of 200 mL/min delivery. Metering shut-off valve is required sep. from controller.

(**g**) *Air*.—Cylinder of compressed air equipped as in (**f**) to deliver up to 100 mL/min. Sep. shut-off valve is not needed.

(**h**) *Oxygen*.—Cylinder of compressed O gas equipped as in (**f**) to deliver up to 50 mL/min. Combine with air using std Swagelok tee before detector inlet.

(**i**) *Flame photometric detector operation*.—Adjust temp. of burner housing to ca 170–180° before igniting flame. Temp. will rise 20–30° after ignition. Do not allow detector to exceed 220°. Adjust gas flows at controllers to ca 150–300 mL/min H, 50–100 mL/min air, and 5–20 mL/min O. Adjust column effluent flow, **970.52R(a)**, to 120 mL N/min. Turn off H flow with metering shut-off valve (**f**). (*Caution:* Before attempting ignition, make certain H has been purged from detector with other gases. One min interval between ignition attempts is adequate.) Apply ca 750 v to photomultiplier tube from power supply. Zero recorder with electrometer set at appropriate sensitivity (ca $1 \times 10^{-8}$ to $1 \times 10^{-9}$ amp full scale). Push ignitor button and then slowly open H metering shut-off valve. Recorder pen will not return to zero baseline if flame ignites. If ignition is not effected, shut off H valve, increase O flow, and repeat ignition procedure. Establish proper baseline with buck-out control after flame is lit. Operate at sensitivity that produces $^1/_2$ full scale recorder deflection for 2 ng parathion. Reduce photomultiplier voltage to reduce sensitivity. Alternatively, use electrometer sensitivity and attenuator controls to achieve proper response. Check linearity of GC system to paraoxon and carbophenothion O analog.

#### C. Preparation of Sample

Blend and filter sample as in **970.52K(a)**, or (**b**). Transfer aliquot of $CH_3CN$ ext (30–35 mL) equiv. to ca 10 g sample from suction flask to 125 mL separator, add equal vol. $CH_2Cl_2$, shake vigorously 30 sec, and set aside 10–15 min to sep. Calc. g sample in aliquot as g sample × [mL aliquot/(mL $H_2O$ in sample + mL extg solv. added − correction in mL for vol. contraction)].

#### D. Charcoal Cleanup

Fit 1-hole No. 5 rubber stopper onto tip of chromatgc tube, **970.52E(b)**, add side-arm vac. adapter and ₮ 24/40 receiving flask, open stopcock, and connect app. to open vac. line. Place 1 g Celite 545 in tube, tamp, add 6 g adsorbent mixt., and tamp again. Add 2 cm glass wool plug on top of adsorbent. Prewash column with 100 mL eluting soln. Close stopcock when eluting soln is ca 2 cm above glass wool and maintain this head to ensure clean column. Disconnect vac., replace flask with 500 mL Kuderna-Danish flask equipped with 10 mL tube, **970.52E(e)** (check calibration at 1 mL), and reconnect to open vac. line.

Drain lower $CH_2Cl_2$ layer in separator onto column, retaining $H_2O$ layer (upper phase) in separator. Open column stopcock to vac. and adjust flow to ca 5 mL/min. Re-ext $H_2O$ layer cautiously (do not shake vigorously) with two 10 mL portions $CH_2Cl_2$ and add exts to column. Discard $H_2O$ phase. Elute column with 120 mL eluting soln (column may be taken to dryness). Disconnect app. and rinse column tip and vac. adapter with several mL EtOAc. Collect all rinses in same Kuderna-Danish concentrator with tube attached. Add 1 or 2 small boiling chips, attach Snyder column, and conc. cautiously over steam bath to ca 1 mL. (*Caution:* Begin heating very gently due to differences in densities and bps of individual solvs.) When cool, disconnect evaporative app. from Mills tube. Substitute column, **970.52E(h)**, on Mills tube, add boiling chips again, and conc. solv. to <1 mL. While app. is still immersed in steam bath, add 3–4 mL isopropanol (to remove $CH_3CN$ azeotropically) and distil under gentle reflux. Repeat isopropanol addn and conc. to ca 0.5 mL. Remove from heat, cool, remove column, and adjust vol. to 1.0 mL with EtOAc.

### E. Gas Chromatography

Proceed as in **968.24F**, using recommended operating conditions *1* specified for column, **968.24B(j)**(*1*). (*See* **974.22B(e)**–**(i)** if using flame photometric detector.

Refs.: JAOAC **54,** 513(1971); **57,** 930(1974).

CAS-786-19-6 (carbophenothion)
CAS-2104-64-5 (EPN)
CAS-56-38-2 (parathion)
CAS-7173-84-4 (carbophenothion O analog)
CAS-311-45-5 (paraoxon)

## 968.24 Organophosphorus Pesticide Residues

### Sweep Codistillation Method

**First Action 1968**
**Final Action 1977**

(Sweep codistillation cleanup for parent organophosphorus residues of carbophenothion, diazinon, ethion, malathion, Me parathion, and parathion in kale, endive, carrots, lettuce, apples, potatoes, and strawberries (fresh or non-sugared frozen); this cleanup is not adequate for electron capture gas chromatgc detector. Use only with KCl thermionic or flame photometric detector.)

### A. Reagents

(**a**) *Ethyl acetate.*—Redistd from glass. Check suitability of reagent by concg 100 mL to 2 mL. Inject 5 μL into GC (KCl thermionic detector) with operating conditions specified in **968.24B(i)**. Chromatogram should show no peaks to 20 min with chart speed of 1″/2 min.

(**b**) *Pesticide std soln.*—Prep. EtOAc soln contg 1 μg/mL of each of following: carbophenothion, diazinon, ethion, malathion, Me parathion, and parathion.

### B. Materials and Apparatus

(**a**) *Glass wool.*—Silane-treated (available from Applied Science).

(**b**) *Anakrom ABS.*—80–90 mesh. Remove fines by stirring with EtOAc, decanting several times, and drying.

(**c**) *Teflon tubing.*—AWG No. 16, std, natural.

(**d**) *Disposable glass capillary pipets.*—145 mm long, 6 mm id, with capillary stem (Thomas Scientific No. 7760-B30, or equiv.).

(**e**) *Syringes.*—1 mL Tuberculin Luer-Lok and 2 mL Luer-Lok with Luer-Lok 2″ No. 25G needles.

(**f**) *High-speed blender.*—400 mL capacity. Omnimixer (available from DuPont Instruments Co, Sorvall Operations, Peck's Ln, Newtown, CT 06470), or equiv.

(**g**) *Sweep codistillation apparatus.*—*See* Fig. **968.24**. Following tubes are required: (*1*) *Storherr tube.* —24.5 cm long, 6 mm id (Kontes Glass Co., No. K-898600, or equiv.). (*2*) *Concentrating tube.*—10 mL calibrated to 0.5 mL. (*3*) *Adapter for extension of concentration tube.*—7 cm long, ₮ 19/22 (Kontes No. K-570100 (K-500750 part 355), or equiv.).

(**h**) *Kuderna-Danish concentrators.*—500 mL with Snyder distilling column and 5 mL vol. and 10 mL graduated (Mills tube) receiving flasks, ₮ 19/22 (Kontes Glass Co., No. K-570000, K-621400, and K-570050, or equiv.).

(**i**) *Gas chromatograph with potassium chloride thermionic detector.*—*See* **970.52H(a)**. Only thermionic detector, **970.52H(d)**, is required.

Following conditions are important in operation of GC and KCl thermionic detector:

(*1*) Every day before starting work change silane-treated glass wool plug insert in injection port of GC column. Remove and replace only that portion affected by syringe.

(*2*) Every week before starting work reheat KCl spiral over gas burner and reinsert into detector. Detector must then equilibrate ca 2 hr before use.

(**j**) *Column.*—10% DC 200 or OV-101 on 80–100 mesh Chromosorb W HP in glass column 1.85 m (6′) × 4 mm id; *see* **970.52H(b)**. Adjust column temp to give retention time for parathion of ca 5 min.

(*1*) *Recommended operating conditions: I.*—Temps (°): injection 225, column 200, detector 210; N flow 120 mL/min. Split column effluent with 1:1 stream splitter so that only 60 mL N/min enters KCITD. (*2*) *Recommended operating conditions II.*—Temps (°): column 220, injector and detector 240; N flow 60 mL/min.

(**k**) *Potassium chloride thermionic detector (KCITD).*—*See* **970.52H(d)**(*1*) or (*2*), (**e**), (**f**), and (**k**).

### C. Preparation of Apparatus

App. is constructed in 3 parts: removable Storherr tube, permanent heating coil, and distillate collector (*see* Fig. **968.24**).

(**a**) *Preparation of removable Storherr tube.*—Pack Storherr tube with silane-treated glass wool. Use silane-treated glass wool as received. Do not pack glass wool too tightly; otherwise removal for cleaning is difficult. Only 13–15 cm portion from injection end requires packing. Insert injection septum and two 1-hole septums. Tube is now ready for use. Use clean tube for each sample. Clean tubes thoroly with soap and $H_2O$ after use, rinse with acetone, and dry. Soak tubes difficult to clean in chromic acid cleaning soln before cleaning with soap and $H_2O$.

(**b**) *Preparation of permanent heating coil.*—Attach bimetallic wires of calibrated pyrometer directly to outside middle area of Cu tube (length 20 cm (8″) × 11 mm ($^7/_{16}$″) id). (Thermometer with stem covered with Al foil may also be used for temp. measurement.) Wrap heating tape (60 × 1.3 cm) uniformly around outside of Cu tubing and over bimetallic wires or thermometer, and secure ends. Cover heating tape with asbestos tape and secure with glass tape or glass thread. Cover asbestos with several layers of Al foil and secure with tape.

Place heating assembly on ring stand, using asbestos-covered 3-prong clamp. Orient and use heating coil in near horizontal position. Attach heating tape leads to variable transformer. Adjust transformer so pyrometer reads 180–185°. Use this setting or temp. for all crop cleanup.

Add N flow, 600 mL/min, to sidearm of Storherr tube. (For N pressure gage to give meaningful readings, add stainless steel capillary tube to reduce gas flow. Connect capillary tube directly to 1-hole septum in sidearm of tube with short length Teflon tubing.) Measure N flow with gas flow gage, and calibrate pressure regulator gage by this means.

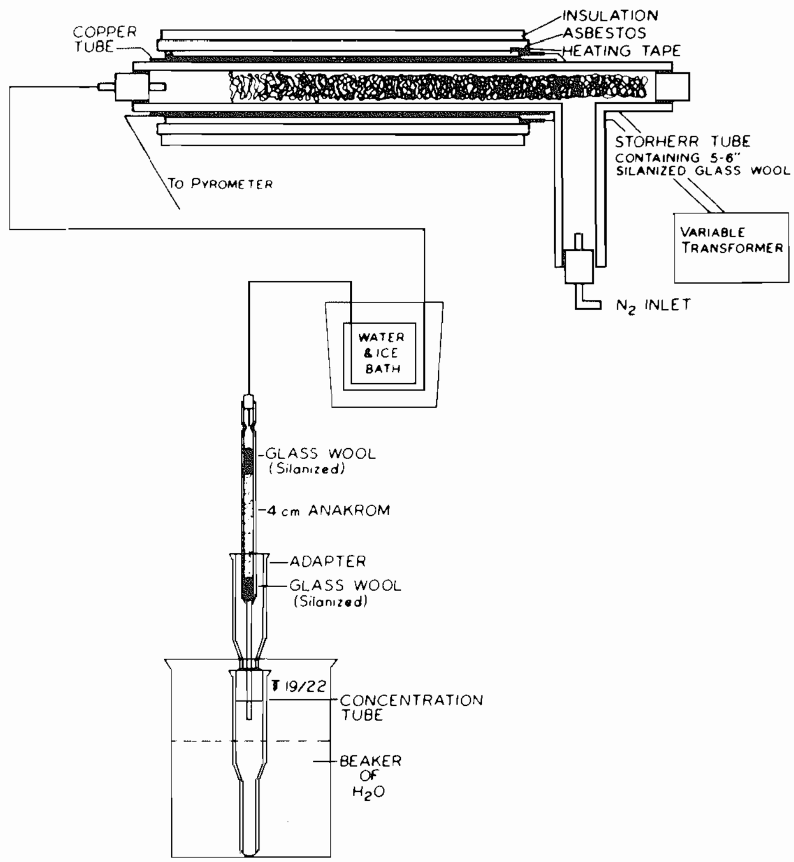


**FIG. 968.24—Sweep codistillation apparatus**

(**c**) *Sample distillate collector.*—Construct in 3 parts: cooling coil, scrubber tube, and concn tube with extended adapter.

(*1*) *Cooling coil.*—Cut 120 cm length of Teflon tubing. Form this tubing into three 7 cm diam. loops having 2 arms of ca 20 cm lengths. Attach Teflon cooling coil and 1-hole septum directly to Storherr tube. Place coils in 250 mL beaker contg ice and $H_2O$. Place 250 mL beaker inside 400 mL beaker for insulation.

(*2*) *Scrubber tube.*—Insert silane-treated glass wool plug in constricted end of disposable pipet. On outside of pipet place marks 4 and 6 cm above top of glass wool plug. Add Anakrom ABS to 6 cm mark and pack Anakrom to 4 cm mark by compressing with 3 mm rod. Place silane-treated glass wool plug on top of packed Anakrom and 1-hole septum in pipet top. Connect exit arm of Teflon cooling coil directly into 1-hole septum in scrubber tube and extending ca 2 cm below septum. Secure scrubber tube on sep. ring stand with 3-prong clamp. Scrubber tube must be lower than cooling bath, especially in rinsing step.

(*3*) *Concentration tubes.*—Use 10 mL calibrated tubes, **968.24B(h)**. Adapter, (**g**)(*3*), is needed for insertion into concn tube to prevent splash during sweep and rinsing steps. Place tip of scrubber thru adapter and into concn tube. If possible, place scrubber tip against wall of concn tube. Hold tube in place with clamp.

Adjust heat to 180–185° and N flow to 600 mL/min (measure before entering Storherr tube). Flush several 0.5 mL EtOAc injections thru entire system, using 2 mL syringe (used for all rinsings). Replace concn tube with clean tube and insert adapter; assembly is ready for use.

### D. Extraction

Ext all crops with EtOAc in *exact* order as follows: To high-speed blender, add 25 g chopped sample, 125 mL EtOAc from pipet, and 25 g anhyd. granular $Na_2SO_4$. Blend 5 min at slow speed; then 5 min at high speed with mixer cup immersed in ice-$H_2O$ bath. Decant liq. thru 2.5 cm silane-treated glass wool plug contained in short glass chromatgc tube. (Do not add solids to glass wool plug.) Collect EtOAc ext (ca 100 mL) in 125 mL flask or bottle. Remove 50 mL aliquot (equiv. to 10 g original sample) and place in Kuderna-Danish concentrator with Snyder column, calibrated Mills tube, or 5 mL vol. receiving flask, and conc. to ca 5 mL. Adjust vol. to exactly 5.0 mL, using air jet or adding EtOAc. Use 1 mL aliquots (2 g sample) for sweep codistn cleanup.

Store all stds and crop solns at ≤0° when not in use. Warm to room temp. ca 1 hr before use.

### E. Sweep Codistillation Cleanup

Assemble app. as in Fig. **968.24**, except position Storherr tube and heating unit so exit end of Storherr tube is ca 10° below horizontal to avoid backup of sample into N inlet arm. If sample backs up, *discard detn.* Check temp. (180–185°), N flow (600 mL/min), and receiver tube. Inject 1 mL (2 g) sample, using 1 mL Luer-Lok tuberculin syringe. Immediately follow sample with injection of 0.5 mL EtOAc sweeping solv. and repeat 0.5 mL EtOAc injection every 3 min for 21 min. After last injection wait 1 min until solv. has cleared cooling coil and scrubber tube; then disconnect cooling coil arm with septum from Storherr tube. Disconnect septum with attached cooling coil arm from scrubber tube and rinse 2 cm Teflon projection, collecting rinse in scrubber tube (still in position in concn tube). With septums in place on disconnected cooling coil arms, *reverse* coil arms and place that end formerly in Storherr tube into scrubber tube. Make certain that Teflon tubing in this arm extends 2 cm into scrubber tube below inserted septum (similar to position when cleaning up sample). Slowly inject 1 mL EtOAc rinse from 2 mL syringe directly into open end of cooling coil arm formerly in scrubber tube. Gently force rinse, using N flow from disconnected Storherr tube, thru cooling coil into scrubber tube and into concn tube. Repeat 1 mL EtOAc rinse 1–2 addnl times. Rinse scrubber tip end and inside of adapter, remove scrubber, disconnect adapter, and rinse ₹ joint. Collect all rinses in concn tube. Rinse down sides of concn tube and conc. to 1 mL, using N or air jet. Prevent $H_2O$ condensation inside tubes by placing tube in room temp. $H_2O$ bath during this step. If cleaned up soln is too concd for GC detn (>2 μg/mL), dil. soln to 5 or 10 mL with EtOAc. If calibrations of Mills tube are incorrect (most usually are except for 1 mL mark), quant. transfer the concd soln to 5 or 10 mL vol. flask, using disposable pipet with attached rubber bulb. Rinse inside of tube with EtOAc and transfer rinse in same pipet. Repeat this rinse of tube and pipet several times; then rinse inside of pipet into flask, using EtOAc. Dil. to vol. Further diln with EtOAc or concn may be necessary to bring concn within measurement range.

Anakrom scrubber tube is used repeatedly without change. Final EtOAc rinses after each run keep it clean. However, if Anakrom becomes discolored, prep. new tube.

### *F. Determination by Gas Chromatography*

Operate chromatograph under conditions specified for column, **968.24B(j)**. Inject 3–8 μL aliquot concd, cleaned-up soln contg amt of pesticide within linear range of gas chromatgc system, **(i)**, using 10 μL syringe. Tentatively identify residue peaks on basis of retention times. Det. amt of pesticide by comparing area under peak with that from known amt of appropriate std pesticide. For accurate detn, baseline current of sample and std must be identical during chromatgy.

Injections <3 μL are difficult to reproduce; injections >8 μL may cause flame blow-out. Sample wt is not critical—use injections equiv. to <1 mg or several hundred mg. Inject appropriate std immediately after every sample. Peak ht also may be used for detn, but only if ht of ref. std is ca same ht as sample unknown (width of base should then be same).

Refs.: JAOAC **51,** 662(1968); **59,** 472(1976).

CAS-786-19-6 (carbophenothion)
CAS-333-41-5 (diazinon)
CAS-563-12-2 (ethion)
CAS-121-75-5 (malathion)
CAS-298-00-0 (methyl parathion)
CAS-56-38-2 (parathion)

## 970.53 Organophosphorus Pesticide Residues

### Single Sweep Oscillographic Polarographic Confirmatory Method

**First Action 1970**
**Final Action 1974**

(Applicable to diazinon, malathion, Me parathion, and parathion)

### *A. Apparatus*

(Wash all glassware with hot $HNO_3$ (1 + 1) and rinse with $H_2O$.)

**(a)** *Polarograph.*—Any voltammetric or polarographic instrument capable of linear sweep voltammetry at 10 ng pesticide/mL cell soln (equiv. to 0.01 ppm based on 1 g sample in 1 mL cell soln).

**(b)** *Silver wire electrode.*—Deposit very thin coating of AgCl on No. 20 or 22 gage Ag wire as follows: Dip wire in 10% $HNO_3$, rinse in $H_2O$, and then let stand 10 min in 1*N* HCl.

### *B. Reagents*

(*See* statement regarding solvs, **970.52A.**)

**(a)** *Acetonitrile.*—Distd in glass at 82 ± 1°.

**(b)** *Acetone.*—Distil at 56.5° with 0.25 g $KMnO_4$/L. Distn must be performed as directed.

**(c)** *Ethyl acetate.*—Distd in glass at 77 ± 1°.

**(d)** *Petroleum ether.*—Distd in glass at 30–60°.

**(e)** *Nitrogen.*—Prepurified, $H_2O$-pumped.

**(f)** *Tetramethyl ammonium bromide.*—Eastman Kodak No. 670, or equiv.

**(g)** *Electrolyte solns.*—*(1) For diazinon.*—Dissolve 7.7 g $Me_4NBr$ in 300 mL $H_2O$. Add 115 mL HOAc and dil. to 500 mL with $H_2O$. *(2) For malathion.*—Dissolve 15.4 g $Me_4NBr$ in 300 mL $H_2O$. Add 0.2 g LiCl and 4.1 mL HCl, and dil. to 500 mL with $H_2O$. *(3) For methyl parathion and parathion.*—Dissolve 2.2 g NaOAc $.3H_2O$ and 1.17 g NaCl in 100 mL $H_2O$ and adjust to pH 4.8 with HOAc, using pH meter.

**(h)** *Pesticide std solns.*—*(1) Stock solns.*—Prep. individual solns contg 1.00 mg pesticide/mL EtOAc. Store at 0°. *(2) Intermediate solns.*—0.2 mg/mL. Transfer 5 mL stock soln to 25 mL vol. flask and dil. to vol. with pet ether for diazinon, MeOH for malathion, and acetone for Me parathion and parathion.

### *C. Preparation of Standard Curves*

**(a)** *Diazinon.*—Transfer 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL intermediate std soln of diazinon to individual 100 mL vol. flasks and dil. to vol. with pet ether. Transfer 1.0 mL of each soln to sep. 50 mL erlenmeyers and evap. to incipient dryness under gentle jet of dry air. Evap. remaining solv. with warmth of hand. Dissolve residue in 5.0 mL electrolyte soln, **(g)**(*1*). Transfer soln to polarographic cell, adjust to 25 ± 1°, and bubble N thru soln 5 min. Polarograph between −0.70 and −1.2 v against either Hg pool or Ag wire ref. electrode.

Peak potential for diazinon at 25° is −0.90 ± 0.05 v against either electrode. Plot μg diazinon/mL cell soln against peak ht in units × instrument sensitivity setting.

**(b)** *Malathion.*—Transfer 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL intermediate std soln of malathion to individual 25 mL vol. flasks and dil. to vol. with MeOH. Transfer 2.0 mL of each soln to sep. 50 mL erlenmeyers and add 1.0 mL 0.1*N* KOH. After 3 min, add 2.0 mL electrolyte soln, **(g)**(*2*), mix well, and let stand 5 min. Transfer to polarographic cell, adjust to 25 ± 1°, and bubble N thru soln 5 min. Polarograph between −0.5 and −1.0 v against either Hg pool or Ag wire electrode.

Peak potential for malathion at 25° is −0.82 ± 0.05 v against Ag wire and −0.85 ± 0.05 v against Hg pool ref. electrodes. Plot μg malathion/mL cell soln against peak ht in units × instrument sensitivity setting. (*Note:* Compd actually polarographed is fumaric acid resulting from basic hydrolysis of malathion.)

**(c)** *Parathion and methyl parathion.*—Transfer 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL intermediate parathion (or Me parathion) std soln to individual 100 mL vol. flasks and dil. to vol. with acetone. Transfer 5.0 mL aliquots of each soln to sep. 50 mL erlenmeyers, add 5.0 mL electrolyte soln, **(g)**(*3*), mix well, and transfer ca 5 mL to polarographic cell. Adjust to 25 ± 1°, bubble N thru soln 5 min, and polarograph between −0.4 and −0.9 v against either Hg pool or Ag wire ref. electrode.

Peak potential for parathion and Me parathion at 25° is −0.68 ± 0.05 v against Hg pool and −0.70 ± 0.05 v against Ag wire ref. electrodes. Plot μg pesticide/mL cell soln (10 mL) against peak ht in units × instrument sensitivity setting. Cell soln vol. = 5 mL sample soln + 5 mL electrolyte soln.

### D. Preparation of Sample Solution

Prep., ext, and clean up samples as in **970.52K–O**. Conc. 15% and 50% eluates from Florisil column to suitable definite vol. in Kuderna-Danish concentrator. All eluting solvs must be peroxide-free by test in *Definitions and Explanatory Terms*.

### E. Determination

(**a**) *Parathion and/or methyl parathion*.—Transfer aliquot of concd 15% Florisil eluate, equiv. to 5 g crop, to 50 mL erlenmeyer. Carefully evap. to dryness under gentle jet of air at room temp. Dissolve residue in 3.0 mL acetone. (*Note:* Since good polarograms can be obtained by using as little as 0.5 mL soln in cell, min. of 0.25 mL acetone can be used to dissolve residue.) Add 3.0 mL electrolyte soln, (**g**)(*3*), mix well, transfer to polarographic cell, and adjust to 25 ± 1°. Bubble N thru soln 5 min and polarograph as in **970.53C(c)**. Measure ht of wave whose peak potential corresponds to that of parathion, and det. concn from freshly prepd std curve or by comparing wave hts of sample soln with those of std soln polarographed immediately before or after sample. (Latter method is recommended for greater accuracy.)

Calc. μg/mL as follows:

$$C_{sample} = [(WH_{sample}) \times (IS_{sample}) \times (C_{std})]/[(WH_{std}) \times (IS_{std})]$$

where C = μg pesticide/mL cell soln; WH = wave ht; IS = instrument sensitivity setting.

$$ppm = (C_{sample} \times \text{mL sample soln})/\text{g sample}$$

Limit of quant. detn is 0.01 ppm based on 1 g crop in 1 mL cell soln.

Me parathion, parathion, and paraoxon polarograph at ca same peak potential. If any one of these pesticides is present as indicated by multiple residue methods, it should be polarographed against that std. If these pesticides are present together, use mixed std contg ratio of pesticides as estd from analysis by multiple residue method. (Paraoxon will not be recovered by cleanup specified.)

Other pesticides known to give polarographic peak potentials similar to parathion are pentachloronitrobenzene (PCNB), 1,2,4,5-tetrachloro-3-nitrobenzene (TCNB, tecnazene), and *O-ethyl O-p*-nitrophenyl phenylphosphonothioate (EPN). PCNB and TCNB are recovered in 6% Florisil eluate and will not interfere. Verify presence or absence of EPN by GC or TLC.

(**b**) *Diazinon*.—Transfer aliquot of concd 15% Florisil eluate, equiv. to 5 g crop, to 50 mL erlenmeyer. Carefully evap. just to dryness, using gentle jet of dry air at room temp. Dissolve residue in 5.0 mL electrolyte soln, (**g**)(*1*). Transfer soln to polarographic cell and adjust to 25 ± 1°. Bubble N thru soln 5 min and polarograph as in **970.53C(a)**. Calc. amt of diazinon present as in (**a**).

Limit of quant. detn is 0.2 ppm based on 1 g crop sample in 1 mL cell soln.

(**c**) *Malathion*.—Transfer aliquot of concd eluate from 50% Florisil eluate, equiv. to 5 g crop, to 50 mL erlenmeyer. Carefully evap. just to dryness under gentle jet of dry air at room temp. Dissolve residue in 2.0 mL MeOH, add 1.0 mL 0.1*N* KOH, and let stand 3 min. Add 2.0 mL electrolyte soln, (**g**)(*2*), mix well, and let stand 5 min. Transfer to polarographic cell, adjust to 25 ± 1°, bubble N thru soln 5 min, and polarograph as in **970.53C(b)**. Calc. amt of malathion present as in (**a**).

Limit of quant. detn is 0.3 ppm based on 1 g crop in 1.0 mL cell soln.

*Note 1:* If polarogram cannot be obtained because of high residual currents, check concd eluate for peroxides. If peroxides are present, transfer 5 mL concd eluate to small separator contg 25 mL 3% $FeSO_4$ soln; shake well and discard aq. layer. Transfer 1.0 mL ether layer to 50 mL erlenmeyer and proceed as in (**a**), (**b**), or (**c**).

*Note 2:* All glassware used for polarographic detns should be thoroly washed with hot $HNO_3$ (1 + 1) and rinsed with distd $H_2O$.

Ref.: JAOAC **52,** 811(1969).

CAS-333-41-5 (diazinon)
CAS-121-75-5 (malathion)
CAS-298-00-0 (methyl parathion)
CAS-56-38-2 (parathion)

## 964.17★ Organophosphorus Pesticide Residues

**Cholinesterase Inhibition Method**

**First Action 1964**
**Surplus 1974**

*See* **29.049–29.055**, 12th ed.

## FUMIGANT RESIDUES

## 977.18 Volatile Fumigants in Grain

**Gas Chromatographic Method**

**First Action 1977**
**Final Action 1981**

(Applicable to $CCl_4$, $CHCl_3$, $BrH_2CCH_2Br$, and $Cl_2CCClH$ in wheat and corn grain)

### A. Apparatus and Reagents

(**a**) *Column*.—4 m × 2.2 (id) mm stainless steel packed with 15% polypropylene glycol (LB 550X, Ucon fluid) on 60–80 mesh Chromosorb W.

(**b**) *Gas chromatograph*.—Isothermal with source-heated electron capture detector and glass-lined heated injection block. (100–200 mCi $^3H$ with Ar as β-ionization detector is more useful for multiresidue detns than $^{63}Ni$ and N.) Use 1 mv recorder with max. response time of 1 sec and chart speed of 0.5 cm/min. Operate electron capture detector with N at 25 psi (173 kPa) at 95° with polypropylene glycol column for $CCl_4$ (retention time, ca 6 min). Use 120° for $CHCl_3$ (3 min), $Cl_2CCClH$ (4 min), and $BrH_2CCH_2Br$ (8 min).

(**c**) *Acetone*.—Check for interfering peaks by gas chromatgy before use.

### B. Determination

(*Caution:* See safety notes on acetone.)

Store sample at ≤5°. Quickly weigh 50 g and immerse in 150 mL acetone-$H_2O$ (5 + 1) in 250 mL g-s flask, and stopper. Let stand 48 hr in dark at 20–25°, swirling at 24 hr. Decant 10 mL supernate into 25 mL g-s graduate, add 2 g NaCl, stopper, and shake vigorously 2 min. Let stand until layers sep. Pour 5 mL clear upper layer into 10 mL g-s graduate, add 1 g anhyd. $CaCl_2$, stopper, and shake 2 min. Let stand 30 min with occasional shaking.

Withdraw 0.5 μL aliquots from upper layer into 1 μL syringe. Inject into gas chromatograph. Dil. 10× or 100× with dry acetone, if necessary to avoid overloading detector. Inject all solns in triplicate and average results.

Construct calibration curve daily of peak hts against ng fumigant/125 mL acetone for suitable range.

Refs.: Analyst **99,** 570(1974). JAOAC **60,** 368, 405(1977).

CAS-56-23-5 (carbon tetrachloride)
CAS-67-66-3 (chloroform)
CAS-106-93-4 (ethylene dibromide)
CAS-79-01-6 (trichloroethylene)

## CARBAMATE RESIDUES

### 975.40 *N*-Methylcarbamate Insecticide Residues

**Gas Chromatographic Method**
**First Action 1975**
**Final Action 1976**

(Carbanolate, Carbaryl, Carbofuran, and Propoxur)

(Applicable to apples, cabbage, collards, corn kernels, green beans, kale, and turnip tops. Rinse all glassware with acetone and then distd $H_2O$ before use.)

#### A. Principle

Residue is extd from crop with $CH_3CN$, and ext is purified by partitioning with pet ether and coagulating with $H_3PO_4$-$NH_4Cl$ soln. Phenolic impurities are largely eliminated by partitioning $CH_2Cl_2$ ext with KOH soln. Carbamate residues are treated with 1-fluoro-2,4-dinitrobenzene to form ether derivative. Residues may be detd at levels ≥0.05 ppm. Recoveries range from 90 to 110%.

#### B. Reagents

(**a**) *Borax*.—5% aq. soln.

(**b**) *Diatomaceous earth*.—Wash thoroly with acetone and dry 2 hr at 110°.

(**c**) *Coagulating soln*.—(*1*) *Stock soln*.—Dissolve 20 g $NH_4Cl$ and 40 mL $H_3PO_4$ in 360 mL $H_2O$. (*2*) *Working soln*.—Dil. 100 mL stock soln to 1 L for coagulation.

(**d**) *1-Fluoro-2,4-dinitrobenzene soln*.—(Eastman Kodak Co.) Redistil at 128° and 1 mm pressure. Dissolve 1.5 mL in 25 mL acetone.

(**e**) *Pesticides*.—Best quality obtainable from manufacturer; anal. grades when available.

(**f**) *Potassium hydroxide soln*.—0.5*N* aq. soln.

(**g**) *Sodium chloride soln*.—30% aq. soln.

(**h**) *Solvents*.—Acetone, $CH_2Cl_2$, isooctane, $CH_3CN$, and pet ether (distd in glass; *see* statement regarding solvs, **970.52A**); acetophenone and MeOH (anal. grade).

#### C. Gas Chromatographic Apparatus

Gas chromatograph equipped with $^3H$ electron capture detector and 46 × 0.64 (od) cm (18 × 1/4″) glass column contg 10% DC-200 (12,500 cst) on 60–70 mesh Anakrom ABS (Analabs, Inc.). Porous Teflon end plugs for 1/4″ od glass tubing (Chemical Research, PO Box 888, Addison, IL 60101) are preferable, but glass wool can be used at outlet and omitted at inlet if necessary. (Glass wool at inlet tends to adsorb derivatives gradually and to release them later, giving rise to "ghost images" of compds.)

Equilibrate column 2 days at 250° and 2 weeks at 212°. Operating conditions: temps (°)—column 212, detector 218, standby temps 190 and 200, resp.; N carrier gas 60 mL/min; sensitivity $1 \times 10^{-9}$ amp full scale; and detector potential either 25 or 50 V, depending on response level needed (1/3 to 2/3 full scale peak ht with injections of 4 ng carbamate).

Alternatively, use instrument with $^{63}Ni$ detector and 1.8 m (6′) × 4 mm id glass column contg 10% DC-200 on 60–70 mesh Anakrom ABS. Do not use glass wool at beginning of column. Operating conditions: temps (°)—column 232, detector 250, N carrier gas 80 mL/min, sensitivity $1 \times 10^{-9}$ amp full scale, and detector potential 50 or 75 V.

#### D. Extraction of Pesticides

(*Caution:* See safety notes on blenders, acetonitrile, and petroleum ether)

Place 100 g sample and 200 mL $CH_3CN$ (add 50 mL $H_2O$ with fruit or other samples contg 5–15% sugar) in sq screw-top jar, and macerate in blender operated 2 min at moderate speed. Filter with suction into 500 mL r-b flask thru rapid paper in 11 cm buchner. Transfer aliquot equiv. to 40 g crop (mL aliquot = (mL $H_2O$ in sample + mL $CH_3CN$ added + mL $H_2O$ added − 5 mL vol. contraction) × 40/100) to 250 mL separator. Shake 10 sec with 25 mL NaCl soln. Drain and discard aq. phase. Repeat with fresh NaCl soln. Add 100 mL pet ether, and shake 30 sec. Drain $CH_3CN$ into 1 L separator. Strip pet ether by shaking 20 sec with 50 and 10 mL portions $CH_3CN$, draining each into the 1 L separator. Add 300 mL $H_2O$, 25 mL NaCl soln, and 50 mL MeOH. Ext mixt. with 100 mL and two 25 mL portions $CH_2Cl_2$, shaking each 20 sec, and drain lower layer into 500 mL r-b flask. Add 2 drops acetophenone, and evap. in rotary evaporator connected to aspirator pump. During evapn, keep $H_2O$ bath within 40–50° range and remove flask from $H_2O$ bath when ext vol. has been reduced to few mL, so that final evapn to dryness takes place at low temp.

Add 5 mL acetone, and swirl flask to dissolve residue. Add 50 mL coagulating soln, swirl to mix, add 1–2 g diat. earth, and swirl again to mix. Pour soln into 150 mL suction filter of medium porosity packed with 6 mm (1/4″) diat. earth, and collect filtrate in 500 mL r-b flask. Break vac. immediately after liq. is drawn into diat. earth layer. Rinse sides of flask with 5 mL acetone, swirl, and repeat coagulation. Rinse flask with 20 mL coagulating soln, and add rinse to filter just after liq. of second coagulation is drawn into diat. earth layer. After filtration is complete (ca 5 min), transfer filtrate to 250 mL separator. Ext carbamates by shaking 20 sec with three 25 mL portions $CH_2Cl_2$, rinsing filter flask with each portion before adding to separator. Drain $CH_2Cl_2$ (lower) ext into another 250 mL separator. Soln may be held overnight at this point. Add 40 mL $H_2O$ and 10 mL 0.5*N* KOH, mix briefly by gentle swirling, and shake 20 sec. Drain $CH_2Cl_2$ thru granular anhyd. $Na_2SO_4$ supported by glass wool in filter funnel, and collect filtrate in 250 mL erlenmeyer. Add 10 mL $CH_2Cl_2$ to separator, swirl gently, and drain org. phase. Repeat once. Rinse filter with two 10 mL portions $CH_2Cl_2$. Add 2 drops acetophenone, and evap. with same technic used in first evapn.

#### E. Determination

Add 100 mL $H_2O$, 2 mL 0.5*N* KOH, and 1 mL 1-fluoro-2,4-dinitrobenzene soln. Stopper, and mix 20 min at high speed on mech. agitator. Add 10 mL 5% borax, swirl to mix, and heat on steam bath 20 min. Cool to room temp. by placing flasks in shallow $H_2O$ bath 10 min. Add 5 mL isooctane, stopper, shake 3 min at high speed, and pour into 250 mL separator. Drain aq. phase, and rinse twice with $H_2O$. Drain isooctane soln thru funnel contg 6 mm glass wool plug into g-s test tube. Soln may be held overnight at this point. Inject 10 μL sample into gas chromatograph. If necessary to dil. sample, transfer 1 mL of isooctane ext to another test tube, dil. to exact vol. with isooctane, and shake to mix. Chromatograph std and sample solns at approx. same level of response.

Methylcarbamates, ppm
= concn std × (peak ht sample/peak ht std)
× (μL std/μL sample) × (diln vol./aliquot vol.) × 5/40

### F. Preparation of Standard Curves

Dissolve 50 mg each carbamate in 100 mL benzene and store in brown bottles. Dil. 5 mL aliquots from these solns to 50 mL with benzene, and store in brown bottles. Transfer 50 μL to 250 mL erlenmeyer, and derivatize as in **975.40E**. After extn of derivatives, solns will contain equiv. of 0.5 ng each carbamate/μL. Chromatograph 4, 6, 8, and 10 μL and plot mm response against ng carbamate. If response is nonlinear, adjust GC parameters and/or prep. more dil. ref. soln, *e.g.*, equiv. of 0.25 μg carbamate/mL, and establish suitable linear working range.

Refs.: JAOAC **56,** 713(1973); **58,** 562(1975).

CAS-671-04-5 (carbanolate)
CAS-63-25-2 (carbaryl)
CAS-1563-66-2 (carbofuran)
CAS-114-26-1 (propoxur)

## 985.23 *N*-Methylcarbamate Insecticide and Metabolite Residues

### Liquid Chromatograhic Method

**First Action 1985**
**Final Action 1986**

(Applicable to residues of aldicarb, bufencarb, carbaryl, carbofuran, methiocarb, methomyl, oxamyl, and metabolites aldicarb sulfone and 3-hydroxycarbofuran in grapes and potatoes)

### A. Principle

Sample is extd with MeOH and cleaned up by liq.-liq. partitioning and Nuchar-Celite column chromatgy. Residues are sepd by reverse phase liq. chromatgy and detected by in-line post-column fluorometric technic.

### B. Reagents

(**a**) *Solvents.*—$CH_3CN$, MeOH, $CH_2Cl_2$, pet ether, and toluene. Distd-in-glass grade (Burdick & Jackson Laboratories, Inc.).

(**b**) *LC acetonitrile.*—UV grade distd-in-glass (Burdick & Jackson Laboratories, Inc.). Before use, degas $CH_3CN$ in glass bottles by applying vac. and slowly stirring solv. with mag. stirrer 5 min.

(**c**) *Ultrapure water.*—Prep. using Milli-Q $H_2O$ purification system (Millipore Corp.). For LC use, degas $H_2O$ as described above for LC $CH_3CN$.

(**d**) *NaOH soln.*—0.05N. Pipet 27 mL clear supernate NaOH in $H_2O$ (1 + 1), **936.16B(b)**, into 100 mL vol. flask. Dil. to vol. with $H_2O$ and mix (5N NaOH). Pipet 10 mL 5N NaOH into 1 L vol. flask. Dil. to 1 L with degassed ultrapure $H_2O$, and mix well but gently to minimize reincorporation of air into soln.

(**e**) *$Na_2SO_4$.*—Anhyd., granular. Heat at 600° overnight and then cool in desiccator.

(**f**) *Sodium tetraborate soln.*—0.05M. Add 19.1 g ACS grade sodium tetraborate decahydrate ($Na_2B_4O_7.10H_2O$) and ca 500 mL degassed ultrapure $H_2O$ to 1 L vol. flask. Heat flask in steam bath to dissolve sodium borate, cool to room temp., and dil. to vol. with degassed ultrapure $H_2O$. Mix well but gently to minimize reincorporation of air into soln.

(**g**) *Reaction soln.*—Weigh 500 mg *o*-phthalaldehyde (Fluoropa, Dionex Corp., 1228 Titan Way, Sunnyvale, CA 94088), transfer to 1 L vol. flask, add 10 mL MeOH, and swirl flask to dissolve *o*-phthalaldehyde. Add ca 500 mL 0.05M sodium tetraborate soln and 1.0 mL 2-mercaptoethanol (Aldrich Chemical Co., Inc., nos. 22, 173-0 and M370-1, resp.) and dil. to vol. with 0.05M sodium tetraborate soln. Mix well but gently to minimize reincorporation of air into soln.

(**h**) *Silanized Celite 545.*—Slurry 150 g Celite 545 (Manville Filtration and Minerals) with 1 L HCl-$H_2O$ (1 + 1) in 2 L beaker, cover with watch glass, and stir mag. while boiling 10 min. Cool slurry, filter, and wash with distd or ultrapure $H_2O$ until filtrate is neut. Wash Celite with 500 mL MeOH followed by 500 mL $CH_2Cl_2$ and then air-dry Celite in hood on watch glass to remove solv. Transfer Celite to 1 L erlenmeyer with g-s joint. Heat unstoppered flask in 120° oven overnight and then cool flask in desiccator. Place flask in hood and carefully pipet 3 mL dichlorodimethylsilane (Pierce Chemical Co.) onto Celite. Stopper flask, mix well, and let flask remain at room temp. 4 h. Add 500 mL MeOH to flask, mix, and let stand 15 min. Filter silanized Celite and wash with isopropanol until neut. Air-dry silanized Celite in hood to remove isopropanol. Dry silanized Celite in 105° oven 2 h, and cool in desiccator. Store silanized Celite in g-s container.

Test Celite for total silanization by placing ca 1 g in 50 mL $H_2O$ and placing ca 1 g in 20 mL toluene saturated with methyl red. Silanized Celite should float on $H_2O$, and appear yellow with methyl red-toluene soln. Repeat silanization of Celite with dichlorodimethylsilane if particles of Celite are dispersed in $H_2O$, and/or appear pink with methyl red-toluene soln, indicating active sites.

(**i**) *Nuchar S-N.*—Slurry 100 g Nuchar S-N (Eastman Kodak no. 1180454) with 700 mL HCl, cover with watch glass, and stir mag. while boiling 1 h. Add 700 mL $H_2O$, stir, and boil addnl 30 min. Cool slurry, filter, and wash with distd or ultrapure $H_2O$ until neut. Then wash Nuchar S-N with 500 mL MeOH followed by 500 mL $CH_2Cl_2$, and air-dry Nuchar S-N in hood to remove solv. Dry Nuchar S-N in 120° oven 4 h. Cool in desiccator. Store Nuchar S-N in g-s container.

(**j**) *Nuchar S-N–silanized Celite 545 chromatographic mixture.*—Mix well 1 part Nuchar S-N with 4 parts silanized Celite 545 (w/w). Test each Nuchar S-N with mixed carbamate soln (carbaryl, methiocarb, methiocarb sulfoxide, methomyl). *Note:* Use freshly prepd mixed std soln because methiocarb sulfoxide degrades in soln. Prep. mixed carbamate soln in MeOH at concn of 5 μg each/mL. Pipet 5 mL of this soln into 250 mL r-b flask and 5 mL into 25 mL actinic vol. flask. Dil. soln in vol. flask to 25 mL with MeOH and use as LC ref. std. Evap. std soln in r-b flask just to dryness with vac. rotary evaporator as described in *Extraction*. After last trace of MeOH has evapd, remove r-b flask from evaporator and dissolve carbamate residue in 10 mL $CH_2Cl_2$. Transfer $CH_2Cl_2$ soln in r-b flask to prepd adsorbent column and elute as described in *Coextractive Removal—Chromatographic*. After evapn of eluate in r-b flask, dissolve residue in 25 mL MeOH. Filter 5–8 mL of this soln thru Swinny filter holder as described in *Coextractive Removal—Chromatographic*. Quantitate recovery of carbamates using LC detn. Nuchar S-N is considered satisfactory if av. recovery of carbamates is ≥95%, with no one compd <90% rec.

(**k**) *Carbamate LC std solns.*—Dissolve carbamate std(s) (EPA/FDA Reference Standards, Environmental Protection Agency, Pesticides and Industrial Chemicals Repository (MD-8), Research Triangle Park, NC 27709) in MeOH to give 1 μg/mL concn or as needed. Store soln(s) in actinic glassware, and when not in use store in refrigerator. Most carbamate stds stored in this manner are stable for several months. However, methiocarb sulfone and sulfoxide degrade within hours and days, respectively, even with storage precautions.

### C. General Apparatus

(**a**) *Homogenizer*.—Polytron Model PT 10-35, with PT 35K generator contg knives (Brinkmann Instruments, Inc.).

(**b**) *Homogenizer jar*.—Four side glass qt jar (Tropicana Products, Inc., Bradenton, FL 33506).

(**c**) *Vacuum rotary evaporator*.—Model RE rotavapor (Brinkmann Instruments, Inc.). Maintain soln in condensing coils and around receiving flask at −15°. (Refrigerated $H_2O$-antifreeze soln works well.) Use vac. pump fitted with manometer and needle valve to control vac. in evaporator.

(**d**) *Chromatographic tubes*.—Chromaflex 30 cm × 22 mm id column (size 233) with coarse porosity frit with Varibor stopcock (size 2) (No. K-420540-9042, Kontes).

(**e**) *Swinny filter holder*.—13 mm filter size (No. XX3001200, Millipore Corp.).

(**f**) *Miltex filters*.—5 μm, 13 mm diam., white, plain (No. LSWP 01300, Millipore Corp.).

### D. LC Apparatus

LC app. (Fig. **985.23A**) must be capable of performing as described in *LC Operating Parameters*. Following specific individual items of app. have been found to meet operating parameters and are listed as guide for analyst:

(**a**) *Mobile phase delivery system*.—Model 322 MP programmable gradient system (replacement Model System Gold Protein II, Beckman Instruments, Inc., 2350 Camino Ramon, PO Box 5101, San Ramon, CA 94583-0701).

(**b**) *Injector*.—Model 16 AS-7000 automatic sampler with 10 μL injection loop.

(**c**) *Guard column*.—7 cm × 2.1 mm id column pellicular ODS (no. 4390-413, Whatman Inc.)

(**d**) *Analytical column*.—25 cm × 4.6 mm id column contg 6 μm Zorbax C-8 spherical particles (DuPont Co.). Equiv. column should contain 5 or 6 μm spherical silica particles that have been bonded with a monofunctional octyl silane reagent to form a monomolecular bond.

(**e**) *Column oven*.—Custom-built forced draft oven (66 × 13 × 11 cm).

(**f**) *Carbamate hydrolysis chamber*.—Column bath (18 × 18 × 13 cm) from Model 5360 Barber-Coleman gas chromatograph with Model 700-115 proportional temp. controller (replacement Model 70A, Dowty Electronics Co., Prospect St, PO Box 250, Brandon, VT 05733-0250) contg 3 m × 0.48 mm id No. 321 stainless steel tubing (Tubesales, 175 Tubeway St, Forest Park, GA 30051).

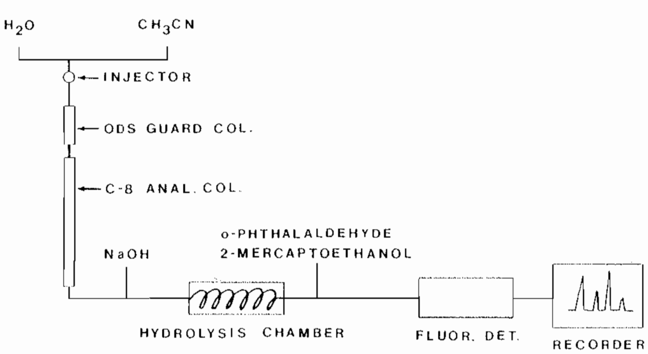


**FIG. 985.23A—LC system with in-line post-column fluorometric detector**

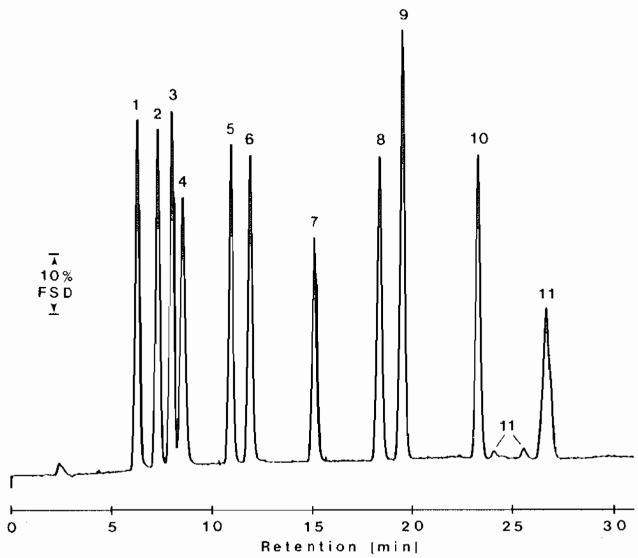


**FIG. 985.23B—Typical chromatogram of separation of carbamates and carbamate metabolites: 1, aldicarb sulfoxide; 2, aldicarb sulfone; 3, oxamyl; 4, methomyl; 5, 3-hydroxy carbofuran; 6, methiocarb sulfoxide; 7, aldicarb; 8, carbofuran; 9, carbaryl; 10, methiocarb; 11, bufencarb (1 and 6 not included in official method).**

(**g**) *Fluorescence detector*.—Model 650-10LC, with 20 μL cell (Perkin-Elmer Corp.).

(**h**) *Recorder*.—Model 4000 microprocessor/printer plotter (Spectra Physics, 3333 N First St, San Jose, CA 95134).

(**i**) *NaOH and reaction soln reservoirs*.—60 cm × 25 mm id glass columns with Teflon fittings (No. 125029, Spectrum Medical Industries, Inc., 60916 Terminal Annex, Los Angeles, CA 90060). Pressurize reservoirs with N. Connect 6 m × 0.5 mm id Teflon restriction coil from reservoir to 15 cm × 0.18 mm id stainless steel tubing. Connect stainless steel tubing to 0.74 mm id stainless steel reaction tee (No. ZVT-062, Valco Instruments Co., Inc., PO Box 55603, Houston TX 77255).

(**j**) *Connecting tubing*.—Use No. 304 stainless steel tubing (1.6 mm od × 0.18 mm id) to connect injector, columns, and first tee.

### E. LC Operating Parameters

Adjust mobile phase flow rate to 1.50 ± 0.02 mL/min at 50% $CH_3CN$ in $H_2O$. Equilibrate system at 12% $CH_3CN$ in $H_2O$ for 10 min, inject sample, and begin 30 min linear gradient to 70% $CH_3CN$ in $H_2O$. Adjust flow rate of 0.05N NaOH and reaction soln to 0.50 ± 0.02 mL/min each. Operate column oven at 35° and hydrolysis chamber at 100°. Set fluorescence detector excitation and emission wavelengths to 340 and 455 nm, resp.; slit widths 15 and 12 nm, resp. Set detector PM gain to low and time const to 1 s. Adjust sensitivity so that 10 ng carbofuran produces 50 ± 5% full scale response on recorder. Baseline noise should be <2%. Carbamates are to elute as shown in chromatogram (Fig. **985.23B**). *Note:* If system will not be used for several days, replace $H_2O$ mobile phase with MeOH and pump thru system, drain NaOH and reaction solns from reservoirs, and wash reservoirs and associated tubing first with $H_2O$ and then MeOH. When starting up system, change mobile phase to $H_2O$, and wash reaction reservoirs and associated tubing with $H_2O$ before adding reaction solns.

### F. Extraction

*High moisture (more than 75% water) products.*—Add 150 g chopped sample and 300 mL MeOH to homogenizer jar. Homogenize sample with Polytron for 30 s at ca half speed (setting of 7) and then 60 s at full speed. Vac.-filter homogenate through 12 cm perforated buchner contg sharkskin or 597 S&S filter, collecting filtrate in 500 mL filter flask. (*Note:* Reduce vac. during filtration if filtrate begins to boil.) Transfer portion of filtrate equiv. to 100 g sample to 2 L ₮ 24/40 r-b flask. (*Note:* Vol. 100 g sample = mL $H_2O$ in 100 g sample + 200 mL MeOH − 10 mL contraction factor.) Add distd or ultrapure $H_2O$ to r-b flask to give total of 100 mL $H_2O$. Also add small star mag. stirrer to r-b flask.

Place 250 mL ₮ 24/40 trap on 2 L r-b flask and attach to vac. rotary evaporator. Apply vac. slowly to minimize frothing. After full vac. is supplied, slowly place flask in 35° $H_2O$ bath. Conc. ext to 75 mL.

### G. Coextractive Removal—Partitioning

Transfer concd ext from r-b flask to 500 mL sep. funnel contg 15 g NaCl. Shake sep. funnel until NaCl is dissolved. Wash r-b flask with three 25 mL portions of $CH_3CN$, transferring each to 500 mL sep. funnel, shake 30 s, and let layers sep. 5 min. Drain aq. phase into 250 mL sep. funnel contg 50 mL $CH_3CN$, shake 20 s, let layers sep., and discard aq. layer.

Add 25 mL 20% aq. NaCl soln to $CH_3CN$ in 500 mL sep. funnel, shake 20 s, let layers sep., and transfer aq. soln to 250 mL sep. funnel. Shake 250 mL sep. funnel 20 s, let layers sep., and discard aq. layer.

Add 100 mL pet ether to 500 mL sep. funnel, shake 20 s, let layers sep. and drain $CH_3CN$ layer into second 500 mL sep. funnel. Transfer $CH_3CN$ in 250 mL sep. funnel to first 500 mL sep. funnel which contains pet ether, shake 20 s, let layers sep., and transfer $CH_3CN$ to second 500 mL sep. funnel. Add 10 mL $CH_3CN$ to first 500 mL sep. funnel, shake, let layers sep., and transfer $CH_3CN$ to second 500 mL sep. funnel. Discard pet ether layer.

Add 50 mL 2% aq. NaCl soln to $CH_3CN$ in second 500 mL sep. funnel. Ext mixt. successively with 100, 25, and 25 mL portions of $CH_2Cl_2$, shaking each 20 s (shake 25 mL portions gently). Drain lower $CH_2Cl_2$-$CH_3CN$ layers thru 22 mm id column contg ca 5 cm anhyd. granular $Na_2SO_4$. Collect eluate in 1 L ₮ 24/40 r-b flask. Evap. soln to dryness with rotary evaporator as described earlier. Remove r-b flask from evaporator immediately after last traces of soln have evapd and then add 10 mL $CH_2Cl_2$ to r-b flask.

### H. Coextractive Removal—Chromatographic

Fit 1-hole No. 5 rubber stopper onto tip of chromatgc tube with Varibor stopcock, add ₮ 24/40 side arm vac. adaptor and 500 mL ₮ 24/40 r-b flask, open stopcock, and connect app. to vac. line. Place 0.5 g silanized Celite 545 in chromatgc tube, tamp, add 5 g Nuchar S-N–silanized Celite 545 (1 + 4) mixt., and tamp again. Add 1–2 cm glass wool plug (Corning) on top of adsorbent. Prewash column with 50 mL toluene-$CH_3CN$ (1 + 3) eluting soln. Close stopcock when prewash soln is ca 0.5 cm from top of glass wool. Disconnect vac., discard eluting soln in r-b flask, and reconnect flask to app. Transfer sample in 10 mL $CH_2Cl_2$ from r-b flask to column and elute column at 5 mL/min. Wash 1 L r-b flask with 10 mL $CH_2Cl_2$ and then with 25 mL eluting soln. Transfer each sep. to column and elute each to top of glass wool before adding next soln. Next, add 100 mL eluting soln and elute column at 5 mL/min. Turn off stopcock when top of eluting soln reaches top of glass wool. Evap. soln in 500 mL r-b flask just to dryness using vac. evaporator as before. Remove flask from evaporator immediately after all soln has evapd. Immediately pipet 5 mL MeOH into 500 mL r-b flask to dissolve residue. Pour MeOH sample soln into 10 mL glass syringe contg Swinny filter holder with 5 μm filter. Push MeOH soln thru filter with syringe plunger, collecting filtrate in 10 mL centrf. tube or other suitable container. *Note:* Approximately 4.5 mL filtrate will be collected. Vol. of filtrate collected is not critical because g sample/mL MeOH is known. If soln needs to be dild, pipet aliquot into another container and dil. to vol. as needed.

### I. Determination

Inject 10 μL MeOH sample soln onto LC column using chromatgc app. and parameters as described. Tentatively identify residue peaks on basis of retention times. Measure peak area or peak ht and det. residue amt by comparison to peak area or peak ht obtained from known amt of appropriate ref. material(s). To ensure valid measurement of residue amt, size of peaks from sample residue and ref. std should match within ±25%. Chromatograph ref. material(s) immediately after sample.

CAS-116-06-3 (aldicarb)
CAS-1646-88-4 (aldicarb sulfone)
CAS-8065-36-9 (bufencarb)
CAS-63-25-2 (carbaryl)
CAS-1563-66-2 (carbofuran)
CAS-16655-82-6 (3-hydroxycarbofuran)
CAS-2032-65-7 (methiocarb)
CAS-16752-77-5 (methomyl)
CAS-23135-22-0 (oxamyl)

Ref.: JAOAC **68**, 726(1985).

## INDIVIDUAL RESIDUES

## 961.12★ Azinphos-Methyl Pesticide Residues

**Spectrophotometric Method**

**First Action 1961**
**Surplus 1974**

(Applicable to cole-type crops and to apples, plums, peaches, grapes, apricots, and cherries.)

*See* **29.102–29.107**, 12th ed.

## 956.05★ Benzene Hexachloride Pesticide Residues

**Colorimetric Method**

**First Action 1956**
**Final Action 1960**
**Surplus 1970**

*See* **24.101–24.105**, 10th ed.

## 958.07★ Lindane and Technical BHC Pesticide Residues

**Distinguishing Method**

**First Action**
**Surplus 1970**

*See* **24.106–24.110**, 10th ed.

## 968.25 Biphenyl Pesticide Residues in Citrus Fruits

### Thin Layer Chromatographic–Spectrophotometric Method

**First Action 1968**
**Final Action 1969**

#### *A. Principle*

Biphenyl is extd from blended peel or pulp by steam-liq.-liq. extn. Ext is subjected to TLC and biphenyl zone is completely scraped from developed plate. Biphenyl is eluted from adsorbent with alcohol for spectrophtric detn.

#### *B. Reagents*

(**a**) *Silica gel.*—GF-254 (Brinkmann Instruments, Inc. No. 7730).

(**b**) *Biphenyl std solns.*—(*1*) *Stock soln.*—Approx. 0.5 mg/mL. Dissolve ca 50 mg accurately weighed biphenyl in *n*-heptane and dil. to 100 mL with *n*-heptane. (*2*) *Limit soln.*—Approx. 0.01 mg/mL. Dil. 5 mL stock std to 250 mL with *n*-heptane.

Use stock std soln for spectrophtric quantitation after TLC step. Limit std soln aids in locating biphenyl zone and in estg small amts.

#### *C. Apparatus*

(**a**) *Applicator.*—For depositing thin layer on glass plates.

(**b**) *Glass plates.*—8 × 8″ or 2 × 8″; of uniform thickness.

(**c**) *Plastic board.*—22 × 113 cm, with retaining edges 1.8 cm wide along short and long sides.

(**d**) *Developing jars or tanks.*—Use equipment, **970.52G(a)**, for 8 × 8″ glass plates and glass cylinders for small plates. Cylinders can be covered with plastic caps.

(**e**) *Spotting pipet.*—100 μL.

(**f**) *Tank liner.*—Whatman 3MM paper cut to fit tank.

(**g**) *Moisture test apparatus.*—Similar to lighter-than-$H_2O$ volatile oil trap, **962.17A(a)**, Fig. **962.17**, with cold finger condenser (Lurex Scientific, No. JM-8590, or equiv.).

#### *D. Preparation of TLC Plates*

Mix 40 g silica gel with 80 mL $H_2O$, shaking vigorously few sec, and finally swirling ca 30 sec to eliminate air bubbles. Spread slurry 0.3 mm thick over 5 plates. Let plates air dry in place ca 1 hr. Put plates in drying rack and place in 100° oven 2 min. Remove plates and store in desiccator over silica gel or $CaCl_2$ until used. Plates may be stored up to 30 days.

#### *E. Preparation of Sample*

Sort out and discard rotten units. Completely peel ≥6 whole fruits (include all white material under peel in peel portion). Weigh peelings and peeled fruit, and calc. wt ratio of peelings to peeled fruit.

(**a**) *Peel.*—Grind combined peel in food grinder. Blend 200 g ground peel with 400 g $H_2O$ at high speed 5 min (or in five 1 min increments if blender becomes very warm), using high-speed blender. (Larger batches may be blended with large blender as long as peel-$H_2O$ ratio is same.)

(**b**) *Peeled fruit.*—Cut peeled fruit into small pieces and blend at high speed 5 min (or in five 1 min increments if blender becomes very warm).

#### *F. Extraction*

Accurately weigh ca 300 g recently blended peel slurry or ca 100 g recently blended peeled fruit, and transfer to 1 L r-b ₸ 29/42 flask with enough $H_2O$ to yield total vol. of ca 500 mL; add few boiling chips (6 mesh granular SiC is convenient). Connect extn unit of moisture test app. to flask and fill side arm with $H_2O$ to overflowing. Place ca 3 mL *n*-heptane on top of $H_2O$ layer and insert cold finger cooled with very rapid flow of cold $H_2O$. Gradually heat flask with mantle (controlled by variable transformer) until even boiling is obtained, then intensely enough to maintain vigorous boiling. Continue extn 3 hr from time mixt. starts boiling. (Wrap exposed portion of flask and connector arm between flask and extn unit with Al foil.) Initial carry-over of froth does not interfere. After 3 hr, discontinue heat and drain entire contents of extractor into 125 mL separator. Discard lower layer and drain heptane ext thru 2.5 cm column of granular anhyd. $Na_2SO_4$ (8–10 mm id column) into 10 mL vol. flask. Rinse separator with 1 mL *n*-heptane and add rinse to column. Rinse cold finger and extn unit with five 2 mL portions alcohol, collecting successive rinses in separator. Add 5 mL *n*-heptane to separator and shake vigorously few sec; add 50–75 mL $H_2O$ and shake moderately few sec. Let layers sep. (lower layer may remain slightly cloudy) and discard lower layer. Pass heptane layer thru same $Na_2SO_4$ column into vol. flask. Rinse separator and column with enough *n*-heptane to dil. to vol.

#### *G. Thin Layer Chromatography*

Pre-sat. tank contg liner with *n*-heptane ≥1 hr before use. Establish imaginary spotting line 3 cm from bottom edge of plate. For each intended spot, use tip of 100 μL pipet to scratch mark in adsorbent layer just size of pipet tip. (Space spots evenly with max. of 7 spots including blank.) Spot 100 μL each stock and limit std solns on extreme spots (one on far right and one on far left of plate). Spot 100 μL *n*-heptane as blank and 100 μL sample between std spots. Use same pipet for all spots, rinsing thoroly with *n*-heptane between applications. Keep size of spots uniform at 1.5–2 cm diam. by using following technic: Fill 100 μL pipet past mark with soln to be spotted. Carefully drain excess into absorbent towel until soln is at exact vol. mark. Press pipet tip against exposed glass in center of spotting mark on plate (hold pipet in vertical position at all times). Regulate size of spot by holding finger over top of pipet and pressing tip tightly against plate. Blow across spot (orally) only when necessary to regulate size of spot and never lift pipet from place once spotting is begun.

Pour 10–15 mL *n*-heptane in tank trough, insert plate, and seal tank. Develop until solv. is within 2.5 cm from top of plate (ca 30 min). Remove plate, air dry few min, and view under UV light. Biphenyl appears as bright blue spot on yellow background.

If no biphenyl appears in sample, end analysis at this point. If biphenyl is found, remove spots from plate without delay. Score upper and lower extremes of biphenyl zone horizontally across plate. Score vertical lines in adsorbent between biphenyl spots to include approx. equal area in each rectangle, scribing same area for ref. spot. Use razor blade to scrape off, and discard all adsorbent below biphenyl zone and outside extreme vertical lines. Use absorbent tissue and alcohol to clean exposed glass thoroly. Carefully scrape adsorbent from one extreme rectangular zone onto glazed paper and transfer to funnel inserted in 10 mL vol. flask; do not use solv. to rinse paper. Rinse off razor blade into funnel with small portion of alcohol. Tip plate at angle to facilitate rinsing of scraped area into funnel and rinse with several small portions alcohol. Rinse funnel and finally dil. to vol. with alcohol. Shake mixt. vigorously and let stand 5 min, shaking occasionally. Remove each biphenyl spot same way, working inward from each side of plate and cleaning and drying each previously removed zone. Filter each mixt. thru Whatman No. 44 paper, or equiv., and store filtrate in stoppered vessel for spectrophtric detn.

#### *H. Spectrophotometry*

Det. *A* of each soln at 248 and 300 nm in 1 cm cell with alcohol as ref.

$$\text{ppm Biphenyl} = (\Delta A_{248} \text{ sample}/\Delta A_{248} \text{ std}) \times (\mu\text{g std spotted/g sample spotted})$$

where $\Delta A_{248} = A_{248} - [A_{300} \times (A_{248} \text{ blank}/A_{300} \text{ blank})]$.

Ref.: JAOAC **50,** 934(1967).

CAS-92-52-4 (biphenyl)

## 959.10★ *2-(p-tert-*Butylphenoxy)-1-Methylethyl 2-Chloroethyl Sulfite (Aramite) Pesticide Residues

**Colorimetric Method**

**Final Action 1965**
**Surplus 1975**

*See* **29.067–29.071**, 12th ed.

## 957.14 Captan Pesticide Residues

**Spectrophotometric Method**

**Final Action**

(Applicable to firm fruits such as apples, pears, peaches, and plums and to green vegetables)

### *A. Principle*

Captan is extd from crop with benzene; $H_2O$, color, and appreciable amts of waxes are removed, and red color is developed by fusion of captan with resorcinol at 135°; color changes to yellow on addn of HOAc.

### *B. Reagents*

(**a**) *Resorcinol.*—Must be free of discoloration and pass following tests: Fuse 0.5 g and dissolve in 25 mL HOAc. $A$ at 425 nm is $\leq$0.015, against HOAc. 1.00 g should not lose >2 mg in 4 hr over $H_2SO_4$; if more is lost, dry over $H_2SO_4$ until test is satisfactory.

(**b**) *Cleanup mix.*—10 parts Nuchar, 5 parts Hyflo Super-Cel, and 5 parts anhyd. $Na_2SO_4$.

(**c**) *Captan std solns.*—(*1*) *Stock soln.*—3 mg/mL. Transfer 150 mg pure captan (available from Chevron Chemical Co., PO Box 4010, Richmond, CA 94804) to 50 mL vol. flask and dil. to vol. with benzene. (*2*) *Intermediate soln.*—300 µg/mL. Pipet 10 mL stock soln into 100 mL vol. flask and dil. to vol. with benzene. (*3*) *Working soln.*—30 µg/mL. Pipet 10 mL intermediate std soln into 100 mL vol. flask and dil. to vol. with benzene.

### *C. Preparation of Sample*

(*Caution:* See safety notes on flammable solvents, toxic solvents, and benzene.)

(**a**) *Fruits.*—Accurately weigh ca 500 g sample into clean, dry jar with screw cap faced with sheet cork gasket covered with wet filter paper, or other solv.-tight lid, and add 500 mL benzene. Multiples of sample-to-benzene ratio can be used. Agitate 15 min, drain benzene into container, and transfer to separator. (Transfer to separator may be omitted where there is no separable aq. layer.)

Transfer ca 100 mL sepd benzene layer to 250 mL g-s flask, and decolorize and dehydrate with 3–4 g cleanup mix, (**b**), by shaking vigorously ca 5 min. Filter thru folded paper, rejecting first 10–15 mL.

(**b**) *Green vegetables.*—Chop sample in food chopper such as Hobart Food Cutter, mix, and transfer 100 g to explosion-proof blender. Add 200 mL benzene and blend 2 min; add 20 g anhyd. $Na_2SO_4$ and blend 2 min more. Pour mixt. into 500 mL centrf. bottle, stopper with *cork*, and centrf. at ca 1400 rpm 5–10 min. Decant benzene layer into 250 mL g-s erlenmeyer, add ca 6 g cleanup mix, (**b**)/100 mL benzene, and shake vigorously ca 5 min. Filter thru folded paper, discarding first 10 mL. If water-white soln does not result, repeat cleanup treatment. Pipet 50 mL into 100 mL vol. flask and dil. to vol. with benzene.

### *D. Determination*

(*Caution:* See safety notes on pipets, toxic solvents, pesticides, and benzene.)

Pipet 5 mL filtrate, **957.14C(a)**, or aliquot, (**b**), into 25 × 200 mm test tube and add 0.5 ± 0.1 g resorcinol. Heat 20 min in oil bath at 135 ± 5°, cautiously at first to evap. benzene; then immerse reaction tubes to depth of ca 5 cm but do not let them touch bottom of bath. Remove, and immediately add 10–15 mL HOAc, followed by rapid immersion in $H_2O$ at room temp. Transfer quant. to 25 mL vol. flask, using HOAc, dil. to vol. with HOAc, and mix.

Det. $A$ at 425 nm in 1 cm cell against HOAc within 1 hr. Calc. ppm from std curve.

### *E. Preparation of Standard Curve*

Prep. std curve simultaneously with samples. Pipet 0, 2, 4, and 5 mL aliquots of working std soln into 25 × 200 mm test tubes and add benzene to make total vol. of 5 mL in each tube. Add 0.5 ± 0.1 g resorcinol and continue as in detn, beginning "Heat 20 min in oil bath . . ."

*Note:* One drop $H_2O$ in reaction tube will cause apparent loss of ca 20% captan. Do not leave benzene aliquots in unstoppered reaction tubes where condensation of moisture will take place.

Refs.: JAOAC **40,** 219(1957); **46,** 143, 241(1963).

CAS-133-06-2 (captan)

## 964.18 Carbaryl Pesticide Residues

**Colorimetric Method**

**First Action 1964**
**Final Action 1965**

### *A. Reagents*

(**a**) *Acetone.*—Redistd.

(**b**) *Coagulating soln.*—Dissolve 0.5 g $NH_4Cl$ in 400 mL $H_2O$ contg 1 mL $H_3PO_4$.

(**c**) *Color reagent.*—Dissolve 25 mg *p*-nitrobenzenediazonium fluoborate in 5 mL MeOH and add 20 mL HOAc. Prep. just before use.

(**d**) *Methylene chloride.*—Redistd $CH_2Cl_2$.

(**e**) *Alcoholic potassium hydroxide soln.*—0.1*N* in MeOH.

(**f**) *Polyethylene glycol soln.*—Dil. 1 mL polyethylene glycol to 100 mL with $CH_2Cl_2$.

(**g**) *Carbaryl std solns.*—Ref. std material is available from Rhone-Poulenc Ag Co., 2 T.W. Alexander Dr, PO Box 12014, Research Triangle Park, NC 27709. (*1*) *Stock soln.*—0.5 mg/mL. Place 50.0 mg in 100 mL vol. flask and dil. to vol. with $CH_2Cl_2$. (*2*) *Intermediate soln.*—50 µg/mL. Transfer 10 mL stock soln to 100 mL vol. flask and dil. to vol. with $CH_2Cl_2$. (*3*) *Working soln.*—5.0 µg/mL. Transfer 10 mL intermediate soln to 100 mL vol. flask and dil. to vol. with $CH_2Cl_2$.

### *B. Apparatus*

*Evaporative concentrator.*—*See* Fig. **964.18**. Vac. manifold connected thru stopcock to antisurge column, 250 × 19 mm od, contg glass marble, or Snyder column, attached to

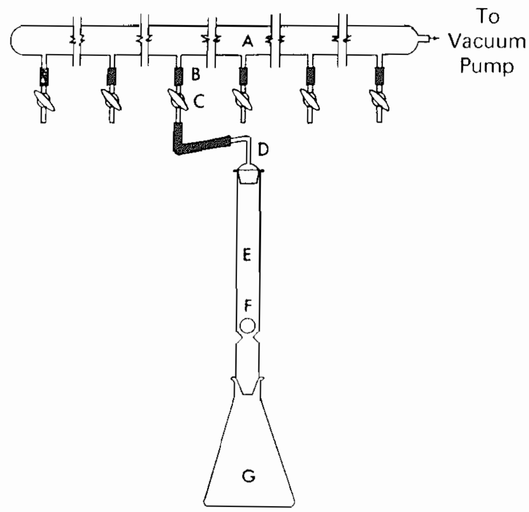


**FIG. 964.18—Evaporative concentrator. A, glass manifold. B, pressure tubing. C, stopcock. D, adapter, ₸ 24/40. E, antisurge column, 25 cm × 19 mm od. F, glass marble. G, erlenmeyer, ₸ 24/40, 250 mL.**

₸ 24/40 erlenmeyer. Use surgical tubing wherever contact with sample is likely.

### *C. Preparation of Sample Solution*

Transfer 50 g sample to high-speed blender and add 150 mL $CH_2Cl_2$ and 100 g powd anhyd. $Na_2SO_4$. Blend at high speed 2 min and let settle 1 min. Decant solv. into 9 cm buchner fitted with Whatman No. 1, or equiv., paper covered with thin coat of Hyflo Super-Cel, or equiv., filter aid. Cautiously apply vac. until all solv. has filtered. Repeat extn with two 100 mL portions $CH_2Cl_2$. Treat combined filtrates as in (**a**) or (**b**):

(**a**) Transfer combined filtrates to 500 mL ₸ erlenmeyer and add 1 mL polyethylene glycol soln. Connect to evaporative concentrator, place flask in $H_2O$ bath at 25–30°, and carefully reduce pressure to ca 20 mm (2.7 kPa). After solv. evaps, immediately disconnect antisurge column from manifold. Rinse down walls of column and flask with 5 mL acetone from pipet, swirl flask, and warm gently under hot $H_2O$ tap 30 sec. Add 50 mL coagulating soln thru column, and swirl. Remove column, let mixt. stand 30 min, and filter with vac. thru 3 mm layer of Super-Cel in No. 1 buchner. Wash flask and pad with two 15 mL portions coagulating soln.

Transfer filtrate to 125 mL separator, add 25 mL $CH_2Cl_2$, shake well, and let sep. completely. Drain lower layer into ₸ 250 mL erlenmeyer. Repeat extn of aq. layer with 25 mL $CH_2Cl_2$, adding ext to same 250 mL erlenmeyer. If combined exts are cloudy, add 5–10 g granular anhyd. $Na_2SO_4$, and shake. Decant solv. into clean 250 mL ₸ erlenmeyer, rinsing with small portion $CH_2Cl_2$. (If residue is expected to be >2 ppm, dil. exts to vol. in 100 mL vol. flask, and use appropriate aliquot.)

(**b**) Add 1 mL polyethylene glycol. Stopper, carefully reduce pressure to ca 150 mm, and warm on steam bath. When vol. is ca 5 mL, remove from steam bath and swirl until dry. Release vac., remove stopper, and let cool. Continue as in (**a**), beginning "Rinse down walls . . ." except column is not present.

### *D. Determination*

To soln in erlenmeyer add 1 mL polyethylene glycol soln and connect to column and evaporator. Evap. solv. as before, immediately disconnect, and remove column. Rinse down walls of flask with 2 mL 0.1*N* KOH in MeOH from pipet, rotating to ensure complete contact. Let stand 5 min, add exactly 17 mL HOAc, and with swirling add 1 mL color reagent. Let stand exactly 1 min and det. *A* in 1 cm cell at 475 nm against reagent blank processed along with sample as ref. Det. μg from std curve.

ppm Carbaryl
= (μg/g sample) × (diln factor if aliquot was used)

### *E. Preparation of Standard Curve*

(*Caution:* See safety notes on pipets and pesticides.)

Pipet 0, 1, 3, 5, and 10 mL aliquots working std soln to 500 mL ₸ erlenmeyers, add 300 mL $CH_2Cl_2$ to each, and proceed as in **964.18C**, beginning "Treat combined filtrates as in (**a**) or (**b**):"

Plot *A* against μg carbaryl to obtain std curve.

Refs.: JAOAC **47,** 283(1964); **48,** 676(1965).

CAS-63-25-2 (carbaryl)

## 968.26 Carbaryl Pesticide Residues
### Qualitative and Semiquantitative Method
### Final Action 1976

(Applicable to apples and spinach)

### *A. Reagents*

(**a**) *Adsorbent.*—$Al_2O_3$ G (contains 10% $CaSO_4$). *See* **970.52C(a)**.

(**b**) *Coagulating soln.—See* **964.18A(b)**.

(**c**) *Chromogenic spray soln.*—Sat. diethylene glycol-alcohol soln (1 + 9) with *p*-nitrobenzene-diazonium fluoborate (practical grade, ca 25 mg/100 mL) by stirring ca 2 min. Filter, keep cold during use, and store in refrigerator. Do not use after 3 days.

(**d**) *Diethylene glycol soln.*—Dil. 10 mL diethylene glycol to 100 mL with redistd $CH_2Cl_2$.

(**e**) *Carbaryl std.*—Mp 141–142°. *See* **964.18A(g)**. Recrystallize from alcohol and $H_2O$, if necessary.

### *B. Apparatus*

(**a**) *TLC apparatus.*—App. suitable for 8 × 8″ plates. *See* **970.52F**.

(**b**) *Evaporative concentrator.*—Two chamber, ₸ 24/25, micro-Snyder column (Kontes Glass Co. K-569001); with 10 mL Mills tube, graduated (Kontes K-570050).

### *C. Extraction and Cleanup of Sample*

Transfer 25 g sample to blender. Add 150 mL $CH_2Cl_2$ and 100 g powd (150 g granular) anhyd. $Na_2SO_4$. Blend 2 min at low speed and let settle. Attach 9 cm buchner contg rapid paper to 500 mL filter flask. Cover paper with thin coat of Hyflo Super-Cel prepd as slurry in $CH_2Cl_2$. Decant ext into buchner and cautiously apply vac. Rinse blender with 50 mL $CH_2Cl_2$ and filter. Return residue to blender. (Complete sepn of residue from Super-Cel is unimportant.) Add 150 mL $CH_2Cl_2$, reext, filter, and rinse again with 50 mL $CH_2Cl_2$. Add 1 mL diethylene glycol soln to filter flask. Place flask with buchner contg original filter pad attached on steam bath and apply vac. When vol. in flask is ca 5 mL, remove flask from steam bath and swirl until dry. Release vac., remove buchner, and let flask cool.

Rinse down side of flask with 3 mL acetone from pipet and swirl to dissolve residue. While gently swirling flask, add 15 mL coagulating soln and let stand >10 min with occasional swirling. Filter, using vac., thru small fritted glass funnel, medium porosity, contg ca 6 mm layer of Hyflo Super-Cel and receive filtrate in 30 mL test tube. Wash ppt with three 2 mL portions acetone-$H_2O$ soln (1 + 9), letting each washing remain in contact with ppt ca 15 sec before applying vac. Transfer filtrate and washings to 25 mL vol. flask, dil. to vol. with acetone-$H_2O$ soln (1 + 9), and mix.

***D. Determination***

(*Caution:* See safety notes on spraying chromatograms.)

Transfer 10 mL sample soln to 125 mL separator. Ext soln with two 5 mL portions $CH_2Cl_2$, shaking 5–10 sec each time. Combine exts in Mills tube, add small SiC chip (<0.01 mL vol.), fit with micro-Snyder column, and evap. to 0.1 mL on steam bath. (*Caution:* Samples may be lost by vigorous ebullition.)

Prepare 8 × 8″ TLC plates coated with 250 μm layer $Al_2O_3$ adsorbent. Dry plates in forced-draft oven 30 min at 80°. Store in desiccator cabinet. Using 1 μL pipet, spot aliquots equiv. to 2 g sample and carbaryl stds (in $CH_2Cl_2$) to cover expected range.

Place trough in chromatgc tank lined with blotting paper. Add ca 50 mL acetone-benzene soln (1 + 4) to bottom of tank to sat. atm., and then add 50 mL same soln to trough. Place plate in trough and seal tank with masking tape. Develop plate until solv. front just reaches line drawn 10 cm from origin. Dry plate ca 15 min in hood. Spray moderately with 1.0*N* alc. KOH soln. Then spray moist plate with chromogenic soln. Blue spot with $R_f$ value same as std carbaryl spot indicates carbaryl ($R_f$ range, 0.52–0.60). Compare size and intensity of sample and std spots for semiquant. estn of amt of pesticide. It is possible to distinguish, for example, between 0.2 and 0.4 μg, but not between 0.3 and 0.4 μg. Optimum range for quant. estn is ca 0.1–0.4 μg. For amts >0.4 μg, spot smaller aliquot of remaining 80 μL soln. Then spot same vol. of std soln for valid comparison.

Ref.: JAOAC **51,** 679(1968).

CAS-63-25-2 (carbaryl)

## 958.08★ *p*-Chlorophenyl Phenyl Sulfone Pesticide Residues

**Spectrophotometric Method**

**Final Action 1965**
**Surplus 1969**

*See* **29.075–29.081**, 11th ed.

## 973.39 Ethylan (Perthane) Pesticide Residues

**Gas Chromatographic Method**

**First Action 1973**
**Final Action 1974**

(For low levels (less than ca 50 ng/GC injection) and for confirmatory quantitation of higher levels of ethylan previously detd by **970.52A–R**)

***A. Principle***

Method is extension of general method for multiple residues, **970.52A–R**. After electron capture GC detn of ethylan and other organochlorine and organophosphorus pesticides, ethylan in 6% mixed ether eluate, **970.52O**, is dehydrochlorinated to its olefin. Ethylan olefin is extd from reaction mixt. into hexane and portion of hexane is injected into gas chromatograph with electron capture detector. Ethylan olefin produces 10-fold increase in electron capture detector response over that of parent compd.

***B. Reagents***

(**a**) *Hexane.—See* **970.52B(j)**.
(**b**) *Carborundum chips.*—SiC, ca 20 mesh.
(**c**) *Alcoholic potassium hydroxide soln.*—Dissolve 2 g KOH in 100 mL alcohol.
(**d**) *Ethylan std soln.*—50 μg/mL hexane.
(**e**) *Ethylan olefin std soln.*—5 μg/mL hexane.

***C. Apparatus***

(**a**) *Graduated centrifuge tube.*—With No. 13 glass stopper (Corning Glass Works, No. 8084, or equiv.).
(**b**) *Oil bath.*—100 mL beaker contg 80 mL paraffin oil. Heat on mag. stirrer hot plate calibrated to maintain oil temp. at 100 ± 5°.

***D. Determination***

After electron capture GC of ethylan and other organochlorine and organophosphorus pesticides, **970.52A–R**, pipet entire 6% eluate, **970.52O**, or aliquot contg ≤30 μg ethylan into reaction tube. Carefully evap. to dryness under gentle air current. Add 2 mL alc. KOH soln and 2–5 SiC chips. Place reaction tube in 100° oil bath to depth of ca 1.0 mL graduation and let soln reflux 15 min. (Conduct reaction in hood. Air flow thru hood will cool upper part of tube, which serves as condenser. Hood also removes odors escaping from hot oil.) Remove tube from oil bath, cool to room temp., and add 3 mL $H_2O$. Pipet vol. hexane (but ≥1 mL) into tube to give concn ca 5 μg ethylan olefin/mL. Shake vigorously ca 30 sec and let layers sep. Det. ethylan olefin in hexane layer as in **970.52R**.

$$\text{ppm Ethylan} = (R/R') \times (W'/W) \times (307.25/270.78)$$

where $R$ and $R'$ = responses to ethylan olefin in sample and std, resp.; $W'$ = ng std injected; $W$ = mg equiv. sample injected; and 307.25 and 270.78 = MW ethylan and ethylan olefin, resp.

Refs.: JAOAC **55,** 1042(1972); **56,** 721(1973).

CAS-72-56-0 (ethylan)

## 960.41★ DDT Pesticide Residues

**Colorimetric Method**

**First Action 1960**
**Final Action 1965**
**Surplus 1980**

*See* **29.097–29.102**, 13th ed.

## 965.36 Dichlone Pesticide Residues

**Spectrophotometric Method**

**First Action 1965**
**Final Action 1966**

(Applicable to fresh fruits and vegetables)

***A. Reagents***

(**a**) *Dichlone std soln.*—0.2 mg/mL. Dissolve and dil. 40 mg dichlone (Eastman Kodak Co. No. 3836, or equiv.) to 200 mL with benzene.
(**b**) *Dimethylamine.*—25% aq. soln (Eastman Kodak Co. P601 or equiv.).

(c) *Florisil.*—60/100 mesh, PR Grade, activated at 1250°F (Floridin Co.). Heat ≥4 hr at 130° and store in stoppered flasks in desiccator prior to use.

### B. Preparation of Standard Curve

Place 0, 1.00, 2.00, 3.00, 4.00, and 5.00 mL dichlone std soln in 25 mL g-s graduates and dil. each to 10 mL with benzene. To each graduate add isopropanol to 20 mL mark and mix. Add 1 mL 25% $Me_2NH$ soln, dil. to 25 mL with isopropanol, and mix.

Read *A* of stds against blank in covered 1 cm cells at 495 nm, and plot *A* against mg dichlone (0–1.0 mg range). Color is stable >1 hr.

### C. Preparation of Column

Fill 15 × 300 mm chromatgc tube, fitted with fritted glass disk or glass wool plug, with Florisil to ca 1/3 its length. (No stopcock is required.) Prewet Florisil with 30 mL benzene.

### D. Determination

(*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, and benzene.)

Strip weighed sample (ca 1 kg) with 500 mL benzene by gently turning or tumbling 10 min in suitable container (ca 4 L; 1 gal.). (Avoid breaking plant tissue.) Drain benzene into 1 L flask thru folded paper (ca 32 cm) contg ca 50 g anhyd. $Na_2SO_4$.

Add 200 mL dried benzene strip soln to prepd chromatgc column. Discard benzene eluate. Elute dichlone from column with 100 mL acetone-benzene eluting mixt. (1 + 99). Collect eluate in beaker and evap. to ca 15 mL. (Do not let sample overheat or go to dryness.) Rinse sample into graduate and dil. to 20 mL with benzene. Develop color in 10 mL of this soln as in **965.36A**.

mg Dichlone from std curve × 5
= ppm dichlone (for 1 kg sample)

If visible color is present in benzene eluate, simultaneously develop color in remaining 10 mL aliquot, omitting $Me_2NH$ and adding 1 mL $H_2O$. Subtract this blank *A* from that of developed sample to correct for sample blank.

Ref.: JAOAC **48,** 759(1965).

CAS-117-80-6 (dichlone)

## 964.19 Dodine Pesticide Residues

**Spectrophotometric Method**

**First Action 1964**
**Final Action 1965**

(Applicable to apples, peaches, pears, pecans, and strawberries)

### A. Reagents

(a) *Bromocresol purple soln.*—Recrystallize indicator-grade bromocresol purple from boiling toluene (ca 2 g/100 mL). Dissolve 0.4 g recrystd material in 75 mL 0.01*N* NaOH; if necessary, add addnl 0.01*N* NaOH to bring pH to 6.0–6.1. Filter, if necessary, and dil. to 500 mL with $CO_2$-free $H_2O$. Store in brown bottle.

(b) *Buffer soln.*—pH 5.5. Dissolve 15.2 g $Na_2HPO_4.7H_2O$ and 74.0 g $NaH_2PO_4.H_2O$ in $CO_2$-free $H_2O$ and dil. to 1 L.

(c) *Dodine (DDGA) std solns.*—(*1*) *Stock soln.*—130 μg/mL. Dissolve 32.5 mg Ref. Std (available from American Cyanamid Co.) in MeOH and dil. to 250 mL with MeOH. (*2*) *Working soln.*—13 μg/mL. Dil. 25 mL aliquot stock soln to 250 mL with MeOH.

### B. Preparation of Sample

Grind sample in high-speed blender with MeOH-$CHCl_3$ (2 + 1) in ratio of 400 mL solv./100 g sample. Filter with suction thru 2 Whatman No. 1, or equiv., papers in buchner, and wash pulp with MeOH-$CHCl_3$ (2 + 1), using 100 mL/100 g sample. Det. vol. of ext and transfer portion equiv. to 50 g sample to 400 mL beaker.

### C. Determination

Add several glass beads and 1 mL HCl to beaker, and evap. to 50 mL on steam bath. Add 30 mL 30% NaCl soln and 100 mL MeOH. Cool, transfer to 500 mL separator, and ext *gently* with 50 mL $CCl_4$ by inverting separator 6–8 times. Let phases sep. and discard $CCl_4$ layer. Repeat with 50 mL $CCl_4$, inverting separator ca twice as many times. Discard $CCl_4$; then ext with 50 mL $CCl_4$, shaking gently 30 sec. Finally, ext with 50 mL $CCl_4$, shaking vigorously 1 min, and again discard $CCl_4$.

Adjust pH of soln to ca 5.5 with 4*N* NaOH (pH meter), and add 20 mL pH 5.5 buffer and 20 mL bromocresol purple soln. Re-adjust pH to 5.5 and ext complex with two 50 mL portions $CHCl_3$, shaking 2 min each time. Shake combined ext 30 sec with 25 mL pH 5.5 buffer, and transfer $CHCl_3$ layer to another separator. Shake 1 min with 25 mL pH 5.5 buffer, let stand 10 min, and transfer $CHCl_3$ to another separator. Shake with 20 mL 0.05*N* NaOH to remove all combined indicator and any org. acids which may persist. Recomplex dodecylguanidine (in $CHCl_3$ as free base) by shaking 3 min with 5 mL bromocresol purple soln and 20 mL pH 5.5 buffer. Wash $CHCl_3$ with three 15 mL portions pH 5.5 buffer, shaking 1 min each time. Transfer $CHCl_3$ to dry 250 mL separator and shake 2 min with 20 mL 0.05*N* NaOH, measured by pipet. Read *A* of indicator in aq. soln at 590 nm, using Beckman spectrophtr, or equiv. Obtain μg DDGA from std curve.

### D. Preparation of Standard Curve

Add 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mL std soln to series of separators contg 100 mL MeOH, 50 mL $H_2O$, 30 mL 30% NaCl soln, 20 mL bromocresol purple soln, and 20 mL pH 5.5 buffer. Adjust pH of each soln to 5.5 and continue as in **964.19C**, par. 2, beginning ". . . and ext complex with two 50 mL portions $CHCl_3$, shaking 2 min each time." Read *A* of each aq. soln at 590 nm and plot against μg DDGA. No blank correction is necessary for stds.

ppm DDGA = μg DDGA in aliquot/g sample in aliquot

Ref.: JAOAC **47,** 300(1964).

CAS-2439-10-3 (dodine)

## 986.20 Ethylene Dibromide in Grains and Grain Products

**Gas Chromatographic Method**

**First Action 1986**
**Final Action 1987**

### A. Principle

Whole grains and intermediate grain-based products are extd by soaking in acetone-$H_2O$; ready-to-eat products are extd by hexane co-distn. Portions of exts are dried and analyzed by gas chromatgy with electron capture detection.

### B. Reagents

(a) *Solvents.*—2,2,4-Trimethylpentane (isooctane), acetone, and hexane (all pesticide quality). Check for interferences by injecting 5 μL into GC system operated as described under *Apparatus*.

**(b)** *Calcium chloride*.—Analyzed reagent grade, anhyd., 8 mesh.

**(c)** *Sodium sulfate*.—Analyzed reagent grade, anhyd., granular.

**(d)** *Sulfuric acid (concd)*.—Analyzed reagent grade.

**(e)** *Std solns*.—(*1*) *Stock soln*.—Prep. in 50 mL vol. flask equipped with Teflon-lined screw cap. Add ca 40 mL isooctane to flask and weigh flask + isooctane to nearest 0.1 mg. Introduce 20 μL pure ref. std (EPA Ref. Std P480) into isooctane and re-weigh to det. wt of EDB. Dil. to vol. with isooctane and calc. concn in μg/mL. Store in freezer. (*2*) *Working std soln*.—Prep. in hexane by serial diln of stock soln to final concn of ca 4 pg/μL. Store in glass container with Teflon-lined screw cap. Store in refrigerator or freezer when not in use.

### C. Apparatus

**(a)** *Volumetric flasks*.—50 mL with Teflon-lined screw caps (Thomas Scientific Co., No. 0319-037).

**(b)** *Soaking vessels*.—250 mL erlenmeyers with Teflon-lined screw caps (Thomas Scientific Co., No. 4903-K23) or 250 mL media bottles with Teflon-lined screw caps.

**(c)** *Teflon liners*.—For erlenmeyers (Thomas Scientific Co., No. 2390-H82).

**(d)** *Test tubes*.—15 mL with Teflon-lined screw caps (Thomas Scientific Co., No. 9212-K42).

**(e)** *Centrifuge*.—For use with test tubes.

**(f)** *Distilling trap*.—Barrett, 20 mL graduated, 24/40 (Thomas Scientific Co., No. 7133-K44).

**(g)** *Condenser*.—Friedrich, 24/40 (Fisher Scientific Co., No. 07-744-5).

**(h)** *Heating mantle*.—To fit 500 mL r-b flask.

**(i)** *Variable electric transformer*.—0–120 V (Powerstat Model 1168, or equiv.)

**(j)** *Magnetic stirrer*.—Thermolyne Model (7200 Barnstead/Thermolyne Corp, 2555 Kerper Blvd, Dubuque, IA 52001) or equiv., with mag. stirring bar, 1 × $^{5}/_{16}$ in.

**(k)** *Gas chromatograph*.—Equipped with 1.8 m × 4 mm glass column packed with 10% SP-1000 on 80-100 mesh Supelcoport (Supelco, Inc., No. 1-1872), and const current $^{63}$Ni electron capture detector (Hewlett-Packard 5730 or equiv.) operated under following conditions: temps—injector 200°, oven 115°, detector 350°, $CH_4$–Ar (5 + 95) carrier gas 40 mL/min. Adjust attenuator to give $^{1}/_{2}$ FSD for 20 pg EDB. Retention time of EDB is ca 4 min. Condition new GC column by holding at 60° for 2 h with 40 mL/min carrier gas flow. Slowly increase temp. to 200° and hold overnight. Cool to 115°, let equilibrate, and check EDB response. If proper sensitivity is not achieved, longer conditioning at 200° may be required.

### D. Extraction and Cleanup

Store all samples in freezer until just before analysis.

**(a)** *Whole grain and intermediate processed grain products*.—Weigh 50 g into appropriate soaking vessel. Add 150 mL acetone-$H_2O$ (5 + 1) and seal with Teflon-lined screw cap. Swirl; let whole grains soak 48 h at 20–25°, with intermittent swirling. Follow same procedure for intermediate products, except soak 16 h. Using disposable pipet, transfer ca 10 mL supernate into 15 mL test tube, add 1–2 g anhyd. 8-mesh $CaCl_2$, secure Teflon-lined screw cap, and shake 2 min. If all $CaCl_2$ dissolves, add more and shake again. Let stand 30 min or centrf. Proceed with *Determination*.

**(b)** *Ready-to-eat products*.—Weigh 20 g into 500 mL r-b flask. Add 150 mL $H_2O$ and stirring bar. While cooling flask under stream of cold tap $H_2O$ or swirling in ice-$H_2O$ bath, slowly add 25 mL concd $H_2SO_4$. Stopper flask to prevent loss of EDB. Pipet 10.0 mL hexane into flask immediately before co-distn. Connect r-b flask to Barrett trap and Friedrich condenser. Place flask in heating mantle on top of stirrer and turn on stirrer. Co-distil hexane and EDB into Barrett trap, using heating mantle with transformer set at 75% full power. Continue distn until 1–2 mL $H_2O$ is collected. Remove flask from heating mantle to prevent further $H_2O$ distn. Drain lower $H_2O$ layer and discard. Drain hexane into 15 mL test tube, add 2–3 g anhyd. $Na_2SO_4$, secure Teflon-lined screw cap, and shake vigorously 1 min. Let stand 30 min or centrf. Proceed with *Determination*. When flask is cool to touch, pipet second 10.0 mL hexane portion into flask and distil as before. Repeat distn third time.

*Calculation of equivalent sample weight*.—(*1*) Whole grains and intermediate processed grain products.

$$\text{mg sample equiv./}\mu\text{L final ext} = 50/125 = 0.4$$

where 50 and 125 = g sample weighed and mL acetone added ($H_2O$ is removed), resp.

(*2*) Ready-to-eat products:

$$\text{mg sample equiv./}\mu\text{L final ext} = 20/10 = 2$$

where 20 and 10 = g sample weighed and mL hexane added (not recovered), resp. Each distn uses same calcn.

### E. Determination

Inject 5 μL dried ext into gas chromatograph operated as specified under *Apparatus*. Quantitate EDB by comparison of peak hts or integrator counts from sample and appropriate std. If EDB response is >100% FDS, quant. dil. sample with hexane to achieve appropriate on-scale response. Sum EDB amts found in each distn of ready-to-eat products to obtain total. Limits of quantitation are 2 ppb EDB for whole grains and intermediate products, 0.4 ppb EDB for ready-to-eat products.

Ref.: JAOAC **69,** 847(1986).

CAS-106-93-4 (ethylene dibromide)

## 978.16 Ethylenethiourea Pesticide Residues

### Gas Chromatographic Method

**First Action 1978**
**Final Action 1980**

(Applicable to potatoes, spinach, applesauce, and milk. *Caution:* See safety notes on chloroform and methanol.)

### A. Apparatus

**(a)** *Chromatographic tube*.—Glass, 300 × 22 (id) mm, with coarse fritted disk and Teflon stopcock.

**(b)** *Filter paper*.—Sharkskin (Thomas Scientific, or equiv.).

**(c)** *Gas chromatograph*.—With flame photometric detector (Meloy Laboratories, Inc., 6715 Electronic Dr, Springfield, VA 22151, or equiv.) contg S filter and 1.8 m × 4 (id) mm coiled glass column packed with 5% Carbowax 20M plus 2.5% KOH (prepd in MeOH) on 80–100 mesh Chromosorb W(HP). Condition new column 2 days at 210°. Typical operating conditions—temps (°): column 180, injection port 185, detector 185; flow rates (mL/min): N carrier gas 60, O 15, air 125, H 200; electrometer sensitivity 1 × $10^{-9}$ amp full scale deflection with 1 mv recorder. Approx. retention time of S-butylated ETU under these conditions is 4 min; 12 ng gives ca 50% full scale deflection. Change glass wool plug in injection port daily before use, and clean out inside of column at injection port weekly.

(**d**) *High-speed blender*.—Waring Blendor, or equiv. (*Caution:* See safety notes on blenders.)

(**e**) *Pipets*.—Disposable glass capillary pipets, 145 × 6 (id) mm (Arthur H. Thomas Co., or equiv.).

(**f**) *Rotary evaporator*.—Use with 150 mm ₮ 24/40 Vigreux column and place vac. release valve in line.

(**g**) *Silanized glass wool*.—Applied Science Laboratories, Inc., or equiv.

### B. Reagents

(**a**) *Aluminum oxide*.—Fisher No. A-540, or equiv., 80–200 mesh, for chromatgc adsorption. (Available from Fisher Scientific Co. as "Alumina, Adsorption, Fisher.") Use as received.

(**b**) *1-Bromobutane*.—Fisher Scientific Co., or equiv. Redistil between 101 and 101.5°.

(**c**) *Diatomaceous earth*.—Celite 545. Do not acid-wash.

(**d**) *Eluant*.—4% alcohol in $CHCl_3$. Dil. 40 mL alcohol to 1 L with $CHCl_3$, and mix well.

(**e**) *Solvents*.—$CHCl_3$, MeOH, and toluene, distd in glass (*see* statement regarding solvs, **970.52A**).

(**f**) *Ethylenethiourea std solns*.—(*1*) *Stock soln*.—10 μg ETU/mL. Transfer 100 mg ETU ref. std (available from Pesticides and Industrial Chemical Repository (MD-8), Environmental Protection Agency, Research Triangle Park, NC 27709) to 100 mL vol. flask, and dil. to vol. with $H_2O$. Pipet 1 mL this soln into another 100 mL vol. flask, and dil. to vol. with $H_2O$. Prep. fresh monthly. (*2*) *Working soln*.—2 μg ETU/mL. Pipet 10 mL stock soln into 50 mL vol. flask, and dil. to vol. with $H_2O$. Prep. fresh weekly.

### C. Extraction

(Samples must be started and completed on same day.)

(**a**) *For crops, canned goods, and milk*.—Blend 100 g chopped crop (vegetables and fruits) or 100 g milk, 150 mL $H_2O$, 15 g NaCl, 10 g diat. earth, (**c**), and 200 mL MeOH in high-speed blender 2 min. Filter with vac. thru 1.3 cm bed of diat. earth spread dry and evenly on 9 cm double sharkskin filter paper in 91 mm (id) buchner. Transfer 87 mL (20 g) aliquot to previously weighed 500 mL ₮ 24/40 r-b flask. Add 50–70 mL MeOH, insert Vigreux column into flask, and conc. on rotary vac. evaporator immersed in 60–65° $H_2O$ bath. If substantial initial frothing occurs, add 4–5 drops octanol. If much frothing occurs during last stages of concn, add addnl 4–5 drops octanol, 25 mL alcohol, or both. Conc. to ca 10 g. Disconnect flask, weigh, and add enough $H_2O$ to bring wt to 13 g. Proceed immediately as in **978.16D**.

(**b**) *Optional extraction for crops*.—(Applicable when presence of parent ethylenebisdithiocarbamate (EBDC) fungicide is suspected in sample. Provides measure of potential ETU residues which may be converted from EBDCs in home cooking.) Place 100 g chopped crop, 150 mL $H_2O$, and 1 mL $NH_4OH$ in 1 L beaker, and record total wt. Cover with large watch glass, place on 600–720 watt hot plate turned to high, and heat 15 min, reducing heat to low after initial boiling. Cool, remove watch glass, and reweigh. Add $H_2O$ to beaker to restore to original wt. Transfer quant. to high-speed blender, using 200 mL MeOH. Add 10 g diat. earth, (**c**), and blend 20 sec. Proceed as in (**a**), beginning "Filter with vac. thru 1.3 cm bed . . .", except conc. sample to ca 8 g and add $H_2O$ to bring wt to 10 g. Proceed immediately as in **978.16D**.

(**c**) *Optional extraction for milk*.—(Applicable when presence of parent EBDC fungicide is suspected in sample.) Place 100 g milk, 25 mL $H_2O$, 5 g NaCl, and 1 mL $NH_4OH$ in 1 L beaker, and record total wt. Proceed as in (**b**), beginning "Cover with large watch glass, . . ." except use 275 mL MeOH, stir thoroly 1 min, and, after filtering, transfer 80 mL (20 g) aliquot to r-b flask. Proceed immediately as in **978.16D**.

### D. Cleanup

Add 10 g Gas-Chrom S to sample ext, stopper, and shake vigorously until lump-free (30–60 sec), tapping on cork ring, if necessary. Add 50 mL eluant, (**d**), stopper, and shake 1–2 min. Pour mixt., including as much of Gas-Chrom S as possible, into chromatgc tube, **978.16A(a)**, contg 4–5 g $Al_2O_3$, (**a**), held in place with 1.3 cm glass wool plug. Rinse flask with 3 addnl 50 mL portions eluant, adding each rinse to tube. Collect eluate in 500 mL ₮ 24/40 r-b flask contg 10 mL $H_2O$ and 20 mL alcohol. Conc. eluate to ca 20 mL using rotary vac. evaporator with Vigreux column. Add 5 mL $H_2O$ and 20 mL alcohol, and conc. to 10 mL to eliminate $CHCl_3$. Rinse column and flask with 5 mL $H_2O$ followed by 20 mL alcohol. Proceed immediately as in **978.16E**.

### E. Derivatization

(**a**) *Sample*.—To sample flask add 1.5 g KOH, 2 mL l-bromobutane, (**b**), and 5–6 boiling chips. Reflux on pre-heated hot plate 10 min, using cold $H_2O$ condenser and clamp to support flask. Cool, and transfer quant. to 250 mL separator with 10 mL $H_2O$ followed by 50–60 mL $CHCl_3$. Shake 1–2 min, and let layers sep. completely (ca 5 min). Collect lower $CHCl_3$ layer in clean 250 mL ₮ 24/40 r-b flask. Add 2 drops HCl and evap. to near dryness on rotary vac. evaporator, using Vigreux column and 60–65° $H_2O$ bath. Rinse neck of flask with 2–3 mL MeOH, and evap. again on rotary evaporator. Remove flask and evap. to dryness, using air jet for 2–3 min. *All MeOH must be removed by this final evapn or it will interfere with GC detn.* Remove flask from air jet, pipet in 1 mL toluene and add 1.0–1.5 mL 10% KOH, stopper, and shake 1–2 min. Remove 0.5–0.7 mL of toluene layer with disposable pipet, (**e**), without collecting any of lower aq. layer. Place toluene sample ext in clean, dry calibrated test tube, and record vol. At this point, 1 mL ext is equiv. to 20 g sample. However, if final ext is dild for GC, vol. recorded with respect to original 1 mL toluene must be used for sample wt. calcns:

Wt sample in final diln
= [(vol. obtained from 1 mL)/(1 mL toluene)] × 20 g

Do not conc. sample ext at this point due to volatility of butyl derivative of ETU.

(**b**) *Standard*.—Pipet 1 mL ETU working std soln into 500 mL ₮ 24/40 r-b flask. Add 15 mL $H_2O$, 20 mL alcohol, 1.5 g KOH, 2 mL l-bromobutane, and 5-6 boiling chips, and proceed as in (**a**), beginning "Reflux on preheated hot plate . . .".

### F. Determination

Initially, inject 4–6 μL std ext, **978.16E(b)**, into gas chromatograph, and then inject 5 μL sample ext, **978.16E(a)**. Adjust injection vol. of sample ext until peak hts of std and sample are approx. equal, and continue with alternate injections of sample and std. (S detector is non-linear; therefore, do not prep. std curve.)

$$\text{ETU, ppm} = (PH/PH') \times (W'/W)$$

where $PH$ = peak ht of sample, $PH'$ = peak ht of std, $W'$ = ng ETU in std aliquot, and $W$ = mg sample represented by sample aliquot.

### G. Regeneration of Gas-Chrom S

Shake all used Gas-Chrom S from cleanup column, **978.16D**, into large beaker or flask. Discard $Al_2O_3$ and glass wool plug. Wash thoroly with $H_2O$, and decant after each wash. Wash thoroly with MeOH, decant, and vac. dry in large buchner.

Air dry in hood and transfer to 80° oven overnight. Gas-Chrom S may now be re-used.

Refs.: JAOAC **60,** 1105, 1111(1977).

CAS-96-45-7 (ethylenethiourea)

## 963.23 Glyodin Pesticide Residues

### Spectrophotometric Method

**First Action 1963**
**Final Action 1964**

(Applicable to apples and pears. Not applicable to fruits with extensive softening or decomposition. All glassware must be free of soap or detergent.)

#### A. Reagents

(**a**) *Bromophenol blue soln.*—Prep. just before use. Transfer 50 mg bromophenol blue powder into 500 mL vol. flask with small amt of $H_2O$. Add 2 mL HOAc and swirl until dye is completely dissolved. Dil. to vol. with $H_2O$, and mix.

(**b**) *Glyodin std solns.*—Prep. from 2-heptadecyl glyoxalidine, purified grade. (*1*) *Stock soln.*—1 mg/mL. Dissolve 100.0 mg 2-heptadecyl glyoxalidine in $CHCl_3$ in 100 mL vol. flask, dil. to vol. with $CHCl_3$, and mix. (*2*) *Working soln.*—0.05 mg free base/mL. Transfer 5.0 mL stock soln to 100 mL vol. flask, dil. to vol. with $CHCl_3$, and mix.

#### B. Preparation of Standard Curve

Add 0, 2, 4, 6, 8, and 10 mL working std soln to six 50 mL vol. flasks. Add exactly 1 mL HOAc to each flask and dil. to vol. with $CHCl_3$. Place 25 mL of each std, measured in graduate or fast-flow pipet, in 125 mL separator. Add 25 mL bromophenol blue soln, (**a**), from graduate or fast-flow pipet to each separator, and shake vigorously 1 min. Let sep. ≥20 min. Filter $CHCl_3$ layer thru pledget of glass wool in stem of separator into small g-s erlenmeyer. Det. *A* at 415 nm in spectrophtr, using 1 cm cells and 0 std as ref. Plot *A* against mg 2-heptadecyl glyoxalidine.

#### C. Preparation of Sample

Fill tared wide-mouth gal. (3.8 L) glass jar with whole fruit so that little or no slack is present (to prevent battering of fruit). Weigh, and add 250 mL isopropanol. Screw cap on tightly with double thickness of cellophane placed over mouth of jar before cap is screwed on to help prevent leakage. Tumble or shake 10 min. Filter into 500 mL vol. flask thru small layer of glass wool in funnel. Drain off as much liq. as possible. Repeat stripping with second 250 mL portion of isopropanol, and filter into vol. flask. Wash glass wool and funnel with small portions of isopropanol and dil. to vol.

#### D. Determination

Transfer 25 mL aliquot of strip soln to 50 mL beaker and evap. to dryness on steam bath under air jet. To residue add exactly 1 mL HOAc, allowing acid to drip slowly down sides of beaker so that all residue is wetted. Cover beaker with watch glass and heat gently on steam bath with swirling, until residue at bottom loosens and disintegrates. Thoroly rinse down sides with few mL $CHCl_3$ and transfer to 50 mL vol. flask. Rinse beaker with 4 addnl small portions $CHCl_3$, and transfer to vol. flask. Dil. to vol. with $CHCl_3$ (disregard turbidity and slight color in soln).

Transfer 25 mL $CHCl_3$ soln, measured in graduate or fast-flow pipet, to 125 mL separator. Proceed as in **963.23B**, beginning, "Add 25 mL bromophenol blue soln, (**a**), . . ."

Perform detns along with prepn of std curve, using 0 std as ref. when detg sample *A*. Det. amt of 2-heptadecyl glyoxalidine in aliquot from std curve.

Glyodin (2-heptadecyl glyoxalidine acetate)
= 2-heptadecyl glyoxalidine × 1.195

Ref.: JAOAC **46,** 238(1963).

CAS-556-22-9 (glyodin)

## 977.19 Hexachlorobenzene and Mirex Pesticide Residues in Fatty Products

### Gas Chromatographic Method

**First Action 1977**
**Final Action 1978**

#### A. Reagents

(**a**) *Solvents.*—Hexane, $CH_2Cl_2$, $CH_3CN$, and pet ether. *See* **970.52B**.

(**b**) *Florisil.*—60–100 mesh PR grade. (*1*) *Unactivated, for partition chromatography.*—Use as received from manufacturer. (*2*) *Activated.—See* **970.52B(i)**.

(**c**) *Eluant mixture.*—For Florisil column cleanup. Dil. 200 mL $CH_2Cl_2$ with hexane. Let reach room temp. and adjust to 1 L with hexane.

#### B. Apparatus

(**a**) *Gas chromatograph.*—With electron capture detector. *See* **970.52H(a)** and (**c**).

(**b**) *Column.*—1.85 m × 4 (id) mm glass column with 80–100 mesh Chromosorb W (HP) support, N flow 120 mL/min, and injection temp. 220°. For HCB analysis, use 15% OV-210 liq. phase; for Mirex, 3% OV-101. For HCB, use column temp. 180°, detector, 200°; for Mirex, use column temp. 210°, detector 220°. Operate $^3H$ electron capture detector at dc voltage which produces half scale deflection for 0.5 ng HCB or Mirex when electrometer sensitivity is $1 \times 10^{-9}$ amp. Or, operate $^{63}Ni$ detector to produce stable, reproducible, linear response and adjust amt of injected sample, **970.52R**, to accommodate difference in instrument sensitivity.

(**c**) *Chromatographic tubes.*—(*1*) *Plain.*—250 × 22 (id) mm. (*2*) *With stopcocks.—See* **970.52E(b)**.

(**d**) *Kuderna-Danish concentrators.—See* **970.52E(e)**.

(**e**) *Micro-Vigreux Column.—See* **970.52E(h)**. Use for concn to vols < 5 mL.

#### C. Extraction of Fat

Ext ≥3 g fat as in **970.52L**. For products reported on fat basis, use 3 g fat. For products reported on as-is basis, record wt, *W*, of fat extd. Corresponding wt sample analyzed = (wt fat taken for cleanup/*W*) × wt original sample.

#### D. Cleanup

(*Caution:* See safety notes on acetonitrile and petroleum ether.)

Weigh 3 g fat into 250 mL beaker, add 20 g unactivated Florisil, and stir with spatula or glass rod until free-flowing powder is obtained. Place glass wool plug in bottom of plain chromatgc tube and add 3 g unactivated Florisil. Completely transfer fat-Florisil mixt. to tube. Settle column by repeatedly tapping tube. Place glass wool plug on top of Florisil. Place 1 L separator under column as receiver. Elute with 150 mL $CH_3CN$ by gravity.

When elution is complete, add exactly 100 mL pet ether to separator, and shake vigorously 1–2 min. Add 10 mL satd NaCl soln and 500–600 mL $H_2O$, and shake vigorously 1 min. Let sep. and discard aq. (lower) layer. Wash pet ether with two 100 mL portions $H_2O$. Discard washings, transfer pet ether to 100 mL g-s graduate, and record vol., *P*. Calc.

Wt sample in eluate
= (wt sample taken for cleanup × *P*)/100

where 100 = mL pet ether added.

Complete cleanup on column of activated Florisil, **970.52O**, using amt Florisil detd from lauric acid test, **970.52B(i)**. Sample ext must be dry and free from polar solvs when placed on column. Elute at ca 5 mL/min with 200 mL eluant mixt., (**c**), and conc. Det HCB and Mirex as in **970.52R**, using column, **977.19B(b)**.

Refs.: JAOAC **58,** 557(1975); **60,** 229(1977); **63,** 277(1980).

CAS-118-74-1 (hexachlorobenzene)
CAS-2385-85-5 (mirex)

## 980.22 Hexachlorobenzene and Mirex Pesticide Residues in Adipose Tissue

**Gas Chromatographic Method**
**First Action 1980**
**Final Action 1982**

(*Caution:* See safety notes on potassium hydroxide, pesticides, hexane, and pyridine.)

### A. Reagents

(**a**) *Hexane, 2-propanol.*—See solv. purity test, **970.52B**.
(**b**) *Sodium sulfate.*—Anhyd. granular, Soxhlet-extd with hexane.
(**c**) *Sodium sulfate.*—2% aq. soln.
(**d**) *Florisil column.*—Packed with 100 mm Florisil and 12 mm $Na_2SO_4$ on top. Hold in 130 ± 2° oven ≥16 hr prior to use. (Remove stopcocks before placing columns in oven.) Prewash with 50 mL hexane immediately before use. Predetermine HCB and mirex elution on each lot of Florisil.
(**e**) *"Keeper" soln.*—1% paraffin oil in hexane.
(**f**) *Pyridine.*—Burdick and Jackson, or equiv., suitable for liq. and gas chromatgy.
(**g**) *Potassium hydroxide soln.*—10% KOH in 2-propanol.

### B. Apparatus

(**a**) *Gas chromatograph.*—With $^3H$ or $^{63}Ni$ electron capture detector and 1.8 m × 4 mm id borosilicate glass columns packed with 1.5% OV-17/1.95% OV-210 or 5% OV-210 on 80–100 mesh Gas-Chrom Q. *Typical operating conditions.*—Carrier gas flow rate 60 mL/min, column temp. 200° (OV-17/OV-210); 180° (5% OV-210).
(**b**) *Glassware.*—300 × 25 mm od Chromoflex column for Florisil (Kontes, K-420530), Kuderna-Danish (K-D) concentrator assembly (K-570000) fitted with 25 mL graduated tube (K-570050, size 2525, 19/22, 25 mL), modified micro Snyder column (K-569250), disposable pipets.

### C. Determination

Accurately weigh ca 0.5 g rendered or extd fat in tube. Dissolve fat in ca 0.5 mL hexane and transfer quant. to Florisil column pre-washed with hexane. Rinse tube with two 0.5 mL portions hexane. Let column elute until solv. level is just at top of $Na_2SO_4$. Rinse column sides with 2–3 mL hexane. Elute with 200 mL hexane at 5 mL/min.

Collect eluate in K-D flask equipped with 25 mL concentrator tube. Evap. on steam bath to ≥10 mL. Use warm (50°) $H_2O$ bath and gentle N stream for further vol. reduction.

Inject 3–8 µL stds and samples into OV-17/OV-210 column. Alternatively, OV-210 column at 180° may be used for mirex quantitation. Adjust sample vol. as required to produce major peak responses. Peak hts of stds and samples should not vary >25%. Do not quantitate <20% full scale deflection. Amts injected must fall within linear range of detector. Work at same attenuation.

### D. Confirmation of HCB

Prep. sample for derivatization by evapg hexane ext to 0.1–0.2 mL with warm $H_2O$ bath and gentle N stream. Derivatize ≥3 stds along with samples. (HCB stds must bracket HCB concn in samples as detd by initial GC analysis. Response of HCB stds must be linear.) Add 2–3 drops 1% paraffin oil in hexane to stds as "keeper" before evapg to 0.1–0.2 mL.

Add 0.5 mL 10% KOH in 2-propanol and 0.2 mL pyridine to each sample and std. Gently tap tube to mix. Place modified micro Snyder columns on concentrator tubes and place tubes in boiling $H_2O$ bath 45 min. Remove tubes and cool under cold tap $H_2O$. Add ca 10 mL 2% $Na_2SO_4$ soln to each tube and exactly 2 mL hexane. Mix vigorously 1 min. Let solv. phases sep. completely. Inject 3–8 µL hexane phase into gas chromatograph for quantitation. Adjust hexane vol. as required. Calc. concn HCB. Mirex will not be recovered thru derivative procedure.

Refs.: JAOAC **63,** 1128(1980); **64,** 531(1981).

CAS-118-74-1 (hexachlorobenzene)
CAS-2385-85-5 (mirex)

## 957.15★ Malathion Pesticide Residues

**Colorimetric Method**
**First Action**
**Surplus 1974**

*See* **29.116–29.120**, 12th ed.

## 963.24 Maleic Hydrazide Pesticide Residues

**Spectrophotometric Method**
**First Action 1963**
**Final Action 1965**

(Applicable to whole, dehydrated mashed, and frozen french fried potatoes, and potato chips; whole cranberries, onions, and peaches; and tobacco dust)

### A. Principle

Sample is boiled in alk. soln to drive off volatile basic interferences. Distn with Zn with N sweep expels hydrazine liberated from maleic hydrazide. Hydrazine is reacted in acid soln with *p*-dimethylaminobenzaldehyde to form yellow compd.

### B. Apparatus

(**a**) *Distillation apparatus.*—*See* Fig. **963.24**. Flask is 300 mL capacity, flat-bottom, double thickness, with thermometer well. Thermometer is 90–220° (Tinius Olsen No. 718636 "Yellow Bak," or equiv. in temp. range and length; available on special order from Accuracy Scientific Instrument Co., 335 E Chew Ave, Philadelphia, PA 19120). Use 5″ wire gauze with 4″ diam. asbestos center. Centrf. tube receiver (50 mL) is graduated in 1 mL divisions.
(**b**) *Spectrophotometer.*—Beckman Model DU, or equiv.

### C. Reagents

(**a**) *p-Dimethylaminobenzaldehyde soln.*—Dissolve 2 g in 100 mL 1*N* $H_2SO_4$. Soln is stable.

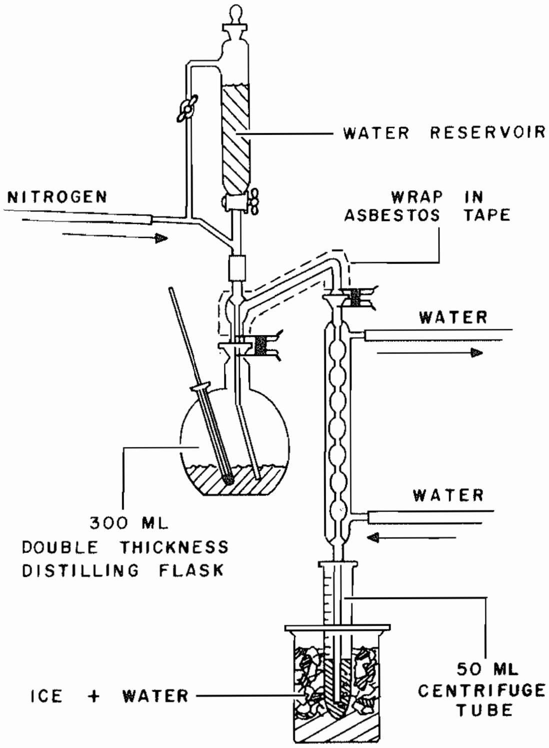


**FIG. 963.24—Distillation apparatus for maleic hydrazide determination**

(**b**) *Zn granules*.—"10 mesh."

(**c**) *Maleic hydrazide std soln*.—10 μg/mL. Dissolve 0.0100 g maleic hydrazide in 100 mL 0.1*N* NaOH and dil. to 1 L. Soln is stable.

### D. Preparation of Sample

Grind sample to soup-like consistency in high-speed blender, adding measured wt of $H_2O$ if necessary. Prepd samples may be frozen for storage.

### E. Determination

Transfer wt ground sample specified in Table **963.24** to 300 mL distn flask. Dry socket neck joint, and add 50 g NaOH pellets, antifoam agent indicated in Table **963.24**, and 40 mL $H_2O$. Add 1 mL high bp oil to thermometer well and insert thermometer. Heat flask on high-temp. hot plate and swirl ca every 20 sec until pellets dissolve and gentle boiling begins. When temp. reads 160°, (180° with cranberries), remove flask and let cool 5 min. Wipe socket joint clean and dry; add 0.5 g *ferrous chloride* and 5 mL (equiv. to ca 5 g) Zn.

Quickly grease socket joint with light film of high-vac. silicone grease and attach flask to app. (Fig. **963.24**). Center flask firmly on asbestos pad. Place 4 mL *p*-dimethylaminobenzaldehyde soln in 50 mL centrf. tube (ice-cooled) and immerse condenser tip. Adjust N flow (*dry N*) to 3 bubbles/sec in receiver. With rapid flow of condenser $H_2O$, heat flask with Bunsen burner, centering tip of outer cone of flame on asbestos pad. When boiling begins, adjust distance of burner so foaming contents fill ca $^2/_3$ of flask. Distil until temp. reads 173°, *slowly* add $H_2O$ from reservoir until temp. drops to 168°, turn off $H_2O$, and distil to 173°. Continue $H_2O$ addn and distn at these temps until receiver contains ca 40 mL. Remove receiver. (If during distn receiver soln becomes turbid or ppt appears, add 2 drops $H_2SO_4$ and shake.)

Record vol. of distillate and det. *A* at 430, 460, and 490 nm, using 1 cm cells and 4 mL *p*-dimethylaminobenzaldehyde soln dild to 40 mL as ref.

After distn, remove hot distg flask from app. with heat-resistant gloves, remove thermometer, and seal well with small cork. Rinse N-$H_2O$ inlet tube free of caustic with HCl from plastic squeeze bottle followed by $H_2O$. Then (with gloves and safety glasses) pour molten contents of distg flask into Fe can in sink to trap Zn granules. Rinse flask 3 times with $H_2O$ and 2 times with HCl to remove encrusted caustic and Zn granules. Fill flask with HCl (1 + 9) to remain until next use. Rinse 3 times with $H_2O$ before reuse. (Careful removal of *all* Zn granules with HCl is essential because residual Zn would cause premature destruction of maleic hydrazide in precook of next sample. Because of corrosion by the caustic soln, flasks may last for only ca 30 detns.)

### F. Preparation of Standard Curve

To clean 300 mL distn flasks, add 50 g NaOH pellets, 40 mL $H_2O$, and std soln equiv. to 0, 5, 10, 20, 30, 50, 100, 150, and 200 μg maleic hydrazide. Precook, distil, and measure *A* as in **963.24E**. Det. Δ*A* for each std as follows:

$$\Delta A = [A_{460} - ((A_{430} + A_{490})/2)] \times \text{mL color soln}/40$$

Plot Δ*A* of each std against μg maleic hydrazide to obtain std curve. If desired, derive simple factor from curve slope, *K*, converting Δ*A* to μg maleic hydrazide; thus, μg maleic hydrazide = Δ*A* × *K*.

### G. Calculations

Multiply Δ*A* of sample by *K* to derive μg maleic hydrazide; ppm = μg/sample wt (g).

Refs.: JAOAC **46,** 261(1963); **48,** 744(1965); **49,** 87(1966); **64,** 394(1981).

CAS-6915-15-7 (maleic hydrazide)

**Table 963.24 Sample Weights of Commodities and Use of Antifoam Agents**

| Commodity | Wt Sample, g | Antifoam Agent |
|---|---|---|
| Cranberries (whole) | 2.0 | 1 g paraffin wax + 1 mL Antifoam A |
| French fries (frozen) | 1.0 | none |
| Mashed potatoes (dehydrated) | 1.5 | 1 g paraffin wax + 1 mL Antifoam A |
| Onions (whole) | 4.2 | 1 mL Antifoam A |
| Peaches (whole) | 5.0 | 1 g paraffin wax + 1 mL Antifoam A |
| Potatoes (whole) | 2.5 | 0.5 mL vegetable oil |
| Potato chips | 1.1 | none |
| Tobacco (dust) | 1.0 | 1 g paraffin wax + 1 mL Antifoam A |

## 954.09★ Methoxychlor Pesticide Residues

**Colorimetric Method**

**Final Action 1965**
**Surplus 1970**

*See* **24.148–24.152**, 10th ed.

## 949.09 Monofluoroacetic Acid Pesticide Residues

### Qualitative Test

### Final Action 1975

### (*Sodium salt, "1080"*)

(Monochloroacetic acid also responds to this test. Confirm presence of org. F by **949.10**.)

#### *A. Reagents*

(**a**) *Decolorizing carbon*.—To 10 g high-grade C (Nuchar C-190-N, Suchar, or Darco G60) in 600 mL beaker, add ca 200 mL $H_2O$ and 30 mL 1*N* HCl, and keep on steam bath 20 min, agitating continuously with air passed thru cotton. Filter on buchner and suck as dry as possible, tamping with flat-end rod. Transfer cake to beaker, add ca 200 mL $H_2O$, mix thoroly, and refilter. Repeat washing and filtering twice, and dry at 100°.

(**b**) *Thiosalicylic acid soln*.—Dissolve 300 mg thiosalicylic acid (Eastman P2805 is suitable) in mixt. of 2 mL 1*N* NaOH and 18 mL $H_2O$.

(**c**) *Potassium ferricyanide soln*.—Dissolve 1 g $K_3Fe(CN)_6$ in $H_2O$ and dil. to 50 mL with $H_2O$.

#### *B. Test*

Prep. sample and ext as in **949.10D–E**. If convenient, ext large enough sample to obtain 2–10 mg 1080. With very low levels of 1080, *e.g.*, 1–5 ppm, ext large enough sample to obtain ≥0.5 mg 1080.

Sep. ether ext from any aq. sludge which may have been carried over in extn, add ca 5 g anhyd. $Na_2SO_4$ and 0.5 g decolorizing C/100 mL ether, and shake vigorously. Let stand ca 15 min at room temp. with occasional shaking, and decant thru fluted paper into separator. Add ca 25 mL $H_2O$ and enough NaOH soln (ca 1*N*) to make aq. layer alk. after vigorous shaking (outside test paper). Drain aq. layer into 125 mL erlenmeyer and aerate to remove dissolved ether. Using pH test paper and ca 1*N* solns of $H_2SO_4$ and NaOH, adjust to pH 4–6. Add 0.5 g C and place on steam bath for 15 min.

Cool under tap and filter thru fluted paper into ca 25 × 150 mm test tube. Add 1 mL thiosalicylic acid soln and 2 drops NaOH (1 + 1), and mix. Conc. soln to small vol. by placing on steam bath under gentle air current. Completely dry residue in oven at 130° or, if time is not factor, in 100° oven. (When convenient, overnight drying is satisfactory, with or without prior concn of soln.)

Dissolve *thoroly* dry residue in 2–3 mL $H_2O$, add 1 mL $K_3Fe(CN)_6$ soln, and mix. Red ppt, which forms at once when ≥1 mg 1080 is present, or upon standing when only fraction of mg is present, is pos. test for 1080.

Employ chromatgc instead of C purification in following cases:

(*1*) With pineapple juice when <2 mg 1080 can be extd.

(*2*) With grape juice even when ≥2 mg of 1080 can be conveniently extd.

(*3*) With any food or material when 1080 is strongly suspected and neg. test is obtained using C purification technic.

For chromatgc purification, follow **949.10F** for sepg 1080 from other acids. Discard forerun, which may contain HOAc and other extraneous materials. Collect fraction large enough to contain all the 1080 as detd by preliminary detn. Ext fluoroacetic acid from eluate with 25 mL $H_2O$ and enough alkali to cause aq. layer to retain alky after vigorous shaking (outside test paper). Drain org. layer and discard. Drain aq. layer into 125 mL erlenmeyer and aerate to remove $CHCl_3$. Pour soln into test tube and continue as above, beginning "Add 1 mL thiosalicylic acid soln . . ."

Refs.: JAOAC **32,** 788(1949); **33,** 608(1950); **34,** 827(1951); **37,** 581(1954).

CAS-144-49-0 (monofluroacetic acid)
CAS-79-11-8 (monochloroacetic acid)

## 949.10 Monofluoroacetic Acid Pesticide Residues

### Quantitative Method

### Final Action

### (*Sodium salt, "1080"*)

#### *A. Principle*

After suitable sample prepn, acid is extd with ether and sepd from inorg. fluorides (partially ether-sol.) by partition chromatgy on silicic acid, using 0.5*N* $H_2SO_4$ as immobile solv. and $CHCl_3$ contg 10% *tert*-amyl alcohol or *n*-BuOH as mobile solv. Monofluoroacetic acid in eluate is converted to its Na salt, and quantity is detd by micro F detn, **944.08E(a)**, **944.08F**, and **944.08G**.

#### *B. Apparatus*

(**a**) *Chromatographic tubes*.—18 mm od × 250 mm long, prepd from Pyrex tubing.

(**b**) *Pressure source*.—Compressed air or cylinder of N or $CO_2$, and means of keeping pressure const, such as Hg column or diaphragm-type pressure regulator.

(**c**) *Mixer*.—High-speed blender.

#### *C. Reagents*

(**a**) *Silicic acid*.—Mallinckrodt analytical reagent grade pptd powder, or equiv.

(**b**) *Mobile solvent*.—Add 100 mL *tert*-amyl alcohol or *n*-BuOH to 900 mL $CHCl_3$, and mix.

(**c**) *Phosphotungstic acid soln*.—Dissolve 20 g in $H_2O$ and dil. to 100 mL.

#### *D. Preparation of Sample*

This will vary with type of material. Dissolve sugars in $H_2O$, acidify with $H_2SO_4$, and ext directly. Following methods for different type materials will be suggestive. Simple $H_2O$ wash may be adequate to prove contamination of certain foods.

(**a**) *Sugar*.—Dissolve 100 g sample in enough $H_2O$ to give ca 350 mL.

(**b**) *Flour*.—Place 100 g sample in mixer, add 400 mL $H_2O$ and 5 g *pancreatin*, and comminute ca 2 min. Adjust to pH 7–8, using *satd $Na_3PO_4.12H_2O$ soln* and suitable indicator paper. Transfer comminuted material to tared 1 L erlenmeyer, washing mixer with three 25 mL portions $H_2O$. Incubate mixt. ≥3 hr at 35–40°. Add 5 mL $H_2SO_4$ (1 + 1) and swirl. Add 20 mL phosphotungstic acid soln and swirl again. Dil. to 750 g with $H_2O$, stopper, and shake vigorously ca 2 min. Filter thru fluted paper or with suction thru buchner (16 cm size is convenient). Or, more quickly, centrf. and decant supernate. Use ≥375 g aliquot of filtrate. (Since sp gr of filtrate is very close to 1, measuring out aliquot in graduate is satisfactory.)

(**c**) *Wheat*.—Finely grind sample in suitable mill, such as Wiley mill. Proceed as in (**b**).

(**d**) *Corn meal*.—Proceed as in (**b**), except omit pancreatic digestion.

(**e**) *Corn*.—Grind sample and proceed as in (**d**).

(**f**) *Peanuts*.—Grind sample finely (like peanut butter) and proceed as in (**d**), except use 100 mL phosphotungstic acid soln. If necessary, refilter thru folded paper to remove oil.

(**g**) *Cheese*.—Proceed as in (**d**), except use 40 mL phosphotungstic acid soln.

(**h**) *Other foods such as chili peppers, cacao beans, etc.*—Treat in manner similar to one of preceding foods.

(**i**) *Biological tissue*.—If material is tough or fibrous, grind it twice thru food chopper. (Soft tissues, *e.g.*, brain and liver, need not be ground.) Place 100 g ground tissue in 800 mL beaker, add ca 300 mL $H_2O$, cover with watch glass, and boil gently ca 30 min. Transfer material to mixer, rinsing beaker with two 25 mL portions $H_2O$, and comminute thoroly (ca 2 min). Transfer comminuted material to tared 1 L erlenmeyer, rinsing mixer with two 25 mL portions $H_2O$. Add 5 mL $H_2SO_4$ (1 + 1) and mix. Add enough phosphotungstic acid soln (50–75 mL) to ppt all proteins, then $H_2O$ to make 600 g. Shake vigorously ca 2 min, and filter thru fluted paper or with suction thru buchner. If material does not filter rapidly, return mixt. to flask, add ca 10 mL addnl phosphotungstic acid soln, shake vigorously, and refilter.

*Alternative method*.—Place 100 g ground tissue in mixer, add 300 mL $H_2O$ and 15 g pancreatin, and comminute thoroly (ca 2 min). Adjust to ca pH 8 with *satd $Na_3PO_4.12H_2O$ soln*, using suitable indicator paper. Transfer comminuted material from mixer to tared erlenmeyer, washing mixer with two 25 mL portions $H_2O$ and incubate ca 3 hr at 35–40°. Ppt proteins and make to wt as directed previously.

### E. Extraction

Transfer soln (of sugar) or wt-aliquot of protein-free filtrate (of protein-contg materials) to 200 mL continuous extractor, Fig. **937.05**. (Tube is 115–120 cm long and 33–34 mm od, side arm, attached ca 63 cm from bottom, is 15–16 mm od; and inner tube is 12–13 mm od flared at top to ca 25 mm diam.; 1.5 L extractors of this type have been used successfully. Extra coarse fritted filter tip on bottom end of inner tube aids in getting smaller droplets of extg solv.) For each 50 g soln, add 1 mL $H_2SO_4$ (1 + 1). Ext with ether until all fluoroacetic acid has been extd (detd by preliminary experiment; usually 3–4 hr with 400 mL extractor). Transfer ether ext to separator of appropriate size.

To extn flask add ca 20 mL $H_2O$, 2 drops phthln, and enough 1.0*N* NaOH from buret to give strong alk. color of indicator after swirling. Pour rinse soln into separator and add addnl alkali until alk. color of indicator persists in aq. phase after vigorous shaking. Record vol. alkali required. Drain aq. layer into 100 mL beaker and wash ether with two 10 mL portions $H_2O$, rinsing extn flask each time with the $H_2O$ before pouring it into separator. Add washings to beaker. Carefully adjust alky of ext just to alk. color of phthln with 0.1*N* $H_2SO_4$ and NaOH solns. Evap. neutzd ext to dryness on steam bath (current of air hastens evapn). If during evapn alk. color of indicator should disappear, add just enough 0.1*N* NaOH to give alk. color again. Do not continue heating after residue is apparently dry. Slightly moist residue is permissible.

### F. Chromatography

To 5 g silicic acid, (**a**), in mortar, add max. amt of 0.5*N* $H_2SO_4$ that it will hold without becoming sticky (50–80% of its wt). Mix well with pestle; then add ca 35 mL of the mobile solv. and work up into smooth slurry. (If $SiO_2$ agglomerates in solv., too much $H_2SO_4$ was used.) Place small cotton plug in bottom of chromatgc tube and pour in slurry, tilting tube slightly to avoid air bubbles. Let silicic acid pack down under 2–10 lb (14–69 kPa) pressure applied thru gas pressure regulator. When excess solv. has drained thru (column firm and viscous enough to resist pouring when tipped), column is ready for use. In prepg column take care to avoid cracking or drying out of the gel caused by leaving pressure on after column packs down and all solv. sinks into gel.

To dry or slightly moist residue in 100 mL beaker add enough $H_2SO_4$ (1 + 1) (ca 18*N*), usually 0.5–1.0 mL, to give excess of ca 0.25 mL over vol. necessary to convert all salts to free acid, as calcd from amt of 1*N* NaOH required to neutze acid extd by the ether. Wet salts *thoroly* with the acid, using small, narrow blade spatula (steel or Monel metal) to loosen salts from glass, and using flat-end glass rod to break up solid particles and mix resulting slurry. Add 5–10 g anhyd. granular $Na_2SO_4$ to take up excess liq. Stir well with tamping rod, breaking up any lumps. Add 10 mL mobile solv., (**b**), stir thoroly, and decant solv. carefully onto column.

Catch eluate in graduate. Apply pressure until all solv. sinks into gel; then release pressure. Add 5 mL mobile solv. to beaker and again stir thoroly. Carefully decant solv. onto column and, with aid of narrow-blade spatula, transfer bulk of material in beaker, mostly $Na_2SO_4$, to column. Renew pressure. When solv. passes ca halfway thru $Na_2SO_4$, release pressure. Rinse out beaker with addnl 5 mL solv. and transfer to column. After this washing sinks ca halfway into $Na_2SO_4$, fill tube with mobile solv. and complete collection, under pressure, of enough eluate to obtain all monofluoroacetic acid, as detd by test run on silicic acid used (ca 50 mL). Collect dropwise; 3–4 mL/min is convenient rate.

Transfer eluate to 125 mL separator; add ca 20 mL $H_2O$ and enough 1.0*N* NaOH to give alk. color of phthln (phthln is present in eluate and no further addn is required) in aq. phase, after vigorous shaking. Drain aq. layer into 125 mL erlenmeyer and return solv. layer to separator. Wash solv. with two 10 mL portions $H_2O$ and add washings to erlenmeyer. Aerate soln with current of air to remove traces of $CHCl_3$. (If excess $CHCl_3$ is not removed, excessive Cl may complicate F distn in next step.)

### G. Determination

Transfer aq. ext to Pt dish with little $H_2O$ and mix with ca 20 mL lime suspension, **944.08D(a)**, evap. to *dryness*, and ash 15–20 min at 600°. (Little C in ash will not interfere in detn.) Proceed as in **944.08E(a)**, beginning "When clean ash is obtained, . . ." and **944.08F–G** (100 mL Nessler tubes are preferable). Convert F results to fluoroacetic acid (×4.11) or to Na monofluoroacetate (1080) (×5.26) as desired, and correct for aliquot taken, if any, in extn. Ignore vol. occupied by insol. solids.

Refs.: JAOAC **32,** 788(1949); **33,** 608(1950); **34,** 827(1951); **37,** 581(1954).

CAS-144-49-0 (monofluoroacetic acid)

## 970.54 Naphthyleneacetic Acid Pesticide Residues

### Spectrophotometric Method

**First Action 1970**
**Final Action 1976**

(Applicable to apples and potatoes)

### A. Apparatus

(**a**) *Spectrophotometer*.—Cary 15 (current Model Cary 219, Varian Instrument Group), or equiv., with 5 cm cells.

(**b**) *Chromatographic tube.*—Glass, 22 mm id × 200 mm.
(**c**) *Food chopper.*—Hobart No. 84141 (Hobart Manufacturing Co., 711 Pennsylvania Ave, Troy, OH 45374), or equiv.
(**d**) *Blender cups.*—Stainless steel, 1 L capacity, with airtight screw cover (Scientific Products, Inc. No. S8390) for high-speed blender.

### B. Reagents

(**a**) *Sodium phosphate soln.*—0.5*M*. 134 g $Na_2HPO_4.7H_2O$ or 70.5 g anhyd. salt/L.
(**b**) *Permanganate soln.*—0.02*M*. 31.6 g $KMnO_4$/L.
(**c**) *Florisil.*—60–100 mesh PR grade activated at 675° (1250° F) (Floridin Co.); use as received.
(**d**) *Naphthyleneacetic acid (NAA) soln.*—0.1 mg α-NAA/mL $CHCl_3$.

### C. Extraction

Chop sample in food chopper and transfer 200 g to blender cup. Add 20 mL 1*N* $H_2SO_4$ and 400 mL $CHCl_3$, screw top on blender, and blend 2 min at low speed. Pour mixt. into 500 mL centrf. bottle and centrf. 10 min at 1600 rpm. Take 200 mL aliquot from $CHCl_3$ layer.

### D. Cleanup

(**a**) *Apples.*—Place glass wool plug into chromatgc tube, add 10 cm Florisil, and top Florisil with glass wool plug. Transfer 200 mL $CHCl_3$ ext to column with min. amt $CHCl_3$. Rinse inside of tube twice with ca 5 mL $CHCl_3$. Elute column, in order, with 100 mL portions of $CH_3CN$, ether, $NH_3$-satd $CHCl_3$, and $CHCl_3$ and discard eluates. Using 500 mL separator as receiver, elute NAA with 100 mL 1% HOAc in $CHCl_3$ followed by 100 mL $CHCl_3$. Discard column, add 50 mL 1*N* $H_2SO_4$ to separator, and shake vigorously. Transfer $CHCl_3$ layer to 250 mL separator contg 50 mL $H_2O$ and shake vigorously. Transfer $CHCl_3$ layer to 250 mL separator contg exactly 50 mL 0.5*M* $Na_2HPO_4$, shake vigorously, and discard $CHCl_3$ layer.
(**b**) *Potatoes.*—Proceed as in (**a**). Add 2 mL 85% $H_3PO_4$ and 2 mL 0.02*M* $KMnO_4$ to separator contg $Na_2HPO_4$ phase, mix, and let stand exactly 5 min. Ext NAA with two 25 mL portions $CHCl_3$, transfer $CHCl_3$ exts to 125 mL separator contg exactly 50 mL 0.5*M* $Na_2HPO_4$, shake vigorously, and discard $CHCl_3$ layer.

### E. Determination

(**a**) *Apples.*—Transfer 1 mL NAA std soln to 125 mL separator, add exactly 50 mL 0.5*M* $Na_2HPO_4$ and 50 mL $CHCl_3$, and shake vigorously. Let layers sep. and discard $CHCl_3$ layer. Obtain UV spectra (230–330 nm) of cleaned up apple ext and NAA std ext, using 5 cm cells, against 0.5*M* $Na_2HPO_4$. Use peak at 283 nm to compare apple ext and NAA std ext, correcting for baseline *A*, and calc. ppm NAA present.
(**b**) *Potatoes.*—Transfer 1 mL NAA std soln to 125 mL separator, add 50 mL 0.5*M* $Na_2HPO_4$ and 50 mL $CHCl_3$, and shake vigorously. Let layers sep. and discard $CHCl_3$ layer. Add 2 mL 85% $H_3PO_4$ and 2 mL 0.02*M* $KMnO_4$ to separator, mix, and let stand exactly 5 min. Ext NAA with two 25 mL portions $CHCl_3$, transfer $CHCl_3$ exts to 125 mL separator contg exactly 50 mL 0.5*M* $Na_2HPO_4$, shake vigorously, let layers sep., and discard $CHCl_3$ layer. Obtain UV spectrum and calc. ppm NAA as in (**a**). (If there is excessive interference in sample spectra, repeat 5 min oxidn for both sample and std, beginning with "Add 2 mL 85% $H_3PO_4$ . . .".)

Ref.: JAOAC **53,** 149(1970).

CAS-86-87-3 (naphthyleneacetic acid)

## 964.20 Nicotine Residues
### Spectrophotometric Method
**First Action 1964**
**Final Action 1965**

(Applicable to apples, cabbage, and spinach)

### A. Reagents

(**a**) *Dilute hydrochloric acid.*—Approx. 0.05*N*. Dil. 4.1 mL HCl to 1 L.
(**b**) *Nicotine std solns.*—(*1*) *Stock soln.*—1 mg/mL. Dil. 100 mg nicotine (Eastman Kodak Co. No. 1242, or equiv.) to 100 mL in vol. flask with ca 0.05*N* HCl. (*Caution:* nicotine is very toxic.) (*2*) *Working soln.*—0.01 mg/mL. Pipet 1 mL stock soln into 100 mL vol. flask and dil. to vol. with ca 0.05*N* HCl.
(**c**) *Stripping soln.*—Dil. 20 mL $NH_4OH$ to 2 L in vol. flask. Prep. at time of use.

#### *Leafy Crops*

### B. Preparation of Sample

(*Caution:* See safety notes on toxic solvents, benzene, and chloroform.)

Weigh 500 g chopped sample (spinach, cabbage) into clean, dry jar (3–5 gal.; 11–20 L). Add 800 mL benzene, 200 mL $CHCl_3$, and 10 mL $NH_4OH$. Close, tumble or roll ca 10 min, and drain soln as completely as possible into 1 L beaker. Filter thru folded 38.5 cm paper into flask and proceed immediately with detn.

### C. Determination

Place 400 mL filtered soln in 500 mL separator. Add 25 mL ca 0.05*N* HCl and 2 mL HCl, and shake vigorously. Let phases sep. (ca 5 min) and drain lower layer into 250 mL separator. Swirl large separator, let stand ca 2 min, and drain any addnl ext into 250 mL separator. Repeat several times. Then ext soln with 25, 25, 15, and 10 mL portions ca 0.05*N* HCl, repeating swirling as above. Drain all acid exts into 250 mL separator. Make exts just alk. to litmus with 10% NaOH soln. Ext with two 50 mL and four 25 mL portions $CHCl_3$, combining exts in 250 mL separator.

Add 2 mL HCl to exts and make sure soln is acid to litmus. Ext with 25, 25, 20, 10, and 5 mL portions ca 0.05*N* HCl, combining all exts in short-stem 125 mL separator. Wash exts with 15 mL pet ether. Drain aq. layer into second 125 mL separator and wash pet ether with 5 mL ca 0.05*N* HCl, adding wash to combined acid soln. Ext soln with another 15 mL pet ether, drain aq. layer into 100 mL vol. flask, and wash pet ether with 5 mL ca 0.05*N* HCl. Drain acid into vol. flask and dil. to vol. with ca 0.05*N* HCl. Mix, pour portion into 50 mL beaker, and let stand 10–15 min. Det *A* at 236, 259, and 282 nm with ca 0.05*N* HCl as ref. Confirm presence of nicotine by reading at 2 nm intervals and plot absorption curve, or use recording spectrophtr. Det. *A* of std nicotine soln against ca 0.05*N* HCl as ref.

#### *Waxy Crops*

### D. Preparation of Sample

Weigh 2–2.5 kg apples into clean, dry, jar (3–5 gal.; 11–20 L). Add 1 L stripping soln, tumble or roll ca 10 min, and drain carefully into 1 L beaker. Filter thru folded 38.5 cm paper into flask and proceed immediately with detn.

### *E. Determination*

Place 400 mL filtered soln in 500 mL separator. Add 50 mL $CHCl_3$, invert separator back and forth gently ca 2 min, and let phases sep. Drain clear portion of ext into 250 mL separator. (With fruits, emulsions may form which are very hard to break. Break emulsions by drawing $CHCl_3$ layer into *dry* 125 mL separator and shaking vigorously. Separator must be dry.) Let phases sep. and drain clear portion into the 250 mL separator. Add 35 mL $CHCl_3$ to the 125 mL separator, shake gently, and drain into the 500 mL separator. Ext as above and combine clear ext in the 250 mL separator. Ext with 35, 35, and 10 mL $CHCl_3$, combining exts in the 250 mL separator. Add ≥1 mL HCl to exts until definitely acid to litmus. Then ext with three 15 mL portions ca 0.05*N* HCl, combining acid exts in a 125 mL separator. Wash the 250 mL separator with 10 mL ca 0.05*N* HCl after each extn and add to 125 mL separator used to break emulsions. Shake, but do not attempt to break any emulsions in this separator. Combine all acid exts in 125 mL separator and shake with 15 mL pet ether. Let stand ca 5 min and drain aq. layer into another 125 mL separator. Wash pet ether with 5 mL ca 0.05*N* HCl (do not shake vigorously) and add washings to separator. Repeat washing with 15 mL pet ether and drain aq. ext into 100 mL vol. flask. Wash pet ether as before, add washings to flask, and let stand 10–15 min. Dil. to vol. with ca 0.05*N* HCl and det. *A* at 236, 259, and 282 nm, against ca 0.05*N* HCl as ref. Confirm presence of nicotine as in **964.20C**.

Take *A* of std soln as:

$$A_{std} = A'_{259} - 0.5(A'_{236} + A'_{282})$$

and *A* of sample soln as:

$$A_{samp.} = A_{259} - 0.5(A_{236} + A_{282})$$

Then:

$$\text{mg Nicotine} = (A_{samp.}/A_{std}) \times 2.5$$

Refs.: JAOAC **44,** 177(1961); **47,** 303(1964).

CAS-54-11-5 (nicotine)

## 960.42★ Parathion Pesticide Residues

**Colorimetric Method**

**First Action 1960**
**Final Action 1965**
**Surplus 1970**

See **29.139–29.144**, 11th ed.

## 985.24 Pentachlorophenol in Gelatin

**Gas Chromatographic Method**

**First Action 1985**

### *A. Principle*

Sample is acid-hydrolyzed and PCP is extd with hexane, partitioned into KOH soln, acidified, and extd into hexane. Compd is sepd and detd by gas chromatgy with electron capture detection.

### *B. Apparatus*

(**a**) *Test tubes.*—25 × 150 mm with Teflon-lined screw caps (Thomas Scientific, no. 9212-K75).

(**b**) *Disposable pipets.*—Pasteur type, 23 cm long (Thomas Scientific).

(**c**) *Centrifuge.*—IEC Model UV (replacement model PR-7000) with head for spinning 25 × 150 mm test tubes at 1000 × g (International Equipment Co., Needham Heights, MA 02194).

(**d**) *Shaker.*—(Optional). Shaker-in-the-Round Model S-500 (Glas-Col Apparatus Co., 711 Hulman St, PO Box 2128, Terre Haute, IN 47802).

(**e**) *Gas chromatograph.*—Equipped with $^{63}$Ni electron capture detector. Conditions: column oven 180°; injector 250°; detector 350°; 5% $CH_4$ in Ar carrier gas flow 60 mL/min. Adjust electrometer setting to obtain 0.5 FSD from 0.1 ng PCP (retention time ca 10 min).

(**f**) *Chromatographic column.*—1.8 m × 4 mm id glass column contg 1% SP-1240DA on 100–120 mesh Supelcoport (Supelco Inc.). Place small plug (2–3 mm) acid-washed glass wool in detector end of column (silanized glass wool may be substituted; however, peaks will be slightly broader). Install packed column for on-column injection. Condition by purging with carrier gas at ambient temp. for 10–15 min. Program from 70 to 190° at 1°/min, holding at 190° for 6–8 h. Lower temp. to 180° for PCP detn. *Note:* Use only recently prepd and thoroly conditioned column.

### *C. Reagents*

Store all reagents in ground glass-stopper or Teflon-lined screw-cap containers.

(**a**) *PCP std solns.*—Dissolve 2.5 mg PCP ref. std (Standard No. 5260, Pesticides and Industrial Chemical Repository (MD-8), Environmental Protection Agency, Research Triangle Park, NC 27709) in 100 mL benzene (pesticide quality). Make appropriate dilns with hexane to give std solns ranging from 0.004 to 0.4 μg PCP/mL.

(**b**) *Extraction solvent mixture.*—Hexane-isopropanol (pesticide quality) (4 + 1).

(**c**) *Acid-washed glass wool.*—Phosphoric acid-treated (Supelco).

### *D. Extraction and Cleanup*

Weigh 2.0 g gelatin into 25 × 150 mm screw-cap test tube and add 10 mL 12N $H_2SO_4$. Tightly cap and heat 1 h in 100° $H_2O$ bath in fume hood. Remove tube periodically during hydrolysis, wrap in cloth towel, and mix sample by carefully shaking.

Prep. reagent blank with each set of samples.

After 1 h, remove tube, let cool, add 10 mL hexane-isopropanol (4 + 1), and shake by hand or by shaker 2 min. Centrf. 2 min at 1000 × g and transfer upper hexane layer to second test tube with Pasteur pipet. Repeat extn and centrfgn 2 addnl times, combining all hexane exts in second test tube. To combined exts, add 5 mL 1.0N KOH, cap, shake 2 min, centrf. as before, remove upper layer with Pasteur pipet, and discard. Add 10 mL hexane, cap, shake, and centrf. as before; remove hexane wash with Pasteur pipet and discard. Add 5 mL 12N $H_2SO_4$, cap, and mix by carefully swirling tube. Ext 3 times by shaking 2 min each with 5, 2, and 2 mL hexane. After each extn, centrf. as before and transfer exts with Pasteur pipet to 10 mL vol. flask. Adjust to vol. for GC detn.

### *E. GC Determination*

Before each injection, rinse syringe by pumping 3–5 times with soln to be injected. Inject 5 μL sample soln (equiv. to 1.0 mg sample) into gas chromatograph. Measure area or ht of PCP peak and det. amt of residue by comparison to peak area or ht obtained from injection of known amt of PCP ref. std. To ensure valid measurement of PCP residue, size of PCP peak from sample and std should be within ± 10%. Make dilns as needed. Following each injection, rinse syringe by pumping 5–10 times with hexane. After each injection of sample or std soln, inject 5 μL hexane. Appearance of ghost PCP peaks may be noted following injection of high PCP soln. Repeat injection of solv. until ghost PCP peak becomes negligible. Repeat sample and std injections until consistent responses are obtained.

Correct sample results by subtracting reagent blank. Max. acceptable reagent blank for satisfactory performance of method is 0.01 ppm.

Ref.: JAOAC **68,** 419(1985).

CAS-87-86-5 (pentachlorophenol)

## 960.43 Piperonyl Butoxide Residues
### Spectrophotometric Method
**First Action 1960**
**Final Action 1963**

(Applicable to Alaska peas, barley, hulled rice, oats, pinto beans, and wheat)

### *A. Principle*

Strong $H_2SO_4$ liberates HCHO, which is detd colorimetrically with chromotropic acid.

### *B. Reagents*

(**a**) *Chromotropic acid reagent.*—Dissolve 100 mg Na 1,8-dihydroxynaphthalene-3,6-disulfonate/mL of $H_2O$, filter, and keep in dark. Prep. daily. (1 mL required for each detn.)

(**b**) *Dilute sulfuric acid.*—Carefully mix 5 vols $H_2SO_4$ with 3 vols $H_2O$. Cool to room temp. and store in tight g-s container.

(**c**) *Methanolic potassium hydroxide.*—Dissolve 1.4 g KOH in 5 mL $H_2O$ and add 95 mL MeOH (HCHO-free).

(**d**) *Methanol.*—If necessary, purify as follows: Reflux 1 L MeOH 1 hr with ca 10 g powd Al and ca 10 g NaOH and distil ca 800–900 mL.

(**e**) *Hexane.*—Redistd.

(**f**) *Chloroform.*—Reagent or redistd (for wheat extn).

(**g**) *Piperonyl butoxide std solns.*—(*1*) *Stock soln.*—1 mg/mL. Dissolve 0.1000 g in 100 mL benzene. (*2*) *Intermediate soln.*—100 μg/mL. Dil. 10 mL stock soln to 100 mL with benzene. (*3*) *Working soln.*—20 μg/mL. Dil. 20 mL intermediate soln to 100 mL with benzene.

### *C. Preparation of Standard Curve*

Add 0, 20, 40, 60, 80, and 100 μg piperonyl butoxide, resp., to each of 6 g-s test tubes (15 × 150 mm) (25–50 mL g-s centrf. tubes are also satisfactory) and evap. on steam bath with small air jet. Evap. last 1–2 mL benzene without heat.

Into each tube pipet both 1 mL chromotropic acid reagent and 5 mL dil. $H_2SO_4$, (**b**). Stopper loosely and place tubes in beaker of boiling $H_2O$ 45 min, remove, and cool in beaker of cold $H_2O$. When cool, pipet 5 mL $H_2O$ into each test tube, mix well, and read *A* in spectrophtr at 575 nm against reagent blank prepd similarly. Plot μg piperonyl butoxide against *A*.

### *D. Determination*

Ext 25 g sample with $CHCl_3$ in soxhlet app. or shake larger samples with suitable amts of $CHCl_3$ in centrf. bottle, centrf. and decant solv. Repeat extn once or twice according to sample size. Measure total vol. of exts. With current of air, evap. 25 mL (or suitable size aliquot) ext in small beaker just to dryness. Add 5 mL methanolic KOH. Warm gently just enough to melt wax (do not boil). Let stand 30 min, swirling vigorously at ca 10 min intervals. Transfer to small separator, rinse beaker with two 5 mL portions $H_2O$, and add to separator. Add 15 mL hexane to separator, shake vigorously 1 min, and let sep. Drain aq. layer and discard. Quant. transfer hexane layer to g-s test tube or centrf. tube and evap. to dryness with air jet. Small amt of heat may be used, but evap. last 1–2 mL with air alone. (Warmth of hand at this point is enough.)

Into dried residue pipet both 1 mL chromotropic acid reagent and 5 mL of the dil. $H_2SO_4$. Swirl vigorously to ensure that reagent contacts all of sample and place test tube in boiling $H_2O$ bath. Stopper tube, lightly at first and then tighten. After 45 min in $H_2O$ bath, remove, and cool to room temp. in beaker of cold $H_2O$. Pipet in 5 mL $H_2O$, mix well, and measure *A* in spectrophtr at 575 nm against reagent blank prepd similarly. From std curve, calc. piperonyl butoxide in aliquot.

Refs.: JAOAC **43,** 707(1960); **46,** 244(1963).

CAS-51-03-6 (piperonyl butoxide)

## 972.29 Thiram Pesticide Residues
### Spectrophotometric Method
**First Action 1965**
**Final Action 1972**

(Applicable to corn, apples, tomatoes, strawberries, celery, and similar fruits and vegetables)

### *A. Principle*

Thiram is extd from sample with $CHCl_3$. Treatment with solid CuI results in formation of brown, $CHCl_3$-sol. Cu dimethyldithiocarbamate, and its *A* is measured at 440 nm. Other commonly used pesticides do not interfere, with exception of metal dithiocarbamates sol. in $CHCl_3$, such as ferbam or ziram. Moderate amts of color, waxes, and other extd plant matter do not interfere.

### *B. Reagents*

(**a**) *Chloroform.*—Either reagent or tech. grade may be used.

(**b**) *Thiram std solns.*—(*1*) *Stock soln.*—500 μg/mL. Dissolve 50.0 mg thiram (available from E. I. du Pont de Nemours & Co., Biochemicals Dept., 1007 Market St, Wilmington, DE 19898) in $CHCl_3$ and dil. to 100 mL with $CHCl_3$. (*2*) *Working soln.*—25 μg/mL. Dil. 5 mL stock soln to 100 mL with $CHCl_3$.

(**c**) *Cuprous iodide.*—If not available, prep. as follows: To soln of 10 g $CuSO_4.5H_2O$ in ca 100 mL $H_2O$, slowly add excess of KI soln. Remove liberated $I_2$ by adding $Na_2S_2O_3$ soln in slight excess. Filter, and wash ppt thoroly with $H_2O$ and with alcohol. Dry at room temp. and crush to fine powder.

(**d**) *Attapulgus clay.*—Available from MC/B Manufacturing Chemists, No. AX1799.

### *C. Apparatus*

(**a**) *Spectrophotometer.*—Suitable for measuring *A* in UV and at 440 nm.

(**b**) *Glassware.*—Avoid contamination by rinsing with $CHCl_3$ and drying before use. Rinse app. that may have contained CuI from previous detns with dil. acid, $H_2O$, alcohol, and $CHCl_3$.

### *D. Preparation of Standard Curve*

(To minimize errors due to evapn of solv., keep flasks closed as much as possible, and cover funnels with watch glasses during filtrations.)

Using buret, add 2.0, 5.0, 10.0, and 15.0 mL working std soln to 25 mL vol. flasks. Dil. to vol. with $CHCl_3$, and mix. Solns contain 2, 5, 10, and 15 μg thiram/mL, resp.

Transfer ca 10 mL portions of std solns to 125 mL g-s erlenmeyers, add 10 mg CuI to each, stopper, and let stand 1 hr with occasional mixing. Filter, using 9 cm quant. paper, and read $A$ at 440 nm against $CHCl_3$ as ref. Plot $A$ against thiram concn in µg/mL.

### E. Isolation

(Avoid contact of solv. with rubber.)

**(a)** *Corn.*—Ext 200 g by shaking with 100 mL $CHCl_3$ 5 min in 500 mL g-s erlenmeyer. Decant ext thru small funnel (to retain corn kernels) into flask.

**(b)** *Apples, pears, and similar firm fruits.*—Weigh 2–3 kg into clean, dry jar (ca 3 gal.; 11 L). Add 500 mL $CHCl_3$ and stopper with tight-fitting cork, wooden bung, or plastic screw cap faced with gasket of sheet cork or other suitable solv.-resisting material. Ext 5 min by tumbling or other agitation. Decant ext into flask.

**(c)** *Tomatoes, berries, and similar soft fruits and vegetables.*—Weigh 1–3 kg into suitable container. Add 500 mL $CHCl_3$ and stopper with solv.-resisting closure. Ext 5 min by gentle shaking and decant into g-s erlenmeyer thru loose plug of glass wool.

**(d)** *Celery.*—Cut 2–3 kg into 3–8 cm pieces. Mix thoroly and ext 500 g sample with 500 mL $CHCl_3$ as above.

Add anhyd. $Na_2SO_4$, ca 5 g/100 mL, to decanted ext. Stopper flask, shake 5 min, and filter thru folded Whatman No. 12, or equiv., paper.

### F. Determination

(Thiram in $CHCl_3$ soln, particularly in presence of plant extractives, may decompose. Make detns as soon as possible.)

Transfer ca 10 mL filtered ext to g-s erlenmeyer and develop color as in **972.29D**, beginning ". . . add 10 mg CuI . . ." As ref., use another portion of filtered ext., untreated with CuI. From std curve, obtain thiram concn in µg/mL. If developed color is too intense, dil. with $CHCl_3$, making similar diln of ref. ext, and multiply thiram value found by appropriate diln factor.

ppm Thiram = (µg thiram/mL) × mL $CHCl_3$ used for extn/g sample.

### G. Qualitative Test

Adjust concn of ext, **972.29E**, if necessary, to 10–15 µg thiram/mL by evapn on steam bath or by diln with $CHCl_3$. Add 0.25–1.0 g Attapulgus clay, depending on color of ext, to 50 mL of adjusted ext in beaker. Mix well and filter thru Whatman No. 12 folded paper, or equiv. Transfer 25 mL filtrate to g-s erlenmeyer, add 0.2 mL ca 0.1$N$ $AgNO_3$ to ppt thiram and other $CHCl_3$-sol. dithiocarbamates, stopper, and shake vigorously 30 sec. Add ca 1 g anhyd. $Na_2SO_4$ and shake 30 sec. Let settle, decant carefully into 1 cm quartz cell, and *use as ref. soln*, adjusting to 0 $A$ at 350 nm. Det. UV absorption curve over range 250–350 nm on clarified and filtered ext untreated with $AgNO_3$. Thiram gives curve with plateau at 270–283 nm, dropping sharply after peaking at ca 283. Ferbam and ziram give characteristic curves distinguishable from thiram.

Refs.: JAOAC **42,** 545(1959); **45,** 410(1962).

CAS-137-26-8 (thiram)

**Common Names and Chemical Names of Pesticides in this Chapter**

| Common Name | Chemical Name |
|---|---|
| Acephate | Acetylphosphoramidothioic acid *O, S*-dimethyl ester |
| Aldicarb | 2-Methyl-2-(methylthio) propanal *O*-[methyl amino)carbonyl]oxime |
| Aldicarb sulfone | 2-Methyl-2-(methylsulfonyl) propanal *O*-[methyl amino)carbonyl]oxime |
| Aldrin | 1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-*exo*-1,4-*endo*-5,8-dimethanonaphthalene, not less than 95% |
| Amitrole | 3-Amino-1,2,4-triazole |
| Aroclor | Polychlorinated biphenyl compound |
| Azinphos-methyl | *O,O*-Dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl)methyl]phosphorodithioate |
| Benfluralin | *N*-Butyl-*N*-ethyl-α,α,α-trifluoro-2,6-dinitro-*p*-toluidine |
| BHC | 1,2,3,4,5,6-Hexachlorocyclohexane (mixture of isomers) |
| Bufencarb | 3-(1-Ethylpropyl)phenyl methylcarbamate mixture with 3-(1-methylbutyl) phenyl methylcarbamate (1:3) |
| Captan | *N*-[(Trichloromethyl)thio]-4-cyclohexene-1,2-dicarboximide |
| Carbanolate | 6-Chloro-3,4-xylyl-methylcarbamate |
| Carbaryl | 1-Naphthyl *N*-methylcarbamate |
| Carbofuran | 2,3-Dihydro-2,2-dimethyl-7-benzofuranol methylcarbamate |
| Carbophenothion | *S*-[(*p*-Chlorophenylthio)methyl] *O,O*-diethyl phosphorodithioate |
| Chloramben | 3-Amino-2,5-dichlorobenzoic acid |
| Chlordane | 1,2,4,5,6,7,8,8-Octachlor-2,3,3a,4,7,7a-hexahydro-4,7-methanoindane |
| Chlorpyrifos | *O,O*-Diethyl *O*-(3,5,6-trichloro-2-pyridyl) phosphorothioate |
| DDE | Dichlorodiphenyldichloroethylene |
| DDT | 1,1′-(2,2,2-Trichloroethylidene)bis[4-chlorobenzene] |
| Diazinon | *O,O*-Diethyl *O*-(2-isopropyl-4-methyl-6-pyrimidinyl) phosphorothiote |
| Dichlone | 2.3-Dichloro-1,4-naphthoquinone |
| Dieldrin | 3,4,5,6,9,9-Hexachloro-1a,2,2a,3,6,6a,7,7a-octahydro-2,7:3,6-dimethanonaphth(2,3-b)oxirene |
| Dodine | *n*-Dodecylguanidine acetate |
| Endosulfan | 6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide |
| Endosulfan sulfate | 1,4,5,6,7,7-Hexachloro-5-norbornene-2,3-dimethanol cyclic sulfate |
| Endrin | Hexachloroepoxyoctahydro-endo, endo-dimethanonaphthalene |
| EPN | *O*-Ethyl *O*-(4-nitrophenyl) phenylphosphonothioate |
| Ethion | *O,O,O,O*-Tetraethyl *S,S*-methylene bisphosphorodithioate |
| Ethylan | 1,1-Dichloro-2.2-bis(4-ethylphenyl) ethane |
| Glyodin | 2-Heptadecyl-2-imidazoline acetate |
| HCB | Hexachlorobenzene |
| Heptachlor | 1,4,5,6,7,8,8-Heptachloro-3*a*,4,7,7*a*-tetrahydro-4,7-methanoindene |
| Heptachlor epoxide | Oxidation product of heptachlor |
| 3-Hydroxycarbofuran | 2,3-Dihydro-2,2-dimethyl-3-hydroxy-7-benzofuranyl methylcarbamate |
| Lindane | Gamma isomer of 1,2,3,4,5,6-hexachlorocyclohexane (BHC) |
| Malathion | *O,O*-Dimethyl *S*-(1,2-dicarbethoxyethyl) phosphorodithioate |
| Maleic hydrazide | 6-Hydroxy-3(2*H*)pyridazinone |
| Methamidophos | Phosphoramidothioic acid *O,S*-dimethyl ester |
| Methiocarb | 3,5-Dimethyl-4-(methythio)phenyl methylcarbamate |
| Methomyl | *N*-[[(Methylamino)carbonyl]oxy]ethanimidothioic acid methyl ester |
| Methoxychlor | 2,2-Bis(*p*-methoxyphenyl)-1,1,1-trichloroethane |

**Common Names and Chemical Names of Pesticides in this Chapter (*Continued*)**

| Common Name | Chemical Name | Common Name | Chemical Name |
|---|---|---|---|
| Methyl parathion | *O-O*-Dimethyl *O-p*-nitrophenyl phosphorothioate | Paraoxon | *O,O*-Diethyl *O-p*-nitrophenyl phosphate |
| Mirex | Dodecachlorooctahydro-1,3,4-metheno-2*H*-cyclobuta(cd)pentalene | Parathion (same as ethyl parathion) | *O,O*-Diethyl *O-p*-nitrophenyl phosphorothioate |
| Monocrotophos | Phosphoric acid dimethyl [1-methyl-3-(methylamino)-3-oxo-1-propenyl] ester | PCBs | Some mixture of chlorinated biphenyl compounds having various percentages of chlorine |
| NAA | Naphthalene acetic acid | Piperonyl butoxide | α-[2-(2-Butoxyethoxy)ethoxy]-4,5-methylenedioxy-2-propyltoluene |
| Nicotine | 3-(1-Methyl-2-pyrrolidyl) pyridine | Propoxur | 2-(1-Methylethoxy) phenol methylcarbamate |
| Omethoate | *O,O*-Dimethyl *S*-[2-(methylamino)-2-oxoethyl] phosphorothioate | Ronnel | *O,O*-Dimethyl *O*-(2,4,5-trichlorophenyl)phosphorothioate |
| Oxamyl | 2-(Dimethylamino)-*N*-[[methylamino)carbonyl]oxy]-2-oxoethanimidothioic acid methyl ester | TDE | 1,1′-(2,2-Dichloroethylidene)bis[4-chlorobenzene] |
| Oxychlor epoxide (also oxychlordane) | 1-*exo*,2-*endo*-4,5,6,7,8,8-Octachloro-2,3-*exo*-epoxy-2,3,3a,4,7,7a-hexahydro-4,7-methanoindene | Tetradifon | 4-Chlorophenyl 2,4,5-trichlorophenyl sulfone |
| | | Tetrasul | 4-Chlorophenyl 2,4,5-trichlorophenyl sulfide |
| | | Thiram | Zinc dimethyldithiocarbamate |

Sources: *The Merck Index* (1983) 10th ed., Merck & Co., Inc., Rahway, NJ; *The Agrochemicals Handbook* (1987) 2nd ed., The Royal Society of Chemistry, Nottingham, UK; *Farm Chemicals Handbook* (1988) 74th ed., Meister Publishing Co., Willoughby, OH.

## SPECIAL REFERENCES

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Burke, "Development of the Food and Drug Administration's Method of Analysis for Multiple Residues of Organochlorine Pesticides in Foods and Feeds." Residue Reviews **34,** 59–90(1971).

# 11. Waters; and Salt

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## WATER

### 955.37 Specific Gravity of Water

**Pycnometer Method**

**Final Action**

Det. sp gr at 20/20°, using pycnometer, as in **945.06C**.

### 973.40 Specific Conductance of Water

**First Action 1973**

***A. Principle***

Conductivity of sample is compared with that of std KCl soln. Method is applicable to drinking, surface, and saline waters, and domestic and industrial wastes.

Synthetic $H_2O$ samples contg increments of inorg. salts analyzed by 41 analysts in 17 laboratories showed following results:

| Increment as sp conductance, μmhos/cm | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | μmhos/cm | % | μmhos/cm |
| 100 | 7.6 | 7.55 | −2.0 | − 2.0 |
| 106 | 7.7 | 8.14 | −0.8 | − 0.8 |
| 808 | 7.5 | 66.1 | −3.6 | −29.3 |
| 848 | 9.4 | 79.6 | −4.5 | −38.5 |
| 1640 | 6.5 | 106 | −5.4 | −87.9 |
| 1710 | 7.0 | 119 | −5.1 | −86.9 |

***B. Apparatus and Reagent***

(**a**) *Conductivity meter*.—Self-contained, Wheatstone bridge-type, capable of being read to ±1%.

(**b**) *Specific conductance cell*.—Choose cell according to expected sp conductance so that measured cell resistance is 500–10,000 ohms. Cell const should be ca 0.1 for solns of low conductivity (<100 μmhos), 1 for moderate, and 10 for highly conducting, such as brines. Check complete assembly with KCl solns of known conductance shown in Table **973.40**. Clean new cells with chromic acid cleaning soln and platinize new electrodes before use. Reclean and platinize electrodes whenever readings become erratic or if inspection shows any Pt black has flaked off. To platinize, connect both electrodes together to neg. terminal of 1.5 v dry cell and immerse in soln of 1 g chloroplatinic acid and 12 mg $Pb(OAc)_2$ in 100 mL $H_2O$. Connect pos. terminal to piece of Pt wire and dip into soln. Control current so that only small amt gas is evolved. Discontinue electrolysis when both electrodes are coated. Soln may be saved for subsequent use. Rinse electrodes thoroly and keep immersed in $H_2O$ when not in use.

(**c**) *Potassium chloride std soln*.—0.01*M*. Dissolve 745.6 mg KCl in freshly boiled double-distd $H_2O$ and dil. to 1 L at 25°. Soln has sp conductance of 1413 μmhos at 25°. It is satisfactory for most waters when using cell with const of 1–2. With other cells, use soln in Table **973.40** and corresponding sp conductance in calcn. Store in g-s Pyrex bottle.

***C. Determination***

Temp. must be const thruout detn since sp conductance varies ca 2%/degree. Use 25° if possible; otherwise use near room temp. but between 20–30°.

Place 4 tubes std KCl soln and 2 tubes of each sample in $H_2O$ bath and let stand 30 min. Rinse cell in 3 tubes of KCl soln and measure resistance of soln 4, $R_{KCl}$. Rinse cell thoroly with tube 1 of sample and measure resistance of tube 2, $R_s$. Do not repeat measurement of KCl soln unless temp. drift of more than few tenths degree occurs. If samples differ in conductivity by factor of ≥5, minimize carry-over by rinsing in 2 tubes of sample and measuring third.

***D. Calculation***

Calc. cell const, $C$, in mhos/cm = $R_{KCl} \times 0.001413$ at 25°. Specific conductance of sample at 25° = $C/R_s$ in mhos/cm. Multiply by $10^6$ to obtain μmhos/cm.

If temp. is not exactly 25°, measure $R_{KCl}$ and $R_s$ at same temp. and calc. sp conductance = $1413 \times R_{KCl}/R_s$ in μmhos/cm.

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). FWPCA Method Study 1; Mineral and Physical Analyses, June 1969 (available from National Environmental Research Center, Environmental Protection Agency, Cincinnati, OH 45268). JAOAC **56,** 295(1973).

### 973.41 pH of Water

**First Action 1973**

***A. Principle***

pH, which is accepted measure of acidity or alky, is detd by change in potential of glass-satd calomel electrodes, as

**Table 973.40 Conductances of KCl Solns at 25°**

| Concn, *M* | Conductance, μmhos/cm | |
|---|---|---|
| | Equiv. | Specific |
| 0 | 149.85 | |
| 0.0001 | 149.43 | 14.94 |
| 0.0005 | 147.81 | 73.90 |
| 0.001 | 146.95 | 147.0 |
| 0.005 | 143.55 | 717.8 |
| 0.01 | 141.27 | 1,413 |
| 0.02 | 138.34 | 2,767 |
| 0.05 | 133.37 | 6,668 |
| 0.1 | 128.96 | 12,900 |
| 0.2 | 124.08 | 24,820 |
| 0.5 | 117.27 | 58,640 |
| 1.0 | 111.87 | 111,900 |

measured by com. app. stdzd against std buffer solns whose pH values are assigned by NIST. pH of most natural $H_2O$ falls within 4–9. Majority of waters are slightly basic from presence of $CO_3$-$HCO_3$ system.

Method is applicable to drinking, surface, and saline waters, and domestic and industrial wastes. Oils and greases, by coating electrodes, may cause sluggish response.

Buffered synthetic $H_2O$ samples analyzed by 44 analysts in 20 laboratories showed following results:

| pH | Std deviation, pH units | Bias, pH units |
|---|---|---|
| 3.5 | 0.10 | −0.01 |
| 3.5 | 0.11 | 0.00 |
| 7.1 | 0.20 | +0.07 |
| 7.2 | 0.18 | −0.002 |
| 8.0 | 0.13 | −0.01 |
| 8.0 | 0.12 | +0.01 |

### B. Apparatus and Reagent

(**a**) *pH meter*.—Com. instrument with flow-type electrodes (preferred for relatively unbuffered samples such as condensates) or immersion electrodes. Operate in accordance with manufacturer's instructions.

(**b**) *Std buffer solns*.—*See* **964.24** and Table **964.24**.

### C. Determination

Thoroly wet electrodes and prep. in accordance with manufacturer's instructions. Stdze instrument with std buffer with pH near that of sample and then with 2 others to check linearity of electrode response.

Analyze sample as soon as possible, preferably within few hr. Do not open sample bottle before analysis. With immersion electrodes, wash 6–8 times with portions of sample, particularly when unbuffered soln follows buffered soln. Equilibrium, as shown by absence of drift, must be established before readings are accepted.

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). FWPCA Method Study 1; Mineral and Physical Analyses, June 1969 (available from National Environmental Research Center, Environmental Protection Agency, Cincinnati, OH 45268). JAOAC **56,** 295(1973).

## 973.42 Acidity of Water

### Titrimetric Method

### First Action 1973

### A. Principle

Sample is titrd to pH 8.3, using phthln as indicator, and results are reported as mg $CaCO_3$/L. Method is applicable to drinking and surface waters, domestic and industrial wastes, and saline waters. Synthetic $H_2O$ contg increments of $HCO_3$ analyzed by 40 analysts in 17 laboratories showed following results:

| Added, mg $CaCO_3$/L | Std deviation % | Std deviation mg $CaCO_3$/L | Bias % | Bias mg $CaCO_3$/L |
|---|---|---|---|---|
| 20 | 9.0 | 1.79 | +2.8 | +0.55 |
| 21 | 8.2 | 1.73 | +0.5 | +0.11 |

### B. Apparatus

(**a**) *Illumination*.—Daytime fluorescent lamps provide uniform lighting conditions.

(**b**) *Potentiometric equipment*.—Automatic titrators and pH meters, suitably calibrated, may be substituted for visual titrn and end point.

### C. Reagents

(**a**) *Carbon dioxide-free water*.—If pH is <6.0, prep. as in **936.16B(a)**. Deionized $H_2O$ may be substituted if conductance is <2 μmhos/cm and pH >6.0.

(**b**) *Sodium hydroxide std soln*.—0.02*N*. Dil. 20.0 mL 1*N* NaOH with $CO_2$-free $H_2O$ to 1 L. Store in tightly stoppered Pyrex bottle protected by soda-lime tube. Prep. weekly. Stdze against 0.0200*N* KH phthalate soln (4.085 g/L) or against stdzd 0.02*N* HCl or $H_2SO_4$. Use vol. soln to give acidity approx. that of samples titrd, dild to vol. of sample, with same vol. indicator, and same time intervals as in detn. 1 mL 0.0200*N* NaOH = 1.00 mg $CaCO_3$/1.00 mL.

### D. Preparation of Samples

Collect and store samples in Pyrex or polyethylene bottles. Refrigerate at 4° and perform detn as soon as possible, preferably within 24 hr.

### E. Determination

Use sample vol. requiring <25 mL titrant. If indicator is used, remove free Cl with 1 drop 0.1*N* $Na_2S_2O_3$, **942.27A**.

To 50 or 100 mL sample in white porcelain casserole or in erlenmeyer over white surface, add 0.15 mL phthln. Titr. with stdzd 0.02*N* NaOH to faint pink (pH 8.3).

$$\text{mg } CaCO_3/L = \text{mL NaOH} \times \text{normality NaOH} \times 50{,}000/\text{mL sample}$$

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). FWPCA Method Study 1; Mineral and Physical Analyses, June 1969 (available from National Environmental Research Center, Environmental Protection Agency, Cincinnati, OH 45268). JAOAC **56,** 295(1973).

## 973.43 Alkalinity of Water

### Titrimetric Method

### First Action 1973

### A. Principle

Unaltered (undild, unconcd, unfiltered) sample is titrd potentiometrically to pH 4.5. Applicable to drinking and surface waters, domestic and industrial wastes, and saline waters. Suitable for all concn ranges.

Synthetic $H_2O$ contg increments of $HCO_3$ analyzed by 40 analysts in 17 laboratories showed following results:

| Added, mg $CaCO_3$/L | Std deviation % | Std deviation mg $CaCO_3$/L | Bias % | Bias mg $CaCO_3$/L |
|---|---|---|---|---|
| 8 | 16 | 1.3 | +10.6 | +0.85 |
| 9 | 12 | 1.1 | +22.3 | +2.0 |
| 113 | 4.7 | 5.3 | − 8.2 | −9.3 |
| 119 | 4.5 | 5.4 | − 7.4 | −8.8 |

### B. Apparatus

*See* **973.42B(b)**.

### C. Reagents

(**a**) *Carbon dioxide-free water*.—*See* **973.42C(a)**.

(**b**) *Acid std soln*.—0.02*N*. Prep. ca 0.1*N* stock soln by dilg 8.3 mL HCl or 2.8 mL $H_2SO_4$ to 1 L. Dil. 200 mL stock soln to 1 L with $CO_2$-free $H_2O$. Stdze against 0.02*N* $Na_2CO_3$ (1.060 g $Na_2CO_3$/L, **936.15F(c)**) or stdzd 0.02*N* NaOH, **973.42C(b)**. Use vol. soln to give alky approx. that of samples titrd, dild to vol. of sample, with same vol. indicator and same time intervals as in detn. 1 mL 0.02*N* acid = 1.00 mg $CaCO_3$/L.

### D. Preparation of Samples

*See* **973.42D**.

### E. Determination

Use sample vol. requiring <25 mL titrant. Titr. potentiometrically to pH 4.5.

$$\text{mg } CaCO_3/L = \text{mL acid} \times \text{normality acid} \times 50{,}000/\text{ mL sample}$$

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). FWPCA Method Study 1; Mineral and Physical Analyses, June 1969 (available from National Environmental Research Center, Environmental Protection Agency, Cincinnati, OH 45268). JAOAC **56,** 295 (1973).

## 973.44 Biochemical Oxygen Demand (BOD) of Water

### Incubation Method

### First Action 1973

### A. Principle

Sample is incubated 5 days at 20° in presence of acclimated biological system. Comparison of O content of sample at beginning and end of incubation is measure of BOD.

Method is applicable to raw or treated domestic wastes, industrial water, and industrial waste water. Following classes of materials exert O demand: (*1*) org. material usable as food by aerobic organisms (source of BOD of many waste waters); (*2*) oxidizable N from nitrites, $NH_3$, and org. N compds which serve as food for specific bacteria (e.g., *Nitrosomonas* and *Nitrobacter*) (a source of some of O demand of biologically treated effluents); (*3*) chemically oxidizable materials (e.g., $Fe^{+2}$, $S^{-2}$, $SO_3^{-2}$) (when present, test must be based upon calcd initial dissolved O content).

Many synthetic org. components of industrial wastes are not degraded by common organisms. Without special seeding material, effect is manifested as retardation of aerobic metabolism because of toxic effect or deficiency or absence of appropriate microorganism. Toxic compds in distd $H_2O$, frequently Cu, may result in low BOD.

Distd $H_2O$ contg known increments of oxidizable org. material analyzed by 74 analysts in 50 laboratories showed following results:

| Increment org. material, mg/L | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | mg/L | % | mg/L |
| 2.2 | 33 | 0.7 | − 4 | − 0.08 |
| 194 | 15 | 26 | −10 | −19 |

### B. Apparatus

(**a**) *Incubation bottles*.—250 or 300 mL with glass stoppers.

(**b**) *Incubator*.—Air or $H_2O$ bath maintained at 20 ± 1° and which excludes light. (*Caution:* Check $H_2O$ bath to assure that it is electrically grounded.)

### C. Reagents

(**a**) *Water*.—Contg ≤0.01 mg Cu/L, obtained by double demineralization of distd $H_2O$ or distn from all-glass or Sn-lined system.

(**b**) *Calcium chloride soln*.—27.5 g anhyd. $CaCl_2$/L.

(**c**) *Ferric chloride soln*.—0.25 g $FeCl_3.6H_2O$/L.

(**d**) *Magnesium sulfate soln*.—22.5 g $MgSO_4$ .$7H_2O$/L.

(**e**) *Phosphate buffer soln*.—pH 7.2. Dissolve 8.50 g $KH_2PO_4$, 21.75 g $K_2HPO_4$, 33.40 g $Na_2HPO_4.7H_2O$, and 1.70 g $NH_4Cl$ in ca 500 mL $H_2O$ and dil. to 1 L.

(**f**) *Seeding material*.—Satisfactory seed may sometimes be obtained or developed from supernate of domestic sewage stored 24–36 hr at 20°, from receiving $H_2O$ downstream from point of discharge, or, in case of industrial wastes contg org. compds not amenable to oxidn by domestic sewage seed, from acclimated seed developed in laboratory.

(**g**) *Sodium hydroxide soln*.—50 g NaOH/L.

(**h**) *Sodium sulfite soln*.—1.575 g $Na_2SO_3$/L. Prep. fresh as needed.

### D. Preparation of Dilution Water

Store $H_2O$, **973.44C(a)**, in cotton-plugged bottles long enough to sat. with atm. O at 20°, or aerate with air filtered to remove any oil from compressor (≤1 hr may be required for 19 L (5 gal.)). Add desired vol. of O-satd $H_2O$ to suitable bottle and add 1 mL each of phosphate buffer, $MgSO_4$, $CaCl_2$, and $FeCl_3$ solns/L. Seed this diln $H_2O$ with seeding material and with vol. found by experience to be most satisfactory for particular waste being examined. Use seeded diln $H_2O$ within 24 hr of prepn.

Periodically check quality of diln $H_2O$, effectiveness of seed, and technic with particular org. compd if known to be present in waste or, for general work, with mixt. of glucose and glutamic acid (150 mg each/ L) which should show BOD ca 220 ± 30 mg/L in 95% of detns. Appreciable divergence requires examination of quality of $H_2O$, viability of seeding material, or technic.

### E. Preparation of Samples

Keep time between collection of sample and start of analysis to absolute min. Protect samples from atm. O. If necessary, pretreat samples as follows:

(**a**) *Acidity or caustic alkalinity*.—Neutze to ca pH 7 with dil. $H_2SO_4$ or 5% NaOH, using pH meter or bromothymol blue as external indicator. pH of seeded diln $H_2O$ should not be changed by diln of sample.

(**b**) *Residual chlorine*.—Let stand 1–2 hr to dissipate Cl. If not effective, use $Na_2SO_3$ treatment. Det. vol. to be used by adding 10 mL HOAc (1 + 1) or $H_2SO_4$ (1 + 49) and 10 mL 10% KI to 1 L sample. Titr. to starch-I end point with $Na_2SO_3$ soln. Add indicated vol. to sample, and test small portion with starch-I soln to check that treatment is complete.

(**c**) *Toxic substances*.—Remove or neutze. Test for toxicity as follows: Add same amt seed to duplicate set of BOD bottles. Add diln $H_2O$ to each bottle, leaving room for amt of sample to give final concns of 0.06, 0.12, 0.25, 0.50, 1.0, 2.5, 5, 10, 20, and 40%. Neutze sample, add required vol. sample to duplicate bottles, and fill with diln $H_2O$. Det. dissolved O in 1 series ca 15 min after prepn of diln. Det. dissolved O in second series after 3 days. Plot consumption of dissolved O against concn. Magnitude of O concn change will depend on amt of food available and toxicity of sample. If toxicity is factor, O consumption will decrease at higher concns.

(**d**) *Supersaturation with oxygen.*—Samples contg >9.2 mg O/L at 20° may be encountered during winter or where algae are actively growing. To prevent loss of O during incubation, reduce O content to satn by transferring sample at ca 20° to partially fill bottle and shake vigorously.

### F. Determination

Sample must be dild with seeded diln $H_2O$ so that at least 1 diln will achieve dissolved O depletion of 1 mg/ L (ppm) during 5 day test period but will not reduce residual dissolved O to <1 mg/L. (Preliminary chemical O demand (COD) detn, **973.46**, may serve as guide to est. range of BOD.)

Carefully siphon seeded diln $H_2O$ into 1 or 2 L graduate, filling it 1/2 full. Add vol. of carefully mixed sample to desired diln and fill to mark with diln $H_2O$. Mix well with plunger-type mixing rod, avoiding entrainment of air. If possible BOD range is large, prep. geometric series of dilns to cover possible range. Siphon, with continued mixing, dild sample to completely fill 3 BOD bottles—1 for incubation, 1 for detn of dissolved O content, and 1 for detn of immediate dissolved O demand (IDOD). Insert stoppers without entrainment of any bubbles. Det. dissolved O by method indicated in **973.45A**.

Alternatively, prep. dild samples directly by pipetting sample with wide-tip pipet into BOD bottles of known capacity and filling bottles with seeded diln $H_2O$. If diln >1:100 is required, prep. in graduate before adding to BOD bottles.

Prep. blank of seeded diln $H_2O$ contg vol. used for diln of samples for detn of initial dissolved O content. Prep. control of 2 BOD bottles with unseeded diln $H_2O$. Stopper and $H_2O$-seal 1 bottle for incubation. (If special $H_2O$-sealed bottles are not used, $H_2O$-seal by immersion in tray of $H_2O$.) Det. dissolved O in other bottle before incubation. Quality of unseeded diln $H_2O$ is satisfactory if depletion obtained is ≤0.2 mg/L, preferably ≤0.1. Do not use this value as blank correction.

If diln $H_2O$ is seeded, det. O depletion of seed used in such diln that will result in 40–70% depletion in 5 days. Use this depletion, not seeded blank, to calc. correction due to small amt of seed in diln $H_2O$.

Incubate prepd mixts, $H_2O$-sealed, 5 days at 20 ± 1° and det. final dissolved O content.

### G. Calculation

Calc. in mg/L (ppm) as follows:
Immediate dissolved O demand ($IDOD$) = $(D_C - D_1)/P$
When seeding is not required, $BOD = (D_1 - D_2)/P$
When using seeded diln $H_2O$, $BOD = [(D_1 - D_2) - (B_1 - B_2)f]/P$

Including $IDOD$, if small or not detd, $BOD = (D_C - D_2)/P$

Where
$D_0$ = dissolved O (DO) of original diln $H_2O$,
$D_1$ = DO in dild sample 15 min after prepn,
$D_2$ = DO of dild sample after incubation,
$S$ = DO of original undild sample,
$D_C$ = DO available in diln at zero time = $(D_0 p) + SP$,
$p$ = decimal fraction of diln $H_2O$ used,
$P$ = decimal fraction of sample used,
$B_1$ = DO of the diln of seed control before incubation,
$B_2$ = DO of the diln of seed control after incubation,
$f$ = ratio of seed in sample to seed in control = (% seed in $D_1$)/(% seed in $B_1$).

### H. Interpretation

Arbitrary std 5 day incubation period is satisfactory measurement of the O load on receiving water for raw or treated domestic sewage. It may be misleading for wastes contg org. compds not easily amenable to biological oxidn. Studies with 3 incubation periods on series of dilns of the waste will provide information on lag periods, suitability of inocula, rate of biochem. oxidn, ultimate O demand, and amenability to biochem. self-purification. Particularly important is ratio of 5 day BOD to ultimate O demand.

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). Method Research Study 3; Demand Analyses, 1971 (available from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, NTIS PB230275/BE). JAOAC **56**, 295(1973).

## 973.45 Oxygen (Dissolved) in Water

**Titrimetric Methods**

**First Action 1973**

(*Caution:* See safety notes on pipets.)

### A. Applications

Azide (Alsterberg) method is ordinarily used; it is not affected by most common interference, nitrite, but most other oxidizing or reducing agents should be absent. Effect of $Fe^{+3}$ is eliminated with $F^-$. Permanganate (Rideal-Stewart) method is used in presence of $Fe^{+2}$ but not org. matter. Pomeroy-Kirshman-Alsterberg method is used for waters supersatd with O or contg high org. matter content.

#### *Method I, Azide Method*

### B. Reagents

(**a**) *Alkaline iodide-sodium azide soln.*—Dissolve 500 g NaOH (or 700 g KOH) and 135 g NaI (or 150 g KI) in $H_2O$, dil. to 950 mL, and cool. Slowly, with stirring, add soln of 10 g $NaN_3$ in 40 mL $H_2O$. Dild and acidified soln must not give color with starch indicator. Store in dark bottle with rubber stopper.

(**b**) *Manganese sulfate soln.*—Dissolve 364 g $MnSO_4.H_2O$ in $H_2O$, filter, and dil. to 1 L. No more than trace of I should be liberated when soln is added to acidified KI soln.

(**c**) *Potassium biiodate std soln.*—0.025*N*. Dissolve 0.8125 g $KH(IO_3)_2$ in $H_2O$ in 1 L vol. flask and dil. to vol.

(**d**) *Potassium fluoride soln.*—40 g $KF.2H_2O$/100 mL. (*Caution:* KF is toxic and corrosive. *See* safety notes on toxic dusts.)

(**e**) *Sodium thiosulfate std solns.*—(*1*) *0.1N.*—Dissolve 25 g $Na_2S_2O_3.5H_2O$ in $H_2O$, add 1 g NaOH or 5 mL $CHCl_3$, and dil. to 1 L. Stdze against $KH(IO_3)_2$ or $K_2Cr_2O_7$, **942.27B**. (*2*) *0.025N.*—Dil. 250 mL 0.1*N* to 1 L. 1 mL = 0.2 mg O.

(**f**) *Starch indicator soln.*—Disperse 5–6 g potato or arrowroot starch in mortar with few mL $H_2O$. Pour into 1 L boiling $H_2O$, boil few min, and let settle overnight. Decant clear soln and preserve with 1.3 g salicylic acid or few drops toluene.

### C. Determination

(Add all reagents, except $H_2SO_4$, well below surface of sample from 10 mL pipets graduated in 0.1 mL, with tips elongated ca 50 mm.)

Add 2.0 mL $MnSO_4$ soln and 2.0 mL alk. I-$NaN_3$ soln to sample in 250 or 300 mL BOD bottle, replace stopper, excluding air bubbles, and invert several times to mix. Let floc settle and repeat mixing. (Water with high chloride concn requires 10 min contact with ppt.) After floc has settled, leaving

≥100 mL clear supernate, remove stopper and add 2.0 mL $H_2SO_4$ down neck of bottle. (If >100 ppm $Fe^{+3}$ is present, add 1.0 mL KF soln before acidifying.) Restopper and mix by inversion until $I_2$ is uniformly distributed. Immediately titr. 203 mL (3 mL is allowance for added reagents) with 0.025*N* $Na_2S_2O_3$ to pale straw yellow. Add 1–2 mL starch indicator and titr. to disappearance of blue. Disregard reappearance of blue.

$$\text{ppm dissolved O} = (\text{mL } 0.025N\ Na_2S_2O_3 \times 0.2/200) \times 1000$$

### *Method II, Permanganate Method*

#### *D. Reagents*

(**a**) *Alkaline iodide soln.*—Prep. as in **973.45B(a)**, except omit $NaN_3$.

(**b**) *Potassium oxalate soln.*—2%. Dissolve 2 g $K_2C_2O_4.H_2O$ in 100 mL $H_2O$. 1 mL equiv. to 1.1 mL $KMnO_4$ soln, (**c**).

(**c**) *Potassium permanganate soln.*—6.3 g/L.

#### *E. Determination*

Add to sample 0.70 mL $H_2SO_4$ and then 1.0 mL $KMnO_4$ soln. If sample is high in Fe, also add 1.0 mL KF soln, **973.45B(d)**. If necessary, add addnl $KMnO_4$ soln to maintain violet tinge 5 min. (If >5 mL is required, prep. stronger $KMnO_4$ soln to avoid diln of sample.) After 5 min, decolorize with just enough oxalate soln (usually 0.5–1.0 mL) within 2–10 min. Add 2.0 mL $MnSO_4$ soln, **973.45B(b)**, and 3.0 mL alk. $I_2$ soln, **973.45D(a)**. Stopper bottle and mix. Let ppt settle, remix 20 sec, and let settle until ≥100 mL clear supernate is present. Acidify with 2.0 mL $H_2SO_4$. Titr., using vol. of 205 mL, and calc. as in **973.45C**.

### *Method III, Pomeroy-Kirshman-Alsterberg Method*

#### *F. Reagent*

*Alkaline iodide-sodium azide soln.*—Dissolve 400 g NaOH in 500 mL freshly boiled and cooled $H_2O$. Cool slightly and then add 900 g NaI; mix. Dissolve 10 g $NaN_3$ in 40 mL $H_2O$. Add slowly, with stirring, to alk. $I_2$ soln, bringing total vol. to ≥1 L.

#### *G. Determination*

To sample add 2.0 mL $MnSO_4$ soln, **973.45B(b)**, and 2.0 mL alk. $I_2$-$NaN_3$ soln, **973.45F**. Stopper and mix by inversion. After ppt has settled, add 2.0 mL $H_2SO_4$ and mix. Titr., using vol. of 203 mL, and calc. as in **973.45C**.

Ref.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). JAOAC **56,** 295(1973).

## 973.46 Chemical Oxygen Demand (COD) of Water

### Titrimetric Methods

### First Action 1973

#### *A. Principle*

Org. substances are oxidized by $K_2Cr_2O_7$ in $H_2SO_4$ (1 + 1) at reflux temp. with $Ag_2SO_4$ as catalyst and $HgSO_4$ to remove Cl interference. Excess dichromate is titrd with $Fe^{+2}$, using orthophenanthroline as indicator. Method is independent detn of org. matter in sample and has no definable relationship to biological oxygen demand (BOD).

Method is applicable to surface and saline waters and industrial wastes. Apply *Method I*, using 0.25*N* reagents, to samples contg >50 mg COD/L; apply low level modification, *Method II*, using 0.025*N* reagents, to samples in range 5–50 mg/L; apply special modification, *Method III*, to saline waters contg >1000 mg Cl/L and >250 mg COD/L.

Org. matter from glassware, atm., and distd $H_2O$ must be excluded. Condition glassware by using it for blank detn to eliminate org. matter.

Distd $H_2O$ contg known increments of oxidizable org. material analyzed by 89 analysts in 58 laboratories showed following results:

| Increment org. material, mg/L | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | mg/L | % | mg/L |
| 12.3 | 34 | 4 | 0 | 0 |
| 270 | 7 | 18 | −2 | −5 |

#### *B. Preparation of Sample*

Collect samples in glass bottles if possible; plastic may be used if it contributes no org. material to sample. Test biologically active samples as soon as possible. Mix or homogenize samples contg settleable materials. Samples may be preserved with $H_2SO_4$, 2 mL/L.

#### *C. Apparatus and Reagents*

(**a**) *Reflux apparatus.*—500 mL erlenmeyer or 300 mL r-b flask with ₸ joint connected to 30 cm (12″) Allihn condenser.

(**b**) *Distilled water.*—Low in org. matter. Ordinary distd $H_2O$ is satisfactory; do not use deionized $H_2O$.

(**c**) *Potassium dichromate std solns.*—(*1*) *0.25N.*—Dissolve 12.259 g $K_2Cr_2O_7$, primary std grade, previously dried 2 hr at 103°, in distd $H_2O$ and dil. to 1 L. (*2*) *0.025N.*—Dil. 100 mL 0.25*N* to 1 L with $H_2O$.

(**d**) *Sulfuric acid reagent.*—Dissolve 23.5 g $Ag_2SO_4$ in 9 lb (4.1 kg) bottle $H_2SO_4$. (1–2 days may be required for dissoln.)

(**e**) *Ferrous ammonium sulfate std soln.*—(*1*) *0.25N.*—Dissolve 98 g $Fe(NH_4)_2(SO_4)_2.6H_2O$ in $H_2O$, add 20 mL $H_2SO_4$, cool, and dil. to 1 L. Stdze daily against 0.25*N* $K_2Cr_2O_7$, (**c**)(*1*). (*2*) *0.025N.*—Dil. 100 mL 0.25*N* to 1 L with $H_2O$. Stdze daily against 0.025*N* $K_2Cr_2O_7$, (**c**)(*2*).

(**f**) *Phenanthroline ferrous sulfate (ferroin) indicator soln.*—Dissolve 1.48 g 1,10-(ortho)-phenanthroline.$H_2O$ and 0.70 g $FeSO_4.7H_2O$ in 100 mL $H_2O$.

#### *D. Standardization of Ferrous Solutions*

(**a**) *Concentrated soln.*—Dil. 25.0 mL 0.25*N* $K_2Cr_2O_7$, (**c**)(*1*), to ca 250 mL with $H_2O$. Add 75 mL $H_2SO_4$ and cool. Titr. with 0.25*N* $Fe(NH_4)_2(SO_4)_2$, using 10 drops ferroin indicator. Normality = (mL $K_2Cr_2O_7$ × normality)/mL $Fe(NH_4)_2(SO_4)_2$.

(**b**) *Dilute soln.*—To 15 mL $H_2O$ add 10.0 mL 0.025*N* $K_2Cr_2O_7$, (**c**)(*2*). Add 20 mL $H_2SO_4$ and cool. Titr. with 0.025*N* $Fe(NH_4)_2(SO_4)_2$, using 1 drop ferroin indicator. Blue-green to reddish brown color change is sharp. Calc. normality as in (**a**).

#### *E. Method I—High Level*

(*Caution:* See safety notes on mercury and mercury salts.)

Place several boiling chips and 1 g $HgSO_4$ in reflux flask. Add 5.0 mL $H_2SO_4$ and swirl until $HgSO_4$ dissolves. Place in ice bath and slowly add, with swirling, 25.0 mL 0.25*N* $K_2Cr_2O_7$. Slowly, and with swirling, add 70.0 mL $H_2SO_4$-$Ag_2SO_4$ reagent. While still in bath, pipet in 50 mL sample (or aliquot dild to 50 mL) with continuous mixing. Attach condenser and reflux 2 hr. (Shorter period may be used on waste $H_2O$ of const or known composition where time of max. oxidn has been detd previously.)

Cool, and wash down condenser with ca 25 mL $H_2O$. If r-b flask has been used, quant. transfer soln to 500 mL erlenmeyer. Dil. to ca 300 mL with $H_2O$, and let cool to ca room temp. Add 8–10 drops ferroin indicator, and titr. excess $K_2Cr_2O_7$ with 0.25*N* $Fe(NH_4)_2(SO_4)_2$ to sharp, reddish end point (*S* mL). Perform blank detn with all reagents, including refluxing, on distd $H_2O$ in place of sample and det. mL 0.25*N* $Fe(NH_4)_2(SO_4)_2$ required (*B* mL).

$$\text{mg COD/L} = (B - S) \times N \times 8000/V$$

where $N$ = normality $Fe(NH_4)_2(SO_4)_2$ soln and $V$ = vol. sample used.

### *F. Method II—Low Level*

Proceed as in high level detn, **973.46E**, except use 0.025*N* $K_2Cr_2O_7$ and $Fe(NH_4)_2(SO_4)_2$.

### *G. Method III—Saline Waters*

Pipet 50 mL sample of 250–800 mg COD/L and $Cl^- > 1000$ mg/L (or aliquot dild to 50 mL with *distd* $H_2O$ having $Cl^-$ concn equal to that of sample) into 500 mL erlenmeyer and add 25.0 mL 0.25*N* $K_2Cr_2O_7$ and 5.0 mL $H_2SO_4$. Add 10 mg $HgSO_4$/mg Cl in sample and swirl until dissolved. Carefully add 70.0 mL $H_2SO_4$-$Ag_2SO_4$ reagent with swirling. Add several boiling chips, attach condenser, and reflux 2 hr. (If volatile org. compds are present in sample, attach condenser prior to addn of $H_2SO_4$-$Ag_2SO_4$ reagent and add reagent thru condenser while cooling flask in ice bath.)

Cool, and proceed as in low level detn, **973.46F**, including blank. Disregard reappearance of blue-green after end point is reached.

For saline waters, prep. std curve of COD against mg $Cl^-$/L, using NaCl solns with intervals of $\leq$4000 up to 20,000 mg $Cl^-$/L, carried thru entire detn.

$$\text{COD, mg/L} = [(B - S) \times N \times 8000 - 50D] \times 1.20/V$$

where $D$ = $Cl^-$ correction from std curve, and 1.20 is compensation factor to account for extent of $Cl^-$ oxidn which is dissimilar in org. and inorg. systems. Other symbols are defined in **973.46E**.

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). Method Research Study 3; Demand Analyses, 1971 (available from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, NTIS PB230275/BE). JAOAC **56,** 295(1973).

## 920.193 Solids in Water

**Final Action**

### *A. Total Solids*

Thoroly shake sample, and pipet 100 mL unfiltered sample into weighed Pt dish. If sample contains much suspended matter, shake, pour rapidly into 100 mL graduate, and immediately transfer to weighed Pt dish. Evap. to dryness and heat to const wt at 100°.

### *B. Solids in Solution*

Let sample stand until all sediment settles and filter if necessary to secure perfectly clear liq. (Occasionally, clear filtrate can be obtained only by use of alumina cream; avoid if possible.) Evap. 100–250 mL to dryness in weighed Pt dish. Heat to const wt at 100°.

*Alumina cream.*—Prep. cold satd soln of alum in $H_2O$. Add $NH_4OH$ with constant stirring until soln is alk. to litmus, let ppt settle, and wash by decantation with $H_2O$ until wash $H_2O$ gives only slight test for sulfates with $BaCl_2$ soln. Pour off excess $H_2O$ and store residual cream in g-s bottle. (Alumina cream is suitable for clarifying light-colored sugar products or as adjunct to other agents when sugars are detd by polariscopic or reducing sugar methods.)

### *C. Ignited Residue*

Ignite residue from **920.193A** at 525–550° in furnace or over burner until dish shows dull red glow and ash is white or nearly so. Note any odor or change in color produced during ignition. Det. wt ignited residue and calc. loss on ignition.

## 973.47 Organic Carbon in Water

**Infrared Analyzer Method**

**First Action 1973**

### *A. Principle*

Carbonaceous material of water sample is oxidized to $CO_2$ in stream of O or air in catalytic combustion tube at 950°. Calibrated IR analyzer measures $CO_2$.

Method is applicable to 1–150 mg org. C in surface and saline waters and domestic and industrial wastes. Preliminary treatment of sample defines type of C measured: (*1*) sol., nonvolatile org. C (e.g., natural sugars); (*2*) sol., volatile org. C (e.g., mercaptans); (*3*) insol., partially volatile C (e.g., oils); (*4*) insol., particulate carbonaceous materials (e.g., cellulose fibers); (*5*) sol. or insol. carbonaceous materials adsorbed or entrapped on insol. inorg. suspended matter (e.g., oily matter adsorbed on silt particles).

Since usefulness of method is in assessing potential O-demanding load of org. material, $CO_3$ and $HCO_3$ carbon must be removed before analysis or subtracted from final result.

Distd $H_2O$ analyzed by 28 analysts in 21 laboratories showed following results on exact increments of oxidizable org. compds:

| Added total org. C, mg/L | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | mg/L | % | mg/L |
| 4.9 | 80 | 3.9 | +15 | 0.75 |
| 107 | 8 | 8.3 | + 1 | 1.1 |

### *B. Preparation of Sample*

Glass bottles are preferable storage containers but polyethylene and Cubitainers (Hedwin Corp., 1209 E Lincoln Way, La Port, IN 46390) may be used if tests show no contribution of C to samples. Keep interval between collection and analysis at min., store at 4°, and protect from light and O. If samples cannot be analyzed within 2 hr, acidify to pH <2 with HCl or $H_2SO_4$.

### *C. Apparatus*

(**a**) *Organic carbon analyzer.*—Dow-Beckman Carbonaceous Analyzer (single channel) or Model No. 915B (dual channel) (Beckman Industrial Corp., 600 S Harbor, LaHabra, CA 90631), or equiv., with air pump, purification train, flow controls, nondispersive-type IR stream analyzer specific for $CO_2$, and recorder.

(**b**) *Syringes.*—(*1*) 0–50 µL, needle opening ca 150 µm, Hamilton No. 705N, or equiv.; (*2*) 0–500 µL, needle opening ca 400 µm, for samples with large particulates, Hamilton No. 750N, or equiv.; or (*3*) push button syringes which ensure uniformity of injection rate, 20 or 200 µL size, Hamilton No. CR700-20 or CR700-200, or equiv.

### D. Reagents

(*Caution:* See safety notes on asbestos.)

(**a**) *Water.*—For diln of samples and prepn of stds, blanks, and reagents. Use $CO_2$-free, double distd $H_2O$; do not use $H_2O$ purified by ion exchange.

(**b**) *Organic carbon std solns.*—(*1*) *Stock phthalate soln.*—1000 mg C/L. Dissolve 0.2128 g KH phthalate, **936.16B(c)**, in $H_2O$, and dil. to 100 mL. (*2*) *Working solns.*—Prep. solns contg 10, 20, 30, 40, 50, 60, 80, and 100 mg C/L by dilg 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0, and 10.0 mL stock soln to 100 mL with $H_2O$.

(**c**) *Carbonate carbon std solns.*—(*1*) *Stock soln.*— 1000 mg C/L. Dissolve 0.3500 g $NaHCO_3$ and 0.4418 g $Na_2CO_3$ in $H_2O$ and dil. to 100 mL. (*2*) *Working solns.*—Prep. identical series with concns as in (**b**)(*2*).

(**d**) *Packing for total carbon tube.*—Dissolve 20 g $Co(NO_3)_2.6H_2O$ in 50 mL $H_2O$ and add to 15 g long fiber asbestos in porcelain evapg dish. Mix, and evap. to dryness on steam bath. Place in cold furnace, heat to 950°, and hold at this temp. 1–2 hr. Cool, break up any lumps, and mix adequately but not excessively. With combustion tube held vertically, taper joint up, add ca 1 cm untreated asbestos and then ca 1 g catalyst transferred in small amts with forceps. As it is added, tap or push material with 6 mm glass rod, using only wt of rod to compress material. Do not force packing. When completed, length of packing should be ca 5–6 cm. Test packed tube by measuring flow rate of gas thru it at room temp. and at 950°. Rate should drop <20%.

(**e**) *Packing for carbonate tube* (*dual channel instrument*).—Place small wad of quartz wool or asbestos near exit of carbonate evolution tube. From entrance end add 6–12 mesh quartz chips to length of 10 cm. Add $H_3PO_4$ while holding tube vertically and let excess drain.

### E. Adjustment of Instrument

Turn on IR analyzer, recorder, and furnaces, setting total C furnace at 950° and carbonate furnace at 175°. Let warm up >2 hr; leave on continuously for daily operation. Adjust O flow to 80–100 mL/min thru total C tube. With recorder set at appropriate mv range, adjust amplifier gain so that 20 μL sample of 100 mg C/L std gives peak ht ca half scale. Noise level should be <0.5% full scale; if higher, analyzer or recorder may need servicing.

If single channel unit is equipped with large diam. combustion tube and dual channel unit with Hastalloy tube for total C channel, use 100 μL sample in range 1–30 mg C/L.

### F. Calibration

(**a**) *Dual channel instrument.*—Rinse syringe several times with std soln, fill, and adjust to 20 μL. Wipe off excess with soft paper tissue, taking care that no lint adheres to needle. Remove plug from syringe holder, insert syringe, and inject soln into tube with single, rapid movement of index finger. Leave syringe in holder until flow rate returns to normal; then replace it with plug. Run duplicate detns on each std soln and on blank. Read ht of each peak. Let recorder return to baseline between injections. Subtract blank from each peak and prep. std curve of corrected peak ht against mg C/L.

Turn 4-way valve to direct flow thru low temp. tube and analyzer. Adjust flow to 80–100 mL/min and let baseline stabilize. Inject in duplicate 20 μL each of 20, 40, 60, 80, and 100 mg inorg. C/L std solns and blank. Prep. std curve of corrected peak ht against mg inorg. C/L.

(**b**) *Single channel instrument.*—Prep. std curve as in (**a**), par. 1.

### G. Determination

(**a**) *Dual channel instrument.*—Mix sample thoroly and dil. to bring C content within range of std curve. Inject 20 μL sample in duplicate as in **973.47F(a)** and det. peak hts corresponding to total and inorg. C. Convert to concn and subtract inorg. C from total C to obtain total org. C. Results may be verified by operating unit as single channel system, injecting acidified, N-purged sample into high temp. furnace, and comparing results.

Filter 100 mL aliquot sample thru prerinsed 0.45 μm fritted glass filter and repeat detn to obtain dissolved C values. Subtract dissolved or inorg. C to obtain dissolved org. C. Results may be verified by operating unit as single channel system, injecting acidified, N-purged, filtered sample into high temp. furnace, and comparing results.

(**b**) *Single channel instrument.*—Transfer 10–15 mL sample to 30 mL beaker. If sample is not acid-preserved, add 2 drops HCl to reduce pH to ≤2 and purge with $CO_2$-free N ca 5–10 min. (Do not use plastic tubing.) Place beaker on mag. stirrer and withdraw 20 μL aliquot while stirring. Inject as in **973.47F(a)**. Prep. and inject filtered samples. Calc. total, inorg., and dissolved C as in (**a**).

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). Method Research Study 3; Demand Analyses, 1971 (available from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, NTIS PB230275/BE). JAOAC **56,** 295(1973).

## 973.48 Nitrogen (Total) in Water

**Kjeldahl Method**

**First Action 1973**

### A. Principle

Sample is digested with $H_2SO_4$ to convert org. N to $NH_3$, which is distd after alkalinization and detd by nesslerization or titrimetry. Preserve samples by addn of 40 mg $HgCl_2$/L and store at 4°. Analyze as soon as possible, as conversion of org. N to $NH_3$ may occur even with preservation.

Method is applicable to surface and saline waters and domestic and industrial wastes. Some industrial wastes contg materials such as amines, nitro compds, hydrazones, oximes, semicarbazones, and some refractory tertiary amines may not be converted to $NH_3$.

Natural $H_2O$ analyzed by 31 analysts in 20 laboratories showed the following results on exact increments of org. N:

| Method | Added, mg N/L | Std deviation | | Bias | |
|---|---|---|---|---|---|
| | | % | mg N/L | % | mg N/L |
| Colorm. | 0.20 | 100 | 0.20 | +15.5 | 0.03 |
| Colorm. | 0.31 | 81 | 0.25 | + 5.5 | 0.02 |
| Titr. | 4.10 | 26 | 1.06 | + 1.0 | 0.04 |
| Titr. | 4.61 | 26 | 1.19 | − 1.7 | −0.08 |

### B. Apparatus

(**a**) *Digestion apparatus.—See* **920.02B(a)**.

(**b**) *Distillation apparatus.—See* **920.02B(b)**; or use all-glass app. with 800 or 1000 mL distg flask and 500 mL g-s erlenmeyers, marked at 350 and 500 mL, as receivers. Prep. for use by distg mixt. of $NaOH-Na_2S_2O_3$ soln and $H_2O$ (1 + 1) until distillate is $NH_3$-free by Nessler reagent, (**j**). Repeat each time app. is out of service ≥4 hr.

(c) *Nessler tubes.*—Matched, ca 300 mm long, 17 mm id, and marked at 225 ± 1.5 mm inside measurement from bottom.

(d) *Spectrophotometer or filter photometer.*—For use at 425 nm.

### C. Reagents

(*Caution:* See safety notes on mercury, mercury salts, and toxic dusts.)

(a) *Water.*—Distd, $NH_3$-free. Pass thru ion exchange column of mixed strongly acidic cation and strongly basic anion exchange resins. Regenerate resins according to manufacturer's instructions.

(b) *Mercuric sulfate soln.*—Dissolve 8 g red HgO in 50 mL $H_2SO_4$ (1 + 5) and dil. to 100 mL with $H_2O$.

(c) *Digestion soln.*—Dissolve 267 g $K_2SO_4$ in 1300 mL $H_2O$ and add 400 mL $H_2SO_4$. Add 50 mL $HgSO_4$ soln, (**b**), and dil. to 2 L.

(d) *Sodium hydroxide-sodium thiosulfate soln.*—Dissolve 500 g NaOH and 25 g $Na_2S_2O_3.5H_2O$ in $H_2O$ and dil. to 1 L.

(e) *Phenolphthalein indicator soln.*—Dissolve 5 g phthln in 500 mL alcohol or isopropanol and add 500 mL $H_2O$. Add 0.02*N* NaOH until faint pink.

(f) *Sulfuric acid std soln.*—0.02*N*. Prep. and stdze as in **890.01**. 1.00 mL = 0.28 mg N.

(g) *Ammonia std solns.*—(*1*) *Stock soln.*—1.00 mg N/mL. Dissolve 3.819 g $NH_4Cl$ in $H_2O$ and dil. to 1 L. (*2*) *Working soln.*—0.01 mg N/mL. Dil. 10 mL stock soln to 1 L.

(h) *Boric acid soln.*—Dissolve 20 g $H_3BO_3$ in $H_2O$ and dil. to 1 L.

(i) *Mixed indicator.*—Mix 2 vols 0.2% alc. Me red with 1 vol. 0.2% alc. methylene blue. Prep. fresh every 30 days. SDA 3-A or 30 denatured alcohol may be used.

(j) *Nessler reagent.*—Dissolve 100 g $HgI_2$ and 70 g KI in small amt $H_2O$. Add slowly, with stirring, to cooled soln of 160 g NaOH in 500 mL $H_2O$, and dil. to 1 L. Reagent is stable 1 year if stored in Pyrex container out of direct sunlight. Reagent should give characteristic color, but no ppt, with 0.04 mg $NH_3$-N in 50 mL $H_2O$ within 10 min.

### D. Digestion and Distillation

Det. sample size as follows:

| *mg N/L* | *mL sample* |
|---|---|
| 0– 5 | 500 |
| 5– 10 | 250 |
| 10– 20 | 100 |
| 20– 50 | 50.0 |
| 50–100 | 25.0 |

Place sample, or residue from $NH_3$ detn (for org. Kjeldahl N only), into 800 mL Kjeldahl flask. Dil., if necessary, to 500 mL and add 100 mL digestion soln, (**c**). Boil until $SO_3$ fumes are evolved and soln becomes colorless or pale yellow. Cool, and dil. with 300 mL $H_2O$. Add NaOH-$Na_2S_2O_3$ soln slowly down neck of tilted flask to underlay acid soln in amt sufficient to make final soln strongly alk. as shown by phthln (60 mL NaOH-$Na_2S_2O_3$ soln will neutze 20 mL $H_2SO_4$). Connect flask to condenser, with tip of condenser dipping into 50 mL 2% $H_3BO_3$ soln in 500 mL g-s erlenmeyer. If soln is to be titrd, 100 or 200 mL $H_3BO_3$ may be used. Mix solns and distil 300 mL at 6–10 mL/min. If $NH_3$ concn is ≥1 mg/L, det. titrimetrically, **973.48E**; if less, det. colorimetrically, **973.48F**.

### E. Titrimetric Determination

Add 3 drops mixed indicator, (**i**), to distillate and titr. with 0.02*N* $H_2SO_4$, (**f**), matching end point against blank contg same vol. $NH_3$-free $H_2O$, $H_3BO_3$ soln, and indicator.

mg Total N/L = [(mL std $H_2SO_4$ for sample − mL std $H_2SO_4$ for reagent blank) × normality std $H_2SO_4$ × 14.01 × 1000]/mL sample digested

### F. Colorimetric Determination

Prep. series of stds contg 0.0, 0.2, 0.5, 1.0, 1.5, 2.0, 3.0, and 4.0 mL $NH_3$ working std soln, (**g**)(*2*), dild to 50 mL with $NH_3$-free $H_2O$ (contains 0.0, 0.04, 0.10, 0.20, 0.30, 0.40, 0.60, and 0.80 mg $NH_3$ N/L). Add 1 mL Nessler reagent, (**j**), and mix. After 20 min, read *A* at 425 nm against 0.0 (blank) std, and plot *A* against concn to obtain std curve. Distil 1 or more high and low std solns daily to ensure adequate recoveries.

As estd by preliminary detn, det. $NH_3$ in 50 mL aliquot, or aliquot dild to 50 mL, as above, and read $NH_3$ concn from std curve.

mg Total N/L = [(mg $NH_3$-N from curve × 1000)/ mL sample taken for distn] × (mL final distillate, including $H_3BO_3$ soln/ mL distillate taken for nesslerization)

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). Method Study No. 2; Nutrient Analyses, Manual Methods, 1970 (available from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, NTIS PB230828/BE). JAOAC **56**, 295(1973).

CAS-7727-37-9 (nitrogen)

## 973.49 Nitrogen (Ammonia) in Water

**Colorimetric Method**

**First Action 1973**

### A. Principle

Sample buffered at pH 9.5 is distd into $H_3BO_3$ soln. Depending upon concn, $NH_3$ is detd colorimetrically (0.05–1.0 mg N/L) by nesslerization or titrimetry (1.0–25 mg N/L). Hg, if present as preservative, and residual Cl must be removed by addn of $Na_2S_2O_3$ before distn.

Method is applicable to surface and saline waters and domestic and industrial wastes. A number of volatile amines will cause turbidity with Nessler reagent. Some volatile compds, such as certain ketones, aldehydes, and alcohols, may cause off color on nesslerization. Some of these, such as HCHO, may be eliminated by boiling at pH 2–3 before distn. Volatile compds, such as hydrazine, influence titrimetric results.

Natural and distd $H_2O$ analyzed by 24 analysts in 16 laboratories showed the following results on exact increments of ammonium salt:

| | | | Std deviation | | Bias | |
|---|---|---|---|---|---|---|
| Method | Type | Added, mg N/L | % | mg N/L | % | mg N/L |
| Colorm. | Distd. | 0.21 | 58 | 0.122 | − 5.54 | −0.01 |
| Colorm. | Nat. | 0.26 | 27 | 0.070 | −18.12 | −0.05 |
| Titr. | Distd. | 1.71 | 14 | 0.244 | + 0.46 | +0.01 |
| Titr. | Nat. | 1.92 | 15 | 0.279 | − 2.01 | −0.04 |

### B. Apparatus

*See* **973.48B**(**b**), (**c**), and (**d**).

### C. Reagents

*See* **973.48C**(**a**), (**f**)–(**j**), and following:

(a) *Borate buffer.*—pH 9.5. Add 88 mL 0.1*N* NaOH to 500 mL 0.025*M* $Na_2B_4O_7$ (5.0 g anhyd. salt/L), and dil. to 1 L.

(**b**) *Sodium hydroxide soln.*—1*N*. Dissolve 40 g NaOH in $NH_3$-free $H_2O$ and dil. to 1 L.

(**c**) *Dechlorinating reagent.*—Dissolve 3.5 g $Na_2S_2O_3$ in $NH_3$-free $H_2O$ and dil. to 1 L. 1 mL will remove 0.5 mg residual Cl in 500 mL sample.

### D. Distillation

Add 500 mL $NH_3$-free $H_2O$ and few boiling chips previously treated with NaOH soln to Kjeldahl distg flask.

Adjust 400 mL sample to pH 9.5 with 1*N* NaOH, using pH meter or short range test paper. If sample contains residual Cl, remove by adding equiv. amt dechlorinating reagent, (**c**). Transfer to distg flask and add 25 mL buffer, (**a**). Distil 300 mL at 6–10 mL/min into 50 mL $H_3BO_3$ soln, **973.48C(h)**. Dil. distillate to 500 mL in receiving flask. Det. $NH_3$ in 50 mL aliquot as in colorimetric detn. If $NH_3$ concn is ≥1 mg/L, det. titrimetrically, **973.49E**; if less, det. colorimetrically, **973.49F**.

### E. Titrimetric Determination

Proceed as in **973.48E**, using remaining 450 mL distillate.

$$\text{mg } NH_3\text{-N/L} = (\text{mL } 0.02N\ H_2SO_4 \times 1000)/\text{equiv. mL sample in aliquot titrd}$$

### F. Colorimetric Determination

Proceed as in **973.48F**.

$$\text{mg } NH_3\text{-N/L} = (NH_3 \text{ concn from std curve} \times 1000)/(0.8 \times \text{mL distillate taken for detn})$$

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). Method Study No. 2; Nutrient Analyses, Manual Methods, 1970 (available from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, NTIS PB230828/BE). JAOAC **56,** 295(1973).

## 973.50 Nitrogen (Nitrate) in Water

**Brucine Colorimetric Method**

**First Action 1973**

### A. Principle

Nitrate ion reacts with brucine in $H_2SO_4$ at 100° to form colored compd whose *A* is measured at 410 nm. Temp. control of reaction is critical. Applicable to 0.1–2 mg $NO_3$-N/L in surface and saline waters and domestic and industrial wastes.

Org. matter developing color with $H_2SO_4$ and natural color are compensated for by blank; effect of salinity is compensated for by addn of NaCl. Strong oxidizing and reducing agents interfere. Det. presence of free Cl with *o*-tolidine reagent. Eliminate residual Cl by addn of $NaAsO_3$ soln. Effect of $Fe^{+2}$, $Fe^{+3}$, and $Mn^{+4}$ is negligible at <1 mg/L.

Natural $H_2O$ analyzed by 27 analysts in 15 laboratories showed the following results on exact increments of inorg. nitrate:

| Added, mg N/L | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | mg N/L | % | mg N/L |
| 0.16 | 58 | 0.092 | −6.8 | −0.01 |
| 0.19 | 44 | 0.083 | +8.3 | +0.02 |
| 1.08 | 23 | 0.245 | +4.1 | +0.04 |
| 1.24 | 17 | 0.214 | +2.8 | +0.04 |

### B. Apparatus

(**a**) *Spectrophotometer or filter photometer.*—Capable of accommodating 25 mm diam. tubes and measuring *A* at 410 nm.

(**b**) *Tubes.*—Matched tubes for conducting reaction and measuring *A*.

(**c**) *Racks.*—Neoprene, wire coated, evenly spaced, to permit uniform flow of bath $H_2O$ between tubes.

(**d**) *Water baths.*—(*1*) *100°.*—Boiling $H_2O$ bath of sufficient size so that when tubes are inserted, temp. drop is ≤1–2°. Should have tight-fit cover, preferably of gable construction, with circulator or stirrer to maintain uniform temp. *Uniform temp. control of this bath is critical.* (*Caution:* Check $H_2O$ bath to assure it is electrically grounded.) (*2*) *10–15°.*—For cooling tubes.

### C. Reagents

(**a**) *Water.*—Use distd or deionized $H_2O$ for prepn of all reagents and stds.

(**b**) *Salt soln.*—Dissolve 300 g NaCl in $H_2O$ and dil. to 1 L.

(**c**) *Sulfuric acid.*—13*N*. Carefully add 500 mL $H_2SO_4$ to 125 mL $H_2O$. Cool, and keep tightly stoppered.

(**d**) *Brucine-sulfanilic acid reagent.*—Dissolve 1 g brucine sulfate.$7H_2O$ and 0.1 g sulfanilic acid.$H_2O$ in 70 mL $H_2O$. Stored in dark bottle at 5°, soln is stable several months. Slowly developing pink does not affect usefulness.

(**e**) *Nitrate std solns.*—(*1*) *Stock soln.*—100 mg N/L. Dissolve 0.7218 g $KNO_3$ in $H_2O$ and dil. to 1 L. (*2*) *Working soln.*—1 mg/L. Dil. 10 mL stock soln to 1 L. Prep. fresh weekly.

### D. Determination

(*Caution:* See safety notes on pipets and sulfuric acid.)

Preserve samples with 40 mg $HgCl_2$/L and store at 4°. Adjust pH to ca 7 with HOAc (1 + 3) and, if necessary, filter thru 0.45 μm filter.

Prep. set of matched tubes for blanks, stds, and samples. If necessary to correct for color or for org. matter which will cause color on heating, add extra set of tubes to which all reagents except brucine will be added.

Pipet 10 mL sample, or aliquot dild to 10 mL, into sample tubes. For saline sample, add 2.0 mL 30% NaCl soln to samples, stds, and blank tubes. Swirl tubes and place in 0–10° bath. Pipet 10 mL 13*N* $H_2SO_4$ into each tube and swirl. Let all tubes come to thermal equilibrium. Pipet 0.5 mL brucine reagent to all tubes except color control tubes and swirl. Then place entire rack contg all tubes in boiling $H_2O$ bath for exactly 25 min. Remove rack and transfer to cold $H_2O$ bath and let cool to 20–25°. Dry tubes and read *A* against reagent blank at 410 nm.

Prep. set of stds contg 0.1–2 mg N/L and conduct stds along with samples. Color may not follow Beer's law. If necessary, subtract *A* of color controls from *A* of samples.

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). Method Study No. 2; Nutrient Analyses, Manual Methods, 1970 (available from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, NTIS PB230828/BE). JAOAC **56,** 295(1973).

## 973.51 Chloride in Water

**Mercuric Nitrate Method**

**First Action 1973**

### A. Principle

Chloride titrd with mercuric ions forms sol., slightly dissociated $HgCl_2$. In pH range 2.3–2.8, diphenylcarbazone indicates end point by forming purple complex with excess $Hg^{+2}$. Xylene cyanol FF serves as pH indicator and background color to facilitate end point detection. $NaHCO_3$ added to both blank and sample followed by const amt of $HNO_3$ added with indicators provides pH of 2.5 ± 0.1. Increasing strength of titrant and modifying indicator mixt. permits detn of high Cl concns common in waste water.

Br and I titr. as chloride. Chromate, $Fe^{+3}$, and $SO_3^{-2}$ interfere when present at >10 mg/L. Sulfites may be removed with 0.5–1 mL $H_2O_2$/50 mL sample. Methods are applicable to drinking, surface, and saline waters, and domestic and industrial wastes at all Cl concns. However, to avoid large titrn vols, use sample contg <20 mg Cl/50 mL.

Synthetic $H_2O$ samples analyzed by 42 analysts in 18 laboratories showed the following results on exact increments of Cl:

| Added, mg Cl/L | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | mg Cl/L | % | mg Cl/L |
| 17 | 9.1 | 1.54 | +2.2 | +0.4 |
| 18 | 7.3 | 1.32 | +3.5 | +0.6 |
| 91 | 3.2 | 2.92 | +0.1 | +0.1 |
| 97 | 3.3 | 3.16 | −0.5 | −0.5 |
| 382 | 3.1 | 11.7 | −0.6 | −2.3 |
| 398 | 3.0 | 11.8 | −1.2 | −4.7 |

### B. Reagents

(**a**) *Sodium chloride std soln.*—0.0141*N*. Dissolve 824.1 mg NaCl, dried at 140°, in Cl-free $H_2O$, and dil. to 1 L. 1 mL = 0.500 mg Cl.

(**b**) *Chlorine-free water.*—Redistd or deionized.

*For Low Chloride Concentration*

(**c**) *Indicator-acidifier reagent.*—(Neutzes 150 mg $CaCO_3$/L in 100 mL sample.) Dissolve, in order given, 250 mg *s*-diphenylcarbazone, 4.0 mL $HNO_3$, and 30 mg xylene cyanol FF in 100 mL alcohol or isopropanol. Store in dark bottle in refrigerator. For routine analysis of samples with very high or low alky, $HNO_3$ concn may be adjusted so that final pH when added to samples is 2.5 ± 0.1.

(**d**) *Mercuric nitrate std soln.*—0.0141*N*. Dissolve 2.3 g $Hg(NO_3)_2$ or 2.5 g $Hg(NO_3)_2.H_2O$ in 100 mL $H_2O$ contg 0.25 mL $HNO_3$. Dil. to just under 1 L. Stdze as in **973.51C(a)**, using 5.00 mL aliquot NaCl std soln, (**a**), and 10 mg $NaHCO_3$, dild to 100 mL. Adjust soln to exactly 0.0141*N* and perform final stdzn. Store in dark bottle away from light. 1 mL = 0.500 mg Cl.

*For High Chloride Concentration*

(**e**) *Mixed indicator.*—Dissolve 5 g *s*-diphenylcarbazone and 0.5 g bromophenol blue in 750 mL alcohol or isopropanol and dil. to 1 L with same solv.

(**f**) *Mercuric nitrate std soln.*—0.141*N*. Dissolve 25 g $Hg(NO_3)_2.H_2O$ in 900 mL $H_2O$ contg 5.0 mL $HNO_3$. Dil. to just under 1 L. Stdze as in **973.51C(b)**, using 25.00 mL aliquots NaCl std soln, (**a**), and 25 mL $H_2O$. Adjust soln to exactly 0.141*N* and perform final stdzn. Store in dark bottle away from light. 1 mL = 5.00 mg Cl.

### C. Determination

(**a**) *For low chloride (drinking water).*—To ≤100 mL sample contg ≤10 mg Cl, add 1.0 mL indicator-acidifier, (**c**). Color should be green-blue. If not, adjust pH of sample to 8 before addn of reagent. Titr. with 0.0141*N* $Hg(NO_3)_2$ to definite purple end point. (Soln becomes blue few drops before end point.) Det. blank by titrn of equal vol. $H_2O$ contg 10 mg $NaHCO_3$.

(**b**) *For high chloride.*—To 50.0 mL sample (5.00 mL if ≥5 mL titrant needed) in 150 mL beaker, add 0.5 mL mixed indicator, (**e**), and mix well. Color should be purple. Add 0.1*N* $HNO_3$ dropwise until just yellow. Titr. with 0.141*N* $Hg(NO_3)_2$ to first permanent dark purple. Det. blank by titrn of equal vol. $H_2O$.

(**c**) *Calculation.*—

$$\text{mg Cl/L} = [(\text{mL sample titrn} - \text{mL blank titrn}) \times \text{normality } Hg(NO_3)_2 \times 35{,}340]/\text{mL sample}$$

$$\text{mg NaCl/L} = (\text{mg Cl/L}) \times 1.65$$

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). FWPCA Method Study 1; Mineral and Physical Analyses, June 1969 (available from National Environmental Research Center, Environmental Protection Agency, Cincinnati, OH 45268). JAOAC **56,** 295(1973).

## 939.11 Fluoride in Water
### Colorimetric Method
### Final Action

### A. Reagents

(**a**) *Fluoride std soln.*—0.01 mg F/mL. Dissolve 2.21 g NaF (min. purity 98%) in 1 L $H_2O$. Dil. 10 mL of this soln to 1 L.

(**b**) *Thorium nitrate soln.*—Dissolve 0.25 g $Th(NO_3)_4.12H_2O$ or 0.2 g $Th(NO_3)_4.4H_2O$ in 1 L $H_2O$.

(**c**) *Alizarin red indicator.*—0.01% aq. soln Na alizarin sulfonate (alizarin red S).

(**d**) *Hydrochloric acid.*—Exactly 0.05*N*.

(**e**) *Sodium hydroxide soln.*—Exactly 0.05*N*.

(**f**) *Hydroxylamine hydrochloride soln.*—1.0 g/100 mL.

### B. Apparatus

(**a**) *Claisen flask.*—250 mL.

(**b**) *Nessler tubes.*—6 long-form 50 mL tubes with double optically plane disks fused to tubes. Match tubes for length and test for optical similarity as follows: Add ca 40 mL $H_2O$, 1 mL indicator, 2 mL 0.05*N* HCl, and $H_2O$ to mark on tube. To 1 tube add amt of $Th(NO_3)_4$ soln such that, after dilg to mark and mixing, color is barely changed to faint pink. Note amt of $Th(NO_3)_4$ soln used. Add same amt of $Th(NO_3)_4$ soln to each of remaining 5 tubes. Reject tubes showing detectable differences in shade or intensity.

*See also* **944.08C**.

### C. Preparation of Sample

If sample has odor of $H_2S$, oxidize with 0.1 mL 30% $H_2O_2$ soln before evapn.

Place 100 mL sample in porcelain or Pt dish, make alk. to phthln with 10% NaOH soln (avoid excess), and evap. to 20 mL over burner at temp. just below bp. During evapn keep sample alk. by adding small amts of 0.05*N* NaOH from time to time. Transfer the 20 mL evapd sample to Claisen flask contg glass beads or boiling tube previously rinsed with boiling 10% NaOH soln to eliminate all traces of gelatinous $SiO_2$ accumulating in flask.

Place flask contg sample on insulating board (15 × 15 × 0.6 cm with 2.5 cm center hole) over burner adjusted for medium flame. Close straight neck of flask with 2-hole rubber stopper thru which pass thermometer and stem of small separator with outlet constricted to 2 mm diam. (Adjust thermometer and outlet tube of separator to extend almost to bottom of flask.) Close other neck of flask with solid rubber stopper. (Alternatively, all-glass distn assembly may be used.)

Connect flask with $H_2O$ condenser; add 20 mL 60% $HClO_4$ (*Caution:* See safety notes on perchloric acid.) to flask, rinsing evapg dish and separator; then add amt of *satd $AgClO_4$ soln* that will ppt chlorides (detd previously by titrn with std $AgNO_3$ soln), and distil at 132 ± 3°, adding $H_2O$ dropwise thru separator to maintain temp. during distn. Collect nearly 200 mL distillate. Dil. to vol. (200 mL) and mix well. To det. acidity, use 40 mL distillate, add 1 mL indicator, mix thoroly, and note mL 0.05*N* NaOH required for neutzn.

Repeat prepn and distn, using 100 mL $H_2O$ in place of sample, to det. blank.

***D. Determination***

Prep. 1 std, 1 color comparison tube, and 1 or more sample tubes as follows:

(**a**) *Color comparison tube.*—To 40 mL $H_2O$ add 2 mL 0.05*N* HCl, 1 mL alizarin red indicator, 1 mL $NH_2OH.HCl$ soln, and enough $Th(NO_3)_4$ soln to give faint but definite pink end point. Compare all end point colors with this color.

(**b**) *Sample tube.*—To sample tube contg 40 mL distillate add 1 mL indicator, 1 mL $H_2NOH.HCl$ soln, and vol. 0.05*N* HCl such that total vol. acid in tube (acidity previously detd plus vol. 0.05*N* HCl added) equals 2 mL 0.05*N* HCl. Dil. to vol. and mix. If in preliminary acidity detn it is found that the 40 mL distillate requires >2 mL 0.05*N* NaOH soln for neutzn, do not add the HCl soln to sample tube, but add to std tube same amt of acid as was found present in sample tube. If 40 mL distillate requires >5 mL 0.05*N* NaOH, repeat distn under conditions favorable to low acidity. From 10 mL buret, graduated to 0.05 mL, add $Th(NO_3)_4$ soln with frequent mixing until faint pink appears, comparable to comparison tube, (**a**). Note vol. $Th(NO_3)_4$ soln used.

(**c**) *Std tube.*—To std tube contg 40 mL $H_2O$ add 1 mL indicator, 1 mL $H_2NOH.HCl$ soln, and ≥2 mL 0.05*N* HCl, as was required in sample tube in (**b**). If aliquot chosen for detn already contains 2–5 mL 0.05*N* acid, add exactly same amt to std tube. Add exactly same amt of $Th(NO_3)_4$ soln as was added to sample tube. To std tube (now more highly colored than sample tube), add std F soln from 10 mL buret with mixing until color matches that of sample tube. Dil. contents of both std and sample tubes to same vol. Mix soln in each tube and let all air bubbles escape before making color comparisons. Check end point by adding 1–2 drops std F soln to std tube. Distinct color change should develop.

***E. Calculation***

Subtract mL F soln required by blank from mL F soln required by sample.

$$\frac{\text{mL F soln} \times \text{mL total distillate} \times 10}{\text{mL aliquot titrd} \times \text{wt sample taken}} = \text{F (ppm)}$$

*Example:* 100 mL sample, evapd and distd to 200 mL, of which 40 mL aliquot corresponds to 5 mL F soln, gives:

$$(5 \times 200 \times 10)/(40 \times 100) = 2.5 \text{ F (ppm)}$$

Ref.: JAOAC **22**, 482(1939).

## 926.15★ Hydrogen Sulfide in Water

**Iodometric Method**

**Final Action**
**Surplus 1965**

*See* **31.016–31.017**, 10th ed.

## 920.194 Carbonate and Bicarbonate in Water

**Titrimetric Method**

**Final Action**

To 100 mL sample add few drops phthln, and if pink is produced, titr. with 0.05*N* HCl or $H_2SO_4$, adding drop every 2–3 sec until color disappears. Multiply buret reading by factor 3 to obtain mg $CO_3$ ion in 100 mL. To colorless soln from this titrn, or to original soln if no color is produced with phthln, add 1–2 drops Me orange, continue titrn without refilling buret, and note total reading. If $CO_3$ is absent, multiply total buret reading by factor 3.05 to obtain value of $HCO_3$ ion in mg/100 mL. If $CO_3$ is present, multiply reading with phthln by 2 and subtract from total reading of buret. Multiply difference by 3.05 to obtain $HCO_3$ ion in mg/100 mL. Express results as mg/L.

## 920.195 Silica in Water

**Gravimetric Method**

**Final Action**

Make preliminary examination, using 100–250 mL sample, to det. approx. amt of Ca and Mg present, in order to det. amt of sample to be evapd for final analysis.

Evap. amt of sample equiv. to 0.1–0.6 g CaO or 0.1–1 g $Mg_2P_2O_7$ (usually 1–5 L). Acidify sample with HCl and evap. on steam bath to dryness in Pt dish. Continue drying ca 1 hr. Thoroly moisten residue with 5–10 mL HCl. Let stand 10–15 min and add enough $H_2O$ to bring sol. salts into soln. Heat on steam bath until salts dissolve. Filter to remove most of $SiO_2$ and wash thoroly with hot $H_2O$. Evap. filtrate to dryness and treat residue with 5 mL HCl and enough $H_2O$ to dissolve sol. salts, as before. Heat, filter, and wash thoroly with hot $H_2O$. Designate filtrate as *Soln X*.

Transfer the two residues to Pt crucible, ignite, heat over blast lamp, and weigh. Moisten contents of crucible with few drops $H_2O$, add few drops $H_2SO_4$ and few mL HF, and evap. on steam bath under hood. Repeat treatment if all $SiO_2$ is not volatilized. Dry carefully on hot plate, ignite, heat over blast lamp, and weigh. Difference between the two wts is wt $SiO_2$. Add wt residue ($Fe_2O_3$ + $Al_2O_3$) to that of $Al_2O_3$ and $Fe_2O_3$ obtained in **920.196**. (If residue weighs >0.5 mg, $BaSO_4$ may be present in sample. If so, make necessary correction and add to wt $Fe_2O_3$ and $Al_2O_3$ in **920.196**.)

## 920.196 Aluminum and Iron in Water

**Gravimetric Method**

**Final Action**

Conc. *Soln X*, **920.195**, to 200 mL; while still hot, slowly add $NH_4OH$, stirring constantly, until alk. to Me orange. Boil, filter, and wash 3 times with hot $H_2O$. Dissolve ppt in hot HCl (1 + 1). Dil. to ca 25 mL, boil, and again ppt with $NH_4OH$. Filter, wash thoroly with hot $H_2O$, dry, ignite, and weigh as $Al_2O_3$ and $Fe_2O_3$. (In presence of $H_3PO_4$, wt of this residue must be corrected for $P_2O_5$ equiv. to $H_3PO_4$ found in **973.55E**, allowing for difference in vols of the water used for these detns.) Designate filtrate as *Soln Y*.

CAS-7429-90-5 (aluminum)
CAS-7439-89-6 (iron)

## 920.197 Iron in Water

**Final Action**

### A. Colorimetric Method

(Iron <1 mg; not applicable in presence of phosphates)

Fuse, in Pt crucible, ignited ppt of $Fe_2O_3$ and $Al_2O_3$, **920.196**, with fused $KHSO_4$, dissolve in $H_2O$, and ppt Fe and Al with $NH_4OH$. Filter, dissolve ppt on filter paper in HCl and $HNO_3$, dil. soln, add 3 mL 5% $NH_4SCN$ soln, dil. to suitable vol., and compare color developed with that of calibrated color disks or stds contg known amts of Fe treated similarly.

### B. Titrimetric Method

(*Caution:* See safety notes on hydrogen sulfide.)

Fuse residue of $Fe_2O_3$ and $Al_2O_3$, **920.196**, in Pt crucible with ca 1 g fused $KHSO_4$. (Fusion takes only few min, and must not be continued beyond time actually needed.) When fusion is complete, set crucible aside to cool. Add $H_2SO_4$ (1 + 4) and heat crucible until fused mass dissolves. Evap. on steam bath as far as possible; then heat gradually until copious fumes of $SO_3$ evolve. Dissolve in $H_2O$ and let stand on steam bath. Cool, transfer to erlenmeyer, and dil. to such vol. that soln contains ≤2.5% free $H_2SO_4$.

Pass *$H_2S$* thru soln to reduce Fe and ppt any Pt contaminating residue from fusion. (Zn may be used instead of $H_2S$ for reducing Fe.) Filter, wash, and again pass $H_2S$ thru soln to reduce all Fe. Expel $H_2S$ by boiling, at same time passing current of *$CO_2$* thru soln. Test escaping gas with $Pb(OAc)_2$ paper to confirm complete removal of $H_2S$. Discontinue boiling and let flask cool without discontinuing current of $CO_2$. Titr. reduced Fe with std $KMnO_4$ soln (1 mL = 1 mg Fe) and calc. as Fe.

CAS-7439-89-6 (iron)

## 973.52 Hardness of Water

**First Action 1973**

### A. Calculation Method

Calc. hardness as sum of $CaCO_3$ equivs (mg/L) obtained by multiplying concn (mg/L) found of following cations by factor shown:

| Cation | Factor | Cation | Factor |
|---|---|---|---|
| Ca | 2.497 | Al | 5.564 |
| Mg | 4.116 | Zn | 1.531 |
| Sr | 1.142 | Mn | 1.822 |
| Fe | 1.792 | | |

#### EDTA Titrimetric Method

### B. Principle

Ca and Mg at pH 10 in presence of dye eriochrome black T are wine red. When completely complexed with EDTA, soln becomes blue. Mg must be present for satisfactory end point and is added as MgEDTA. End point sharpness increases with pH, but high pH may cause pptn of $Ca(OH)_2$ or $Mg(OH)_2$ and cause color changes of dye. pH of 10.0 ± 0.1 is satisfactory compromise. Limit of 5 min for titrn minimizes pptn. Heavy metal interference is minimized by complexing with cyanide.

Method is applicable to drinking and surface waters and domestic and industrial wastes. To avoid large titrn vols, use aliquot contg <25 mg $CaCO_3$.

Synthetic $H_2O$ samples contg exact increments of Ca and Mg salts analyzed by 43 analysts in 19 laboratories showed the following results:

| Increment, total hardness as mg $CaCO_3$/L | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | mg $CaCO_3$/L | % | mg $CaCO_3$/L |
| 31 | 9.4 | 2.9 | −0.87 | − 0.003 |
| 33 | 7.6 | 2.5 | −0.73 | − 0.24 |
| 182 | 2.7 | 4.9 | −0.19 | − 0.4 |
| 194 | 1.5 | 3.0 | −1.04 | − 2.0 |
| 417 | 2.3 | 9.7 | −3.35 | −13.0 |
| 444 | 2.0 | 8.7 | −3.23 | −14.3 |

### C. Reagents

(**a**) *Buffer soln.*—Dissolve 16.9 g $NH_4Cl$ in 143 mL $NH_4OH$, add 1.25 g MgEDTA, and dil. to 250 mL with $H_2O$. (1.179 g $Na_2$ EDTA.$2H_2O$ and 0.780 g $MgSO_4.7H_2O$ or 0.644 g $MgCl_2.6H_2O$ dissolved in 50 mL $H_2O$ may be substituted for 1.25 g MgEDTA.) Store in tightly stoppered Pyrex or plastic bottle. Dispense from bulb-operated pipet. Discard after 1 month or when 1–2 mL added to sample fails to produce pH 10.0 ± 0.1 at end point of titrn.

(**b**) *Indicator.*—Mix 0.5 g eriochrome black T and 100 g NaCl to prep. dry powd mixt. If end point change is not clear and sharp, prep. new mixt.

(**c**) *EDTA std soln.*—0.01*M*. Weigh 3.723 g $Na_2EDTA.2H_2O$ and dil. to 1 L with $H_2O$. Stdze against Ca std soln as in **973.52D**. Store in polyethylene bottle and restdze periodically.

(**d**) *Calcium std soln.*—1.000 mg $CaCO_3$/mL. Weigh 1.000 g $CaCO_3$ (primary std or special reagent low in heavy metals, alkalis, and Mg) into 500 mL erlenmeyer. Place funnel in neck and add, little at a time, HCl (1 + 1) until all $CaCO_3$ has dissolved. Add 200 mL $H_2O$ and boil few min to expel $CO_2$. Cool, add few drops Me red indicator, and adjust to intermediate orange with 3*N* $NH_4OH$ or HCl (1 + 1), as required. Transfer quant. to 1 L vol. flask and dil. to vol.

### D. Determination

Dil. 25 mL sample (or such vol. as to require <15 mL titrant) to ca 50 mL with $H_2O$ in porcelain casserole, add 1–2 mL buffer soln, 250 mg NaCN (pH of soln should be 10 ± 0.1), and ca 200 mg indicator powder, and titr. with EDTA std soln slowly, with continuous stirring, until last reddish tinge disappears, adding last few drops at 3–5 sec intervals. Color at end point is blue in daylight and under daylight fluorescent lamp. Complete titrn within 5 min from time of buffer addn.

For waters of low hardness (<5 mg/L), use 100–1000 mL sample, proportionately larger amts of reagents, microburet, and blank of distd $H_2O$ equal to sample vol.

$$\text{Hardness (EDTA) as mg } CaCO_3/L = T \times B \times 1000/\text{mL sample}$$

where $T$ = mL EDTA std soln and $B$ = mg $CaCO_3$ equiv. to 1.00 mL EDTA std soln.

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). FWPCA Method Study 1; Mineral and Physical Analyses, June 1969 (available from National Environmental Research Center, Environmental Protection Agency, Cincinnati, OH 45268). JAOAC **56,** 295(1973).

## 920.198 Aluminum in Water
### Gravimetric Method
### Final Action

To obtain wt $Al_2O_3$, in absence of phosphates, subtract from wt $Fe_2O_3$ and $Al_2O_3$, **920.196**, the Fe, **920.197A** or **B**, calcd as $Fe_2O_3$. Calc. as Al.

CAS-7429-90-5 (aluminum)

## 920.199 Calcium in Water
### Gravimetric Method
### Final Action

Conc. *Soln Y*, **920.196**, to 150–200 mL, and to this soln, contg equiv. of $\leq$0.6 g CaO or 1 g $Mg_2P_2O_7$, add 1–2 g $H_2C_2O_4.2H_2O$ and enough HCl (1 + 1) to clear soln. Heat to bp and neutze with $NH_4OH$, stirring constantly. Add $NH_4OH$ in slight excess and let stand 3 hr in warm place. Filter supernate and wash ppt once or twice by decantation with 1% $(NH_4)_2C_2O_4$ soln. Dissolve ppt in HCl (1 + 1), dil. to 100–200 mL, add little more $H_2C_2O_4$, and ppt as above. After letting ppt stand 3 hr, filter, wash with 1% $(NH_4)_2C_2O_4$ soln, dry, ignite, heat over blast lamp at $\geq$950°, and weigh as CaO and SrO. From this wt subtract wt SrO equiv. to the Sr, **911.03**. Difference is wt CaO. Calc. as Ca. Designate combined filtrates and washings as *Soln Z*.

As check on CaO, evap. to dryness filtrate from the $Sr(NO_3)_2$ in **911.03**, beginning "Filter, and wash with ether-alcohol mixt. . . ." Dissolve the $Ca(NO_3)_2$ in $H_2O$, ppt as oxalate, filter, wash, ignite at 950°, and weigh as CaO. CaO × 0.7147 = Ca.

CAS-7440-70-2 (calcium)

## 911.03 Strontium in Water
### Spectroscopic Method
### Final Action

Dissolve oxides, **920.199**, in $HNO_3$ (1 + 1) and test with spectroscope for Sr. If Sr is present, transfer $HNO_3$ soln to small erlenmeyer. Evap. nearly to dryness over low flame, and heat 1–2 hr at 150–160° after $H_2O$ is evapd. Break up dried material with stirring rod and add 10–15 mL mixt. of absolute alcohol and ether (1 + 1) to dissolve the $Ca(NO_3)_2$. Cork flask and let stand with frequent shaking $\geq$2 hr. Decant soln thru 5.5 cm filter, reserving filtrate. Wash residue several times by decantation with small portions of the ether-alcohol mixt. Dry residue and paper, and repeatedly wash paper with small portions of hot $H_2O$, collecting filtrate in flask contg main portion of $Sr(NO_3)_2$ residue. Add 1 or 2 drops $HNO_3$ (1 + 1), evap., dry, pulverize, and treat with 10–15 mL ether-alcohol mixt. Cork flask and let stand ca 12 hr, shaking occasionally.

Filter, and wash with ether-alcohol mixt. until few drops filtrate evapd on watch glass leave practically no residue. Dry paper and ppt. Dissolve $Sr(NO_3)_2$ in few mL hot $H_2O$. Add few drops $H_2SO_4$ and then add vol. alcohol equal to vol. soln and let stand 12 hr. Filter, ignite, weigh as $SrSO_4$, and calc. to Sr. Test spectroscopically for Ca and Ba. If these elements are present, det. amt and make necessary correction.

Refs.: Chem. Ztg. **35**, 337(1911). JAOAC **1**, 97, 458(1915); **2**, 113(1916).

CAS-7440-24-6 (strontium)

## 920.200 Magnesium in Water
### Gravimetric Method
### Final Action

Conc. *Soln Z*, **920.199**, to 200 mL, acidify with HCl (1 + 1), and add 2–3 g $(NH_4)_2HPO_4$ and enough HCl (1 + 1) to produce clear soln when all $(NH_4)_2HPO_4$ is dissolved. When cold, make slightly alk. with $NH_4OH$, stirring constantly. Add 2 mL excess of $NH_4OH$ and let stand ca 12 hr. Filter supernate and wash 4 times by decantation with $NH_4OH$ (1 + 10). Dissolve ppt in HCl (1 + 1), dil. to ca 150 mL, add little $(NH_4)_2HPO_4$, and ppt with $NH_4OH$ as before. Let stand 12 hr, filter, wash Cl-free with $NH_4OH$ (1 + 10), place in porcelain crucible, ignite, heat over blast lamp, and weigh as $Mg_2P_2O_7$. Calc. to Mg. $Mg_2P_2O_7 \times 0.21842 = Mg$.

CAS-7439-95-4 (magnesium)

## 974.27 Cadmium, Chromium, Copper, Iron, Lead, Magnesium, Manganese, Silver, Zinc in Water
### Atomic Absorption Spectrophotometric Method
### First Action 1974
### Final Action 1984

***A. Principle***

Metals in soln are detd directly by AA spectrophotometry; suspended metals are sepd by membrane filtration, or suspension is dissolved and analyzed; Pb and Cd in low concn are chelated, concd, and then extd with org. solv. prior to AA detn. Applicable to surface and saline waters, and domestic and industrial wastes. Three synthetic water samples contg between 0.05 and 1.0 mg each metal/L analyzed by 8–23 laboratories showed results given in Table **974.27A**.

**Table 974.27A Bias and Standard Deviations of Determination of Metals by Atomic Absorption**

| Metal | Added, mg/L | Std deviation % | Std deviation mg/L | Bias % | Bias mg/L |
|---|---|---|---|---|---|
| Cd | 0.01 | 53 | 0.007 | +27.5 | +0.003 |
| | 0.01 (extn) | 61 | 0.006 | 0.0 | 0.0 |
| | 0.05 | 8 | 0.004 | + 2.0 | +0.001 |
| | 0.05 (extn) | 10 | 0.005 | + 1.2 | +0.001 |
| | 0.10 | 8 | 0.008 | + 3.4 | +0.003 |
| | 0.10 (extn) | 52 | 0.045 | −15.0 | −0.015 |
| Cr | 0.05 | 26 | 0.013 | − 2.3 | −0.001 |
| | 0.10 | 22 | 0.021 | − 2.9 | −0.003 |
| | 0.20 | 12 | 0.024 | − 3.0 | −0.006 |
| Cu | 0.05 | 42 | 0.023 | + 8.3 | +0.004 |
| | 0.25 | 8 | 0.020 | + 2.2 | +0.006 |
| | 1.00 | 6 | 0.060 | + 0.6 | +0.006 |
| Fe | 0.10 | 34 | 0.032 | − 5.3 | −0.005 |
| | 0.30 | 18 | 0.050 | − 5.0 | −0.015 |
| | 0.50 | 6 | 0.031 | + 1.1 | +0.006 |
| Pb | 0.05 | 76 | 0.036 | − 5.0 | −0.002 |
| | 0.05 (extn) | 53 | 0.028 | + 3.0 | +0.002 |
| | 0.10 | 67 | 0.057 | −16.0 | −0.016 |
| | 0.10 (extn) | 55 | 0.053 | − 5.0 | −0.005 |
| | 0.20 | 30 | 0.052 | −14.0 | −0.028 |
| | 0.20 (extn) | 48 | 0.088 | − 8.0 | −0.017 |
| Mg | 0.05 | 10 | 0.006 | + 8.5 | +0.004 |
| | 0.10 | 10 | 0.011 | + 8.2 | +0.008 |
| | 0.20 | 7 | 0.014 | + 5.0 | +0.010 |
| Mn | 0.05 | 14 | 0.007 | + 6.0 | +0.003 |
| | 0.25 | 12 | 0.030 | + 4.4 | +0.011 |
| | 0.50 | 8 | 0.043 | + 1.3 | +0.007 |
| Ag | 0.05 | 17 | 0.010 | +10.6 | +0.005 |
| | 0.10 | 11 | 0.010 | − 7.1 | −0.007 |
| | 0.20 | 8 | 0.016 | + 7.3 | +0.015 |
| Zn | 0.05 | 46 | 0.021 | − 9.3 | −0.005 |
| | 0.50 | 3 | 0.016 | + 1.4 | +0.007 |
| | 1.00 | 5 | 0.051 | − 0.1 | −0.001 |

### B. Apparatus

(Use Pyrex, quartz, or Teflon labware exclusively; clean thoroly with detergent and $H_2O$; soak in $HNO_3$ (1+1) for 1 week; rinse with $H_2O$, dil. $HNO_3$, and $H_2O$, in that order. Use deionized, distd $H_2O$ whenever $H_2O$ is specified.)

*Atomic absorption spectrophotometer.*—Spectrophtr capable of operating at conditions given in Table **974.27B**. Operator must become familiar with settings and operations of his app., using table only as guide. Use Boling burner for aq. solns, and premix burner with solv. (*Caution:* See safety notes on AAS.)

### C. Reagents

(**a**) *Deionized distilled water.—See* **973.48C(a)**.

(**b**) *Nitric acid.*—Dil. 500 mL redistd $HNO_3$ to 1 L with $H_2O$. (*Caution:* Perform distn in hood with protective sash in place.)

(**c**) *Hydrochloric acid.*—Dil. 500 mL HCl to 1 L with $H_2O$ and distil in all-Pyrex app.

(**d**) *Metal std solns.*—(*1*) *Stock solns.*—Accurately weigh amt of metal specified in Table **974.27C** into beaker and add dissolving medium. When metal is completely dissolved, transfer quant. to 1 L vol. flask and dil. to vol. with $H_2O$. (*2*) *Working solns.*—Prep. daily. Dil. aliquots of stock solns with $H_2O$ to make $\geq$4 std solns of each element within range of detn, Table **974.27B**. Add 1.5 mL $HNO_3$/L to all working std solns before dilg to vol. Add 1 mL $LaCl_3$/10 mL Mg working std soln.

(**e**) *Lanthanum stock soln.*—50 g La/L ca 5% HCl. Slowly add 250 mL HCl to 58.65 g $La_2O_3$ (99.99%, Ventron Corp., Alfa Products, 8 Congress St, Beverly, MA 01915, or equiv.), dissolve, and dil. to 1 L.

(**f**) *Ammonium pyrrolidine dithiocarbamate (APDC) soln.*—Dissolve 1 g APDC in 100 mL $H_2O$. Prep. fresh daily.

### D. Preparation of Sample

(**a**) *Dissolved metals.*—As soon as practicable after collection, filter known vol. sample thru 0.45 μm membrane. Use first 50–100 mL to rinse flask and discard. Collect filtrate and preserve soln by adding 3 mL $HNO_3$ (1+1)/L.

(**b**) *Suspended metals.*—Transfer residue and membrane from (**a**) to 250 mL beaker and add 3 mL $HNO_3$. Cover with watch glass and heat gently to dissolve membrane. Increase heat and evap. to dryness. Cool, and add 3 mL $HNO_3$, and heat until digestion is complete, generally indicated by light colored residue. Add 2 mL HCl (1+1), and heat gently to dissolve residue. Wash watch glass and beaker with $H_2O$ and filter. Wash filter and discard. Dil. filtrate with $H_2O$ to concn within range of instrument.

(**c**) *Total metal.*—Transfer aliquot of well mixed sample to beaker and add 3 mL $HNO_3$. Heat, and evap. to dryness. (Do not boil.) Continue as in (**b**), beginning "Cool, and add 3 mL $HNO_3$, . . ."

**Table 974.27B Operating Parameters**

| Metal | Wavelength, nm | Flame | Optimum range, mg/L |
|---|---|---|---|
| Cd | 328.1 | Oxidizing air–$C_2H_2$ | 0.1 – 2 |
| Cr | 357.9 | Sl. reducing air–$C_2H_2$ | 1 –200 |
| Cu | 324.7 | Oxidizing air–$C_2H_2$ | 0.1 – 10 |
| Fe | 248.3 | Oxidizing air–$C_2H_2$ | 0.1 – 20 |
| Pb | 217.0 | Sl. oxidizing air–$C_2H_2$ | 1 – 10 |
| Mg[a] | 285.2 | Reducing air–$C_2H_2$ | 0.01– 2 |
| Mn | 279.5 | Oxidizing air–$C_2H_2$ | 0.1 – 20 |
| Ag | 328.1 | Oxidizing air–$C_2H_2$ | 0.1 – 20 |
| Zn | 213.9 | Oxidizing air–$C_2H_2$ | 0.1 – 2 |

[a] With 1% La soln.

**Table 974.27C Preparation of Metal Standard Solutions**

| Metal[a] | Wt, g | Compd | Dissolving medium (1 L total) |
|---|---|---|---|
| Cd | 1.142 | CdO | 5 mL redistd $HNO_3$ |
| Cr | 1.923 | $CrO_3$ | $H_2O$ + 10 mL redistd $HNO_3$ |
| Cu | 1.000 | Cu, electrolytic | 5 mL redistd $HNO_3$ |
| Fe | 1.000 | Fe wire | 5 mL redistd $HNO_3$ |
| Pb | 1.599 | $Pb(NO_3)_2$ | $H_2O$ + 10 mL redistd $HNO_3$ |
| Mg | 0.829 | MgO | 10 mL redistd $HNO_3$[b] |
| Mn | 1.583 | $MnO_2$ | 10 mL HCl |
| Ag | 1.575 | $AgNO_3$ | $H_2O$ + 10 mL redistd $HNO_3$ |
| Zn | 1.000 | Zn | 10 mL $HNO_3$ |

[a] Final concn = 1000 mg/L except for Mg (500 mg/L).
[b] Add 1 mL La stock soln to 10 mL working std soln.

### E. Determination

(P interference in Mg detn is eliminated by adding La stock soln to sample and working std solns so that final dilns contain 1% La.)

(**a**) *General method.*—Set up instrument as in Table **974.27B**, or previously established optimum settings. Secondary or less sensitive lines (*Spectrochim Acta* **17**, 710(1961)) may be used to reduce necessary diln, if desired. Read 4 std solns within range before and after each group of 6–12 samples, and re-establish 0 *A* each time. Prep. calibration curve from av. of each std before and after sample group. Read sample concn from plot of *A* against mg/L.

(**b**) *Special extraction method.*—When Pb or Cd concn is too low for direct detn, transfer sample aliquot to 250 mL beaker and dil. to 100 mL with $H_2O$. Prep. blank and stds in same manner. Adjust pH of sample and std solns to 2.5 with HCl, using pH meter. Transfer quant. to 200 mL vol. flask, add 2.5 mL APDC soln, and mix. Add 10 mL methyl isobutyl ketone and shake vigorously 1 min. Let layers sep.; then add $H_2O$ until ketone layer is in neck of flask. (Centrfg may be necessary.) Aspirate ketone layer and record readings of stds and samples against blank. (Fuel-to-air ratio should be adjusted to as blue a flame as possible, since org. solv. adds to fuel supply.) Prep. calibration curve from av. of each std and read sample concn from plot (mg/L).

### F. Calculations

(**a**) *General method.*—

$$\text{mg Metal/L} = (\text{mg metal in aliquot/L}) \times F$$

where $F$ = final diln/mL aliquot.

(**b**) *Special extraction method.*—

$$\text{mg Metal/L} = \text{mg metal in aliquot/L}$$

Refs.: Water Metals No. 4, Study No. 30 (1968), Analytical Reference Service, Public Health Service (available from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, NTIS PB215673/BE). JAOAC **67**, 421(1984).

CAS-7440-43-9 (cadmium)
CAS-7440-47-3 (chromium)
CAS-7440-50-8 (copper)
CAS-7439-89-6 (iron)
CAS-7439-92-1 (lead)
CAS-7439-95-4 (magnesium)
CAS-7439-96-5 (manganese)
CAS-7440-22-4 (silver)
CAS-7440-66-6 (zinc)

## 977.22 Mercury in Water

### Flameless Atomic Absorption Spectrophotometric Method

**First Action 1977**
**Final Action 1978**

### *A. Principle*

Org. Hg is oxidized to inorg. Hg by $KMnO_4$, $K_2S_2O_8$, and heat. The Hg is reduced to elemental state with stannous ion, and Hg is aerated from soln thru measuring cell in closed system. *A* is measured in AA spectrophtr.

Method is applicable to detn of 0.2–10 μg Hg/L of drinking, surface, and saline waters and domestic and industrial wastes. Interference from Cl or ≤20 mg S/L is eliminated by oxidn with $KMnO_4$; 10 mg Cu/L does not interfere. Analysis without reagents will det. if absorbing interfering volatile org. compds are present.

Natural waters analyzed by 76–82 laboratories showed the following results on exact increments of org. and inorg. Hg compds:

| Added, μg Hg/L | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | μg Hg/L | % | μg Hg/L |
| 0.21 | 79 | 0.28 | +66 | +0.14 |
| 0.27 | 67 | 0.28 | +53 | +0.14 |
| 0.51 | 79 | 0.54 | +32 | +0.16 |
| 0.60 | 55 | 0.39 | +18 | +0.11 |
| 3.4 | 44 | 1.5 | + 0.34 | 0.0 |
| 4.1 | 29 | 1.1 | − 7.1 | −0.3 |
| 8.8 | 42 | 3.7 | − 0.4 | 0.0 |
| 9.6 | 39 | 3.6 | − 5.2 | −0.5 |

### *B. Apparatus*

(Rinse all glassware with chromic acid cleaning soln or $HNO_3$ (1 + 1) and Hg-free $H_2O$ before use.)

(**a**) *Atomic absorption spectrophotometer.*—Equipped with Hg hollow cathode lamp and gas flow-thru cell (Fig. **977.22**), 115 × 25 (id) mm with quartz windows cemented in place. Use at 253.7 nm with operating conditions specified by manufacturer.

(**b**) *Air pump.*—Peristaltic pump capable of delivering ca 1 L air/min. Use Tygon tubing for all connections.

(**c**) *Flowmeter.*—Capable of measuring air flow of 1 L/min.

(**d**) Lamp.—Small reading lamp contg 60 watt bulb to prevent condensation of moisture inside cell. Position lamp to warm cell (*See* Fig. **977.22.**). Alternatively, use $Mg(ClO_4)_2$-filled drying tube, **977.22C(a)**.

(**e**) *Digestion flask.*—250 mL flat-bottom boiling flask fitted with 2-hole rubber stopper. Use straight glass frit of coarse porosity for gas inlet.

### *C. Reagents*

(**a**) *Magnesium perchlorate.*—Place 20 g $Mg(ClO_4)_2$ in 150 × 18 (id) mm drying tube (Fig. **977.22**); replace as needed. (*Caution:* See safety notes on magnesium perchlorate.)

(**b**) *Mercury absorbing media.*—Add one of following to 250 mL gas-washing bottle fitted with 2-hole rubber stopper and attach to aeration app. as by-pass (*See* Fig. **977.22**): (*1*) *Potassium permanganate-sulfuric acid soln.*—Equal vols 0.1*N* $KMnO_4$ and $H_2SO_4$ (1 + 9). (*2*) *Iodine-potassium iodide soln.*—0.25% $I_2$-3% KI soln. Alternatively, vent Hg vapor into exhaust hood.

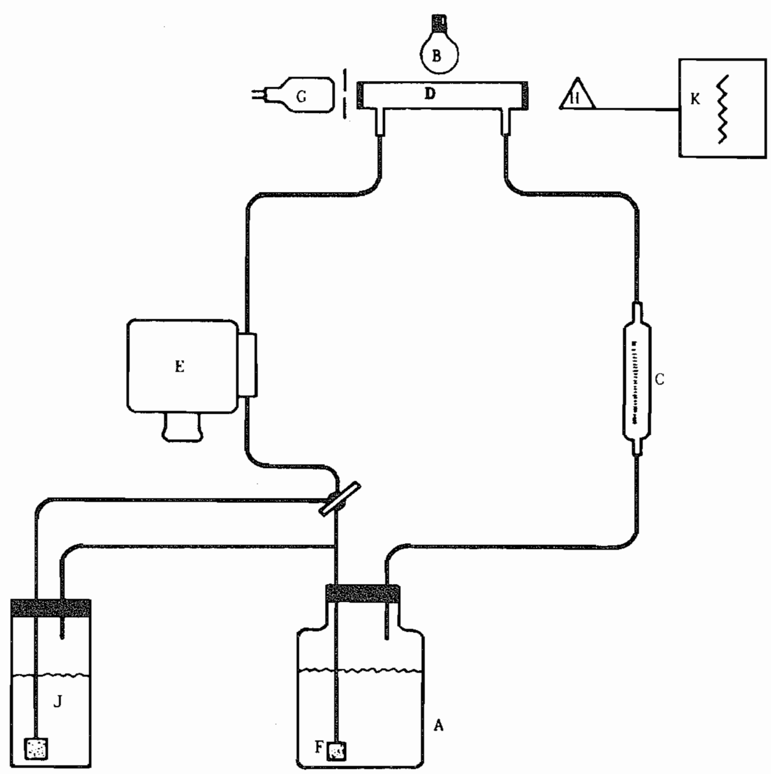


**FIG. 977.22—Apparatus for determination of mercury by flameless atomic absorption closed recirculating system: A, reaction flask; B, 60 watt light bulb; C, rotameter, 1 L air/min; D, absorption cell with quartz windows; E, air pump, 1 L air/min; F, glass tube with fritted end; G, hollow cathode Hg lamp; H, atomic absorption detector; J, gas washing bottle contg 0.25% $I_2$ in 3% KI soln; K, recorder, any compatible model.**

(c) *Sodium chloride-hydroxylamine sulfate soln.*—Dissolve 120 g NaCl and 120 g $(NH_2OH)_2.H_2SO_4$ in $H_2O$ and dil. to 1 L.

(d) *Stannous sulfate soln.*—Disperse 100 g $SnSO_4$ in $H_2O$ contg 14 mL $H_2SO_4$ and dil. to 1 L. Stir suspension with mag. stirrer continuously during use.

(e) *Mercury std solns.*—(*1*) *Stock soln.*—1000 μg/mL. Dissolve 0.1354 g $HgCl_2$ in 75 mL $H_2O$, add 10 mL $HNO_3$, and dil. to 100 mL with $H_2O$. (*2*) *Intermediate soln.*—10 μg/mL. Pipet 10 mL stock soln into 500 mL $H_2O$, add 2 mL $HNO_3$, and dil. to 1 L with $H_2O$. Prep. fresh daily. (*3*) *Working soln.*—0.1 μg/mL. Pipet 10 mL intermediate soln into 500 mL $H_2O$, add 2 mL $HNO_3$, and dil. to 1 L with $H_2O$. Prep. fresh daily.

### D. Preparation of Sample

(a) *Dissolved mercury.*—Proceed as in **974.27D(a)**.

(b) *Suspended mercury.*—Transfer residue and membrane from (a) to digestion flask and proceed as in **977.22E**.

(c) *Total mercury.*—Preserve soln by adding 2 mL $HNO_3$/L and proceed as in **977.22E**.

### E. Determination

Transfer 100 mL sample or aliquot dild to 100 mL, contg ≤1.0 μg Hg, to digestion flask. Slowly add 5 mL $H_2SO_4$ and 2.5 mL $HNO_3$, with mixing. Add 15 mL *5% $KMnO_4$ soln,* shake, and add addnl $KMnO_4$ until purple color lasts ≥15 min. Add 8 mL *5% $K_2S_2O_8$ soln,* heat 2 hr in 95° $H_2O$ bath, and cool to room temp.

Adjust output of pump to ca 1 L/min and connect app. as in Fig. **977.22**, except for gas inlet. With pump working and spectrophtr zeroed, add 6 mL NaCl-$(NH_2OH)_2.H_2SO_4$ soln to reduce excess $KMnO_4$ (purple color disappears); let stand 30 sec, and add 5 mL $SnSO_4$ soln. Immediately connect digestion flask gas inlet to aeration app. and aerate without manual agitation. *A* will reach max. within 30 sec. Record *A*. When pen levels off (ca 1 min), open by-pass valve and continue aeration until *A* returns to min. value. Close bypass valve, remove stopper and frit from digestion flask, and continue aeration to flush system.

Check for interfering volatile org. compds by placing same vol. sample or dild sample into digestion flask. Connect flask to aeration app. and aerate without manual agitation and measure *A* after 30 sec. Subtract *A* from reading obtained on sample with reagents added.

Prep. std curve by dilg 0, 0.5, 1.0, 2.0, 5.0, and 10.0 mL aliquots Hg working std soln to 100 mL, and adding to series of digestion flasks. Proceed as in par. 1, beginning "Slowly add 5 mL $H_2SO_4$ . . ."

Plot *A* against μg Hg. Det. μg Hg in sample from curve.

$$\mu g\ Hg/L = W \times (1000/V)$$

where $W$ = μg Hg in sample and $V$ = mL sample.

Refs.: ASTM STP 573, 1975, pp. 566–580. JAOAC **60,** 474(1977).

CAS-7439-97-6 (mercury)

## 920.201 Barium in Water

### Final Action

(It is not necessary to look for Ba if sulfate is present in appreciable amt unless sample contains large amt of bicarbonate or chloride, which may hold in soln small amts of both sulfate and Ba.)

### *Gravimetric Method*

### A. Reagents

(a) *Ammonium dichromate soln.*—Dissolve 100 g of the $SO_4$-free salt in $H_2O$ and dil. to 1 L.

(b) *Ammonium acetate soln.*—Dissolve 300 g of the salt in $H_2O$, neutze with $NH_4OH$, and dil. to 1 L.

(c) *Dilute ammonium acetate wash soln.*—Dil. 20 mL (**b**) to 1 L.

(Reaction of acetate solns should be alk. rather than acid.)

### B. Determination

Acidify 1–5 L portion of sample with HCl and conc. to ca 200 mL. (If ppt forms, filter off and test for Ba.) Add ca 0.5 g $NH_4Cl$, and ppt Fe and Al with $NH_4OH$. Boil, filter, and wash. To filtrate, add excess (10 mL) $NH_4OAc$ soln, (**b**), keeping total vol. ca 200 mL. Heat to bp, and add, with stirring, ca 5 mL $(NH_4)_2Cr_2O_7$ soln. Let settle and cool. Decant clear liq. thru filter and wash ppt by decantation with dil. $NH_4OAc$ soln until filtrate is no longer perceptibly colored (ca 100 mL wash soln).

Place beaker under funnel, dissolve ppt on paper with warm $HNO_3$ (1 + 1), using as little as possible, and wash paper. Add little more acid to dissolve ppt in beaker, and then $NH_4OH$ until ppt that forms no longer redissolves. Heat to bp; add, with stirring, 10 mL $NH_4OAc$ soln, (**b**), and 2 mL $(NH_4)_2Cr_2O_7$ soln; let cool slowly, and wash ppt free of chromate with dil. $NH_4OAc$ soln by decantation and filtration. Dry ppt, ignite moderately to const wt, and weigh as $BaCrO_4$. Calc. as Ba, using factor 0.5421.

Ref.: Morse, "Exercises in Quantitative Chemistry," p. 417. JAOAC **4,** 86(1920).

### *Titrimetric Method*

### C. Determination

Proceed as in **920.201B** thru ". . . wash ppt free of chromate with dil. $NH_4OAc$ soln . . ." (after second pptn). Dissolve ppt in ca 10 mL HCl (1+1) and hot $H_2O$. Wash filter, dil. soln to ca 400 mL, and add ca 50 mL freshly prepd 10% KI soln. Mix carefully and titr. liberated $I_2$ after 3 or 4 min with 0.1*N* $Na_2S_2O_3$. 1 mL 0.1*N* $Na_2S_2O_3$ = 4.578 mg Ba.

## 973.53 Potassium in Water

### Atomic Absorption Spectrophotometric Method

### First Action 1973

### A. Principle

Method is applicable to detn of 0.01–2 mg K/L of surface and saline waters and domestic and industrial wastes. Na may interfere if present at much higher levels than K but effect may be avoided by approx. matching Na concn of stds with that of sample.

Synthetic $H_2O$ analyzed by 19 analysts in 10 laboratories showed the following results on exact increments of K salt:

| Added, mg K/L | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | mg K/L | % | mg K/L |
| 1.5 | 11 | 0.17 | +4.8 | 0.07 |
| 1.4 | 16 | 0.22 | +6.6 | 0.09 |
| 8.0 | 8 | 0.64 | +7.6 | 0.60 |
| 7.5 | 9 | 0.66 | +8.7 | 0.64 |
| 20.0 | 6 | 1.11 | +7.4 | 1.5 |
| 19.0 | 8 | 1.58 | +7.4 | 1.4 |

### B. Apparatus

*Atomic absorption spectrophotometer*.—Equipped with Boling-type burner, set at 766.5 nm.

### C. Reagents

(**a**) *Deionized distilled water*.—*See* **973.48C(a)**. Use for prepn of reagents and stds, and as diln $H_2O$.

(**b**) *Potassium std solns*.—*(1) Stock soln*.—100 mg K/L. Dissolve 0.1907 g KCl, dried at 110°, in $H_2O$, and dil. to 1 L. (*2*) *Working solns*.—Prep. dil. std solns in range of interest at time of analysis. If $HNO_3$ is used to preserve samples, add corresponding amt to working std solns.

### D. Determination

(*Caution:* See safety notes on AAS.)

Follow manufacturer's instructions for app. operation. Optimize conditions for max. absorption and stability. Beginning with blank and working toward highest std, aspirate solns and record readings. Repeat std solns and samples enough times to secure reliable av. reading for each soln. If necessary, dil. sample with $H_2O$ to bring into range for direct reading.

For instruments which read directly in concn, set curve corrector to read out proper concn. Otherwise, plot calibration curve, using concn range producing absorption of 0–80%. Before plotting, convert % absorption to absorbance: $A = \log(100/\%T) = 2 - \log\%T$, where $\%T = 100 - \%$ absorption. Curves are frequently nonlinear. Increase number of stds in that portion of curve.

Read mg K/L from calibration curve or directly from readout system.

$$\text{mg K/L in sample} = (\text{mg K/L}) \text{ in aliquot} \times D$$

$$D = (\text{mL aliquot} + \text{mL } H_2O \text{ added})/\text{mL aliquot}$$

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). FWPCA Method Study 1; Mineral and Physical Analyses, June 1969 (available from National Environmental Research Center, Environmental Protection Agency, Cincinnati, OH 45268). JAOAC **56,** 295(1973).

CAS-7440-09-7 (potassium)

## 973.54 Sodium in Water

### Atomic Absorption Spectrophotometric Method

### First Action 1973

### A. Principle

Method is applicable to detn of 1–200 mg Na/L in surface and saline waters and domestic and industrial wastes.

Synthetic $H_2O$ analyzed by 22 analysts in 12 laboratories showed the following results on exact increments of Na salts:

| Added, mg Na/L | Std deviation % | Std deviation mg Na/L | Bias % | Bias mg Na/L |
|---|---|---|---|---|
| 4.1 | 3.4 | 0.14 | +1.9 | 0.07 |
| 3.8 | 5.0 | 0.19 | +2.9 | 0.11 |
| 55.0 | 3.6 | 1.99 | +0.9 | 0.5 |
| 52.0 | 3.7 | 1.93 | +0.8 | 0.4 |
| 155 | 2.4 | 3.75 | +0.0 | 0.0 |
| 149 | 2.7 | 3.97 | −0.1 | 0.0 |

### B. Apparatus

*Atomic absorption spectrophotometer*.—*See* **973.53B**. Use Na hollow cathode lamp, 330.2 nm, Boling burner, and oxidizing air-$C_2H_2$ flame. For greater sensitivity (0.005–0.2 mg/L), use 589.0 nm line.

### C. Reagents

(**a**) *Deionized distilled water*.—*See* **973.48C(a)**.

(**b**) *Sodium std solns*.—(*1*) *Stock soln*.—1000 mg Na/L. Dissolve 2.542 g NaCl, dried at 140°, in $H_2O$, and dil. to 1 L. (*2*) *Working solns*.—Prep. dil. std solns in range of interest at time of analysis. If $HNO_3$ is used to preserve samples, add corresponding amt to working std solns.

### D. Determination

Proceed as in **973.53D**, using Na parameters and std solns.

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). FWPCA Method Study 1; Mineral and Physical Analyses, June 1969 (available from National Environmental Research Center, Environmental Protection Agency, Cincinnati, OH 45268). JAOAC **56,** 295(1973).

CAS-7440-23-5 (sodium)

## 973.55 Phosphorus in Water

### Photometric Method

### First Action 1973

### A. Definitions

(**a**) *Phosphorus*.—(*P*). All P present in sample, regardless of form, measured by persulfate digestion method. (*1*) *Orthophosphate*.—(*P, ortho*). Inorg. P, ($PO_4^{-3}$), in sample as measured by direct colorimetric analysis. (*2*) *Hydrolyzable phosphorus*.—(*P, hydro*). P as measured by $H_2SO_4$ hydrolysis method minus orthophosphate; includes polyphosphates, $(P_2O_7)^{-4}$, $(P_3O_{10})^{-5}$, and some org. P. (*3*) *Organic phosphorus*.— (*P, org*) = *P* − [(*P, ortho*) + (*P, hydro*)].

(**b**) *Dissolved phosphorus*.—(*P-D*). P present in filtrate of sample filtered thru 0.45 μm pore filter, measured by persulfate digestion method. (*1*) *Dissolved orthophosphate*.—(*P-D, ortho*). Inorg. P as measured by direct colorimetric method. (*2*) *Dissolved hydrolyzable phosphorus*.—(*P-D, hydro*). P as measured by $H_2SO_4$ hydrolysis − (*P-D, ortho*). (*3*) *Dissolved organic phosphorus*.—(*P-D, org*) = (*P-D*) − [(*P-D, ortho*) + (*P-D, hydro*)].

(**c**) *Insoluble phosphorus*.—(*P-I*). When sufficient amt is present, calc. following: (*1*) *P-I* = (*P*) − (*P-D*). (*2*) *Insoluble orthophosphate*.—(*P-I, ortho*) = [(*P, ortho*) − (*P-D, ortho*)]. (*3*) *Insoluble hydrolyzable phosphorus*.—(*P-I, hydro*) = [(*P, hydro*) − (*P-D, hydro*)]. (*4*) *Insoluble organic phosphorus*.—(*P-I, org*) = [(*P, org*) − (*P-D, org*)].

### B. Principle

Ammonium molybdate and K antimonyl tartrate react in acid soln with dil. solns of $PO_4^{-3}$ to form Sb phosphomolybdate complex which is reduced to intensely blue complexes by ascorbic acid. Method is specific for orthophosphate and for compds that can be converted to orthophosphate. Various forms of P are detd, depending on pretreatment, in range 0.01–0.5 mg P/L.

Method is applicable to surface and saline waters and domestic and industrial wastes. Most commonly measured forms are total P, dissolved P, orthophosphate, and dissolved orthophosphate. Hydrolyzable P is normally found only in sewage-type samples. Concns of Cu, Fe, silicate, and arsenate many times greater than those in sea water do not interfere. Interference of $HgCl_2$, used as preservative, is overcome by adding min. of 50 mg NaCl/L to samples.

Natural $H_2O$ analyzed by 33 analysts in 19 laboratories showed following results on exact increments of org. phosphate:

| Added, mg P/L | Std deviation % | Std deviation mg P/L | Bias % | Bias mg P/L |
|---|---|---|---|---|
| 0.110 | 30 | 0.033 | + 3.1 | +0.003 |
| 0.132 | 39 | 0.051 | +12.0 | +0.016 |
| 0.772 | 17 | 0.130 | + 3.0 | +0.023 |
| 0.882 | 15 | 0.128 | − 0.9 | −0.008 |

Natural $H_2O$ analyzed by 26 analysts in 16 laboratories showed following results on exact increments of orthophosphate:

| Added, mg P/L | Std deviation % | Std deviation mg P/L | Bias % | Bias mg P/L |
|---|---|---|---|---|
| 0.029 | 34 | 0.010 | −5.0 | −0.001 |
| 0.038 | 21 | 0.008 | −6.0 | −0.002 |
| 0.335 | 5.4 | 0.018 | −2.8 | −0.009 |
| 0.383 | 6.0 | 0.023 | −1.8 | −0.007 |

### C. Apparatus

(**a**) *Glassware*.—Wash all glassware with hot HCl (1 + 1) and rinse with $H_2O$. Remove last traces of P by filling with $H_2O$ contg all color-developing reagents. Use treated glassware only for P detns and after use, rinse with $H_2O$ and keep covered until used again. Under such conditions, hot HCl and reagent treatment need be applied only occasionally. *Never use com. detergents on glassware.*

(**b**) *Photometer*.—Spectrophtr or filter photometer measuring at 880 nm, using ≥2.5 cm light path.

### D. Reagents

(**a**) *Dilute sulfuric acid*.—5*N*. Dil. 70 mL $H_2SO_4$ to 500 mL.

(**b**) *Potassium antimonyl tartrate soln*.—Weigh 1.3715 g $K(SbO)C_4H_4O_6.0.5H_2O$, dissolve in ca 400 mL $H_2O$, and dil. to 500 mL. Store in dark g-s bottle at 4°.

(**c**) *Ammonium molybdate soln*.—Dissolve 20 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in 500 mL $H_2O$. Store in plastic bottle at 4°.

(**d**) *Ascorbic acid soln*.—0.1*M*. Dissolve 1.76 g in 100 mL $H_2O$. Stable 1 week at 4°.

(**e**) *Combined reagent*.—Warm reagents (**b**)–(**d**) to room temp., and add with mixing in following order: 50 mL 5*N* $H_2SO_4$, 5 mL K antimonyl tartrate soln, 15 mL $NH_4$ molybdate soln, and 30 mL ascorbic acid soln. If turbidity forms, shake, and let stand few min before proceeding. Stable 1 week at 4°.

(**f**) *Hydrolyzing acid soln*.—Slowly add 310 mL $H_2SO_4$ to 600 mL $H_2O$, cool, and dil. to 1 L.

(**g**) *Phosphorus std solns*.—(*1*) *Stock soln*.—50 mg P/L. Dissolve and dil. 0.2197 g $KH_2PO_4$, dried at 105°, to 1 L. (*2*) *Intermediate soln*.—0.5 mg/L. Dil. 10.0 mL stock soln to 1 L. (*3*) *Working solns*.—Dil. 0.0, 1.0, 3.0, 5.0, 10.0, 20.0, 30.0, 40.0, and 50.0 mL intermediate soln to 50 mL to prep. std solns contg 0.0, 0.01, 0.03, 0.05, 0.10, 0.20, 0.30, 0.40, and 0.50 mg P/L.

### E. Determination

Store samples in plastic or Pyrex containers. If analysis cannot be performed on day of collection, preserve with 40 mg $HgCl_2$/L and refrigerate at 4°. In such case, add 50 mg NaCl/L before analysis.

(**a**) *Phosphorus*.—Add 1 mL hydrolyzing acid soln to 50 mL sample in 125 mL erlenmeyer. Add 0.4 g $NH_4$ persulfate, and boil gently on preheated hot plate 30–40 min or until vol is 10 mL. Do not let sample evap. to dryness. Alternatively, autoclave 30 min at 121°. Cool, add few drops phthln, adjust to pink with 1*N* NaOH, and then to colorless with 1 drop hydrolyzing acid soln. Cool, and dil. to 50.0 mL. If turbid, filter. Proceed as in (**c**), beginning "Add 8.0 mL combined reagent, . . ."

(**b**) *Hydrolyzable phosphorus*.—Proceed as in (**a**), except omit addn of $NH_4$ persulfate.

(**c**) *Orthophosphate*.—Add 1 drop phthln to 50.0 mL sample; if red develops, add hydrolyzing acid soln dropwise until color is discharged. Add 8.0 mL combined reagent, and mix thoroly. After specific time within 10–30 min, measure *A* at 880 nm against reagent blank as ref.

(**d**) *Std curve and calculation*.—Process stds and blank as in (**c**) and plot *A* against mg P/L. Include blank and ≥2 std solns with each series of samples. If stds do not agree with std curve within ± 2%, prep. new std curve. Obtain mg P/L sample directly from std curve.

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7, $5). Method Study No. 2; Nutrient Analyses, Manual Methods, 1970 (available from National Technical Information Service, 5285 Port Royal Rd, Springfield, VA 22161, NTIS PB230828/BE). JAOAC **56**, 295(1973).

CAS-7723-14-0 (phosphorus)

## 973.56 Phosphorus in Water

### Automated Method

### First Action 1973

### A. Principle

*See* **973.55**. Developed color is measured automatically.

Natural $H_2O$ analyzed by 6 laboratories showed following results on exact increments of orthophosphate:

| Added, mg P/L | Std deviation % | Std deviation mg P/L | Bias % | Bias mg P/L |
|---|---|---|---|---|
| 0.04 | 47 | 0.019 | +16.7 | +0.007 |
| 0.04 | 35 | 0.014 | − 8.3 | −0.003 |
| 0.29 | 30 | 0.087 | −15.5 | −0.05 |
| 0.30 | 22 | 0.066 | −12.8 | −0.04 |

### B. Apparatus

(**a**) *Glassware*.—*See* **973.55C(a)**.

(**b**) *Automatic analyzer*.—AutoAnalyzer with following modules (Technicon Instruments Corp.): Sampler I, manifold, proportioning pump, 50° bath, colorimeter with 50 mm tubular flowcell and 650 nm filter, and recorder. *See* Fig. **973.56**.

### C. Reagents

Prep. reagents **973.55D(a)**, (**d**), and (**f**), and following:

(**a**) *Potassium antimonyl tartrate soln*.—Weigh 0.3 g $K(SbO)C_4H_4O_6.0.5H_2O$, dissolve in ca 50 mL $H_2O$, and dil. to 100 mL. Store in dark g-s bottle at 4°.

(**b**) *Ammonium molybdate soln*.—Dissolve 4 g $(NH_4)_6Mo_7O_{24}.4H_2O$ in 100 mL $H_2O$. Store in plastic bottle at 4°.

(**c**) *Combined reagent*.—Prep. as in **973.55D(e)**. 100 mL is enough for 4 hr operation. Prep. fresh for each series.

(**d**) *Wash water*.—Add 40 mL hydrolyzing acid soln, **973.55D(f)**, to ca 1 L $H_2O$ and dil. to 2 L (not used when only orthophosphate is detd).

(**e**) *Phosphorus std solns*.—(*1*) *Stock soln*.—0.1 mg P/L. Dissolve and dil. 0.4393 g $KH_2PO_4$, dried at 105°, to 1 L. (*2*) *Intermediate soln 1*.—0.01 mg P/L. Dil. 100 mL stock soln

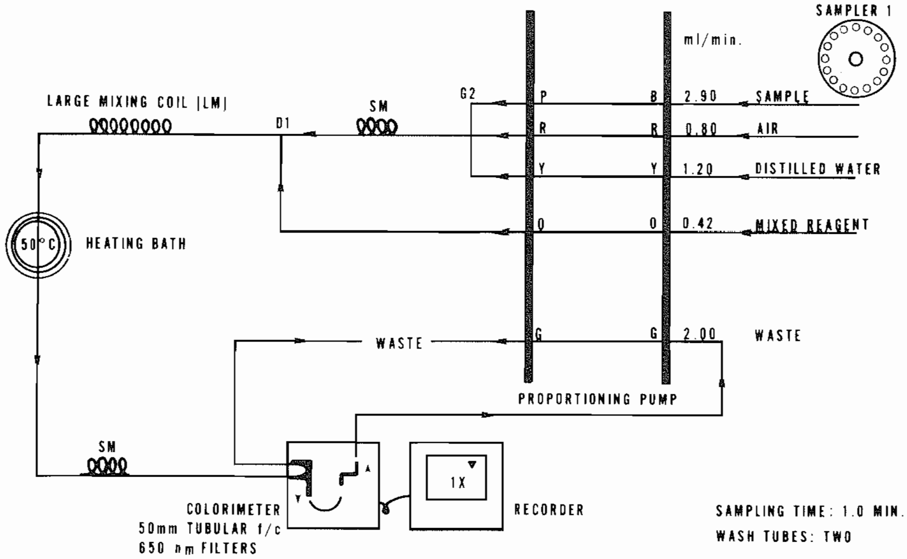


**FIG. 973.56—Phosphorus manifold**

to 1 L. (*3*) *Intermediate soln 2.*— 0.001 mg P/L. Dil. 100 mL intermediate soln *1* to 1 L. (*4*) *Working solns.*—Dil. 0.0, 2.0, 5.0, and 10.0 mL intermediate soln *2* and 2.0, 5.0, 8.0, and 10.0 mL intermediate soln *1* to 100 mL to prep. std solns contg 0.00, 0.02, 0.05, 0.10, 0.20, 0.50, 0.80, and 1.00 mg P/L, resp.

### *D. Determination*

Store and prep. samples as in **973.55E**.

(**a**) *Phosphorus.*—Proceed as in **973.55E**(**a**), but det. orthophosphate as in (**c**), below.

(**b**) *Hydrolyzable phosphorus.*—Proceed as in **973.55E**(**a**), omitting addn of $NH_4$ persulfate, and det. orthophosphate as in (**c**), below.

(**c**) *Orthophosphate.*—Set up manifold as in Fig. **973.56**. Let colorimeter and recorder warm up 30 min. Run baseline with all reagents but with $H_2O$ thru sample line. Adjust dark current and operative opening on colorimeter to obtain stable baseline. Place wash $H_2O$, (**d**), in sampler in pairs, for other than ortho-P, and $H_2O$ for ortho-P, leaving every third position vacant. Set sample timing at 1 min. Place std solns in sampler in vacant positions in order of decreasing concn and complete filling of sampler tray with unknown samples. Change sample line from $H_2O$ to sampler and begin analysis.

(**d**) *Std curve and calculation.*—Prep. std curve by plotting peak hts against mg P/L. Obtain sample concn from peak ht. Reanalyze any sample whose computed value is <5% of its immediate predecessor.

Ref.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC 20402, Stock No. 055-001-00067-7). JAOAC **56**, 295(1973).

CAS-7723-14-0 (phosphorus)

## 973.57 Sulfate in Water

### Turbidimetric Method

### First Action 1973

### *A. Principle*

Sulfate is pptd in dil. HCl with $BaCl_2$ under controlled conditions to form $BaSO_4$ crystals of uniform size. *A* of suspension is measured and sulfate concn is obtained from std curve.

Method is applicable to drinking and surface waters and domestic and industrial wastes. Dil. samples to concn of ≤40 mg $SO_4$/L. Color and suspended matter interfere. Some suspended matter is removed by filtration. Remaining interference is corrected by blank which omits $BaCl_2$.

Synthetic $H_2O$ samples contg exact increments of inorg. $SO_4$ analyzed by 34 analysts in 16 laboratories showed following results:

| Increment as mg $SO_4$/L | Std deviation | | Bias | |
|---|---|---|---|---|
| | % | mg $SO_4$/L | % | mg $SO_4$/L |
| 8.6 | 27 | 2.3 | −3.7 | −0.3 |
| 9.2 | 20 | 1.8 | −8.3 | −0.8 |
| 110 | 7.1 | 7.9 | −3.0 | −3.3 |
| 122 | 6.1 | 7.5 | −3.4 | −4.1 |
| 188 | 5.1 | 9.6 | 0.0 | +0.1 |
| 199 | 5.9 | 11.8 | −1.7 | −3.4 |

### *B. Apparatus*

(**a**) *Magnetic stirrer.*—Adjustable, but once set must operate at const speed. Stirring bars must be of identical shape and size. Exact speed is not critical, but it should be const for each series of samples and stds and should be at max. at which no splashing occurs. Timing device to permit operation for exactly 1 min is desirable.

(**b**) *Photometer.*—Nephelometer, spectrophtr set at 420 nm with 4–5 cm cell, or filter photometer with filter having max. *T* near 420 nm with 4–5 cm cell.

### *C. Reagents*

(**a**) *Conditioning reagent.*—Mix 50 mL glycerol with soln of 30 mL HCl, 300 mL $H_2O$, 100 mL alcohol or isopropanol, and 75 g NaCl.

(**b**) *Barium chloride.*—Crystals, 20–30 mesh. Dispense from 0.2–0.3 mL measuring spoon.

(**c**) *Sulfate std soln.*—100 μg $SO_4$/mL. Dil. 10.41 mL 0.0200*N* $H_2SO_4$ to 100 mL, or dissolve 147.9 mg anhyd. $Na_2SO_4$ in $H_2O$ and dil. to 1 L.

### *D. Determination*

Pipet 5 mL conditioning reagent into 100 mL sample or aliquot dild to 100 mL in 250 mL erlenmeyer, and mix on mag. stirrer. While stirring, add spoonful of $BaCl_2$ crystals and begin timing. Stir exactly 1 min at const speed. Immediately transfer some soln into cell and measure turbidity at 30 sec intervals for 4 min. Record max. reading. Conduct blank detn without $BaCl_2$ and subtract reading.

Prep. std curve by carrying 0–40 mg $SO_4$/L, in 5 mg increments, thru entire detn. Introduce std soln with every 3–4 samples.

$$\text{mg } SO_4/L = \text{mg } SO_4 \text{ from curve} \times 1000/\text{mL sample}$$

Refs.: Methods for Chemical Analysis of Water and Wastes, 1971 (available from Superintendent of Documents, Washington, DC. 20402, Stock No. 055-001-00067-7). FWPCA Method Study 1; Mineral and Physical Analyses, June 1969 (available from National Environmental Research Center, Environmental Protection Agency, Cincinnati, OH 45268). JAOAC **56,** 295(1973).

## 925.54 Sulfate in Water

### Gravimetric Method

### Final Action

Make preliminary examination, using 100–250 mL sample, to det. approx. amt of sulfates. (Alkali salts present can be approximated by calcg amt of Na necessary to combine with excess of acids—HCl, $H_2SO_4$, and $H_2CO_3$—over Ca and Mg.)

Take enough sample (usually 1–5 L) to yield ≤1 g $BaSO_4$ and ≤0.5 g mixed chlorides. Acidify with HCl (1 + 1), evap. to dryness in Pt dish, and remove $SiO_2$ by 2 evapns as in **920.195**, using ≤2 mL HCl for final soln. Combine filtrate and washings from $SiO_2$ detns and conc. to 150–200 mL. Heat to bp and ppt with slight excess of 10% $BaCl_2.2H_2O$ soln, added very slowly and with const stirring. Cover, and let stand on steam bath ca 12 hr. Filter, thoroly wash $BaSO_4$ ppt with hot $H_2O$ until Cl-free, dry, ignite over Bunsen burner and weigh.

If sulfate content of sample is unusually large, proceed as far as concn of $SiO_2$ filtrates, as above. Add 50 mL HCl, heat to bp, and ppt with $BaCl_2$ soln as before. Evap. to dryness, take up in $H_2O$ and few drops HCl, digest until ppt settles, wash by decantation, filter, ignite, and weigh. Calc. to $SO_4$ ion.

## 920.202 Manganese, Iodine, Bromine, Arsenic, and Boric Acid in Water

### Preparation of Sample

### Final Action

Evap. 0.5–2 L sample to dryness after addn of small amts of solid $Na_2CO_3$. Boil residue thus obtained with $H_2O$, transfer to filter, and wash thoroly with hot $H_2O$. Use residue remaining on filter for detn of Mn. Dil. alk. filtrate to definite vol. and use for detn of I, Br, As, and $H_3BO_3$.

## 920.203 Manganese in Water

### Final Action

### *Persulfate Method*

#### *A. Reagents*

**(a)** *Silver nitrate soln.*—Dissolve 2 g $AgNO_3$ in $H_2O$ and dil. to 1 L.

**(b)** *Manganese std soln.*—0.1 mg/mL. Dissolve 0.2877 g $KMnO_4$ in ca 100 mL $H_2O$, acidify soln with $H_2SO_4$ (1 + 1), and slowly heat to bp. Slowly add enough 10% $H_2C_2O_4.2H_2O$ soln to discharge color. Cool, and dil. to 1 L.

#### *B. Determination*

Dissolve insol. residue, **920.202**, in excess $HNO_3$ (1 + 1), evap. to dryness, treat with $H_2O$, and add ca 1 mL $HNO_3$ and little of the $AgNO_3$ soln. If ppt of AgCl appears, add addnl $AgNO_3$ soln until all Cl is pptd. Add excess of ca 10 mL $AgNO_3$ soln for each mg Mn present in sample. Filter, add 1 g $(NH_4)_2S_2O_8$ to filtrate, and place beaker or flask contg soln on steam bath until pink color develops (ca 20 min). Compare color developed with stds similarly prepd by treating solns contg known amts of std Mn soln with dil. $HNO_3$, $AgNO_3$ soln, and $(NH_4)_2S_2O_8$.

#### *C. Bismuthate Method★ —Surplus 1965*

*See* **31.037–31.038**, 10th ed.

CAS-7439-96-5 (manganese)

## 920.204 Bromide and Iodide in Water

### Colorimetric Method

### First Action

(This method is qual. and approx. quant. For accurate quant. methods for iodides, *see* **925.56C**.)

(*Caution:* See safety notes on flammable solvents, toxic solvents, chlorine, and carbon disulfide.)

Evap. aliquot of alk. filtrate, **920.202**, to dryness; add 2–3 mL $H_2O$ to dissolve residue and enough alcohol to make ca 90% alcohol. (This ppts chlorides.) Heat to bp, filter, and repeat soln and pptn once or twice. Add 2 or 3 drops 10% NaOH soln to combined alc. filtrates and evap. to dryness. Dissolve last residue in 2–3 mL $H_2O$ and repeat pptn with alcohol, heating, and filtering. Add drop of 10% NaOH soln to this alc. filtrate and evap. to dryness.

Dissolve residue in little $H_2O$, acidify with $H_2SO_4$ (1 + 5), using 3 or 4 drops excess, and transfer to small flask. Add 4 drops *0.2% $NaNO_2$ soln* and ca 5 mL $CS_2$. Shake until all I is extd and filter off acid soln from $CS_2$. Wash flask, filter, and contents with cold $H_2O$ and transfer $CS_2$ contg the I in soln to Nessler tube, using ca 5 mL $CS_2$. In washing filter, make contents of tube to definite vol., usually 12–15 mL, and compare color with that of other tubes contg known amts of I dissolved in $CS_2$. Prep. these std tubes by treating measured amts of soln of known KI content as above, beginning ". . . acidify with $H_2SO_4$ (1 + 5), . . ."

Sep. transfer acid soln of sample and stds from which I has been removed to small flasks. To stds add definite measured amts of bromide soln of known concn, and to each flask contg sample and stds add 5 mL $CS_2$. Add *satd and freshly prepd Cl-$H_2O$*, 1 mL at time, shaking after each addn until all Br is set free. Avoid large excess of Cl, as a bromo-chloride may form and change color reaction.

Filter off aq. soln from $CS_2$ thru moistened filter, wash contents of filter 2 or 3 times with $H_2O$, and then transfer to Nessler tube with ca 1 mL $CS_2$. Repeat extn of filtrate twice, using 3 mL $CS_2$ each time. Combined $CS_2$ exts usually total 11.5–12 mL. Add enough $CS_2$ to tubes to make definite vol., usually 12–15 mL, and compare sample with stds. If, when using this method near its upper limit, amts of $CS_2$ recommended do not ext all Br, make 1 or 2 addnl extns with $CS_2$, transfer exts to another tube, and compare color with some of lower stds. Add readings thus obtained to others.

Results closely approximating true values for I and Br can be obtained in shorter time on most samples by omitting extns with alcohol and comparing color of $CS_2$ solns directly in extn flasks.

## 950.58★ Bromide in Water

**First Action**
**Surplus 1965**

(In presence of chloride but not iodide)

*See* **31.040–31.043**, 10th ed.

## 950.59★ Bromide in Water

**Final Action**
**Surplus 1965**

(In presence of chloride and iodide)

*See* **31.044–31.046**, 10th ed.

## 920. 205 Arsenic in Water

**Final Action**

### A. Reagents and Apparatus

*See* **963.21A** and **B**.

### B. Determination

Take portion of alk. filtrate, **920.202**, contg $\leq$0.03 mg $As_2O_3$. If amt taken is >10 mL, evap. soln to ca that vol. on steam bath. Transfer soln into generator of app., **963.21B**, with aid of ca 10 mL $H_2O$, add 20 mL $H_2SO_4$ (1 + 2), and proceed as in **942.17A** or **952.13A**, beginning with addn of KI reagent.

## 920.206★ Boric Acid in Water

**Surplus 1965**

### A. Qualitative Test—Procedure

*See* **31.049**, 10th ed.

### B. Quantitative Method—First Action

*See* **31.050—31.051**, 10th ed.

## 965.37 Lead in Water

*See* **934.07A** and **B**.

## 922.12 Constituents in Water

**Method of Reporting Results**
**Procedure**

Report radicals and anhyd. salts in mg/L or, in case of highly concd waters, in g/L. For benefit of physicians, in case of medicinal waters, also report salts in terms of grains/qt, using factor 0.014600 to convert mg/L to grains/qt. In reporting salts in terms of grains/qt, convert salts that have $H_2O$ of crystn to hydrated form as expressed in USP and in NF, and convert $Mg(HCO_3)_2$ to $MgCO_3$ and $Ca(HCO_3)_2$ to $CaCO_3$. Use following factors in these calcns:

$$Na_2SO_4 \times 2.2683 = Na_2SO_4.10H_2O$$
$$MgSO_4 \times 2.0477 = MgSO_4.7H_2O$$
$$CaSO_4 \times 1.2647 = CaSO_4.2H_2O$$
$$Mg(HCO_3)_2 \times 0.5762 = MgCO_3$$
$$Ca(HCO_3)_2 \times 0.6174 = CaCO_3$$

When complete analysis is made, report error of analysis and state how it is distributed. Report only significant figures.

Report Fe and Al together when present in unimportant amts and in calcns consider them as Fe. When Fe and Al are present in larger amts, make sepn and report each sep.

In calcg hypothetical combinations of anions and cations, join $NO_2$, $NO_3$, $BO_3$, and $AsO_4$ to Na; I and Br to K; and $PO_4$ to Ca. Assign residual cations in following order: $NH_4$, Li, K, Na, Mg, Ca, Sr, Mn, Fe, and Al; to residual anions in following order: Cl, $SO_4$, $CO_3$, and $HCO_3$. When not enough $HCO_3$ is present to join with all Ca, residual Ca is joined to $SiO_2$ to form $CaSiO_3$, and Mn, Fe, and Al are calcd to oxides $Mn_3O_4$, $Fe_2O_3$, and $Al_2O_3$, resp.

Use equiv. combining wts or their reciprocals in uniting radicals and, when necessary for purpose of comparison, in reducing salts to radicals and reuniting radicals in order specified above. *See* Table **922.12**.

Equiv. combining wt of radical is obtained by dividing its wt by its valence. Equiv. combining wt of salt is obtained by dividing its MW by product of valence of basic element and number of atoms of basic element in the salt.

Procedure in calcg hypothetical combinations by use of equiv. combining wts and their reciprocals is as follows:

Multiply wts obtained, expressed in mg/L, or, for highly concd waters, in g/L, for each radical to be combined, by corresponding reciprocal of equiv. combining wts. If Na and K are to be detd by calcn, as is frequently the case, subtract sum of values obtained (reacting values) for basic radicals from sum of reacting values for acid radicals. Difference represents reacting value of undetd Na and K.

When all constituents in water have been detd, sums of reacting values of acid and basic radicals should be very nearly equal. In this case, if difference is reasonable and well within limit of accuracy of methods used, it may be distributed equally among all radicals detd, or among those believed to be less accurately detd than others. If difference is unreasonably great, repeat analysis in whole or in part. Sums of reacting values of acid and basic radicals must be equal before calcn is made. Obtain reacting values of the salts by subtracting in succession reacting values of radicals in specified order. To convert these values to mg/L of respective salts, multiply each of them by the equiv. combining wt of respective salt.

Ref.: JAOAC **5**, 385(1922).

## 986.22 TNT, RDX, HMX, and 2,4-DNT in Wastewater and Groundwater

**Liquid Chromatographic Method**
**First Action 1986**

### A. Principle

Presence of 2,4,6-trinitrotoluene (TNT), 1,3,5-trinitro-1,3,5-triazacyclohexane (RDX), 1,3,5,7-tetranitro-1,3,5,7-tetraazacyclooctane (HMX), and 2,4-dinitrotoluene (DNT) in wastewater from munitions manufacturing and processing facilities and in groundwater is identified and measured by comparison of liq. chromatgc peak areas and retention times. Analytical detection limits for TNT, RDX, HMX, and 2,4-DNT are estd to be 14, 22, 26, and 10 $\mu$g/L, resp.

### B. Apparatus

(**a**) *Liquid chromatograph.*—With high pressure pump and 254 nm fixed wavelength UV detector, or variable wavelength detector set at 254 nm, strip chart recorder, stand-alone digital or computer-controlled integrator, and 100 $\mu$L sample loop injector.

**Table 922.12 Equivalent Combining Weights and Their Reciprocals Based on International Atomic Weights, 1973**

| Neg. Radicals | Equiv. Combining Wts | Reciprocals of Equiv. Combining Wts | Pos. Radicals | Equiv. Combining Wts | Reciprocals of Equiv. Combining Wts |
|---|---|---|---|---|---|
| $NO_3$ | 62.0049 | 0.01613 | $NH_4$ | 18.0383 | 0.05544 |
| $BO_2$ | 42.81 | 0.02336 | Li | 6.941 | 0.14407 |
| $AsO_4$ | 46.3064 | 0.02160 | K | 39.0983 | 0.02558 |
| I | 126.9045 | 0.00788 | Na | 22.98977 | 0.04350 |
| Br | 79.904 | 0.01252 | Mg | 12.153 | 0.08228 |
| $PO_4$ | 31.6571 | 0.03159 | Ca | 20.04 | 0.04990 |
| HS | 33.07 | 0.03024 | Sr | 43.81 | 0.02283 |
| S | 16.03 | 0.06238 | Ba | 68.67 | 0.01456 |
| $SiO_3$ | 38.042 | 0.02629 | Mn | 27.4690 | 0.03640 |
| O | 7.9997 | 0.12500 | $Fe^{++}$ | 27.924 | 0.03581 |
| Cl | 35.453 | 0.02821 | $Fe^{+++}$ | 18.616 | 0.05372 |
| $SO_4$ | 48.03 | 0.02082 | Al | 8.9938 | 0.11119 |
| $CO_3$ | 30.005 | 0.03333 | Cu | 31.773 | 0.03147 |
| $HCO_3$ | 61.017 | 0.01639 | | | |
| **Salts** | **Equiv. Combining Wts** | **Reciprocals of Equiv. Combining Wts** | **Salts** | **Equiv. Combining Wts** | **Reciprocals of Equiv. Combining Wts** |
| $NH_4Cl$ | 53.491 | 0.01869 | $MgCl_2$ | 47.606 | 0.02101 |
| LiCl | 42.394 | 0.02359 | $MgSO_4$ | 60.18 | 0.01662 |
| $Li_2SO_4$ | 54.97 | 0.01819 | $MgCO_3$ | 42.157 | 0.02372 |
| $Li_2CO_3$ | 36.946 | 0.02707 | $Mg(HCO_3)_2$ | 73.170 | 0.01367 |
| $LiHCO_3$ | 67.958 | 0.01471 | $Mg(NO_3)_2$ | 74.157 | 0.01348 |
| KCl | 74.551 | 0.01341 | $CaCl_2$ | 55.49 | 0.01802 |
| $K_2SO_4$ | 87.13 | 0.01148 | $CaSO_4$ | 68.07 | 0.01469 |
| $K_2CO_3$ | 69.103 | 0.01447 | $CaCO_3$ | 50.04 | 0.01998 |
| $KHCO_3$ | 100.115 | 0.00999 | $Ca(HCO_3)_2$ | 81.06 | 0.01234 |
| KI | 166.003 | 0.00602 | $CaSiO_3$ | 58.08 | 0.01722 |
| KBr | 119.002 | 0.00840 | $Ca_3(PO_4)_2$ | 51.70 | 0.01934 |
| NaCl | 58.443 | 0.01711 | $SrSO_4$ | 91.84 | 0.01089 |
| NaBr | 102.894 | 0.00972 | $SrCO_3$ | 73.81 | 0.01355 |
| NaI | 149.8942 | 0.00667 | $Sr(HCO_3)_2$ | 104.83 | 0.00954 |
| $Na_2SO_4$ | 71.02 | 0.01408 | $BaSO_4$ | 116.70 | 0.00857 |
| $Na_2CO_3$ | 52.994 | 0.01887 | $Ba(HCO_3)_2$ | 129.69 | 0.00771 |
| $NaHCO_3$ | 84.007 | 0.01190 | $MnSO_4$ | 75.50 | 0.01325 |
| $NaNO_2$ | 68.9952 | 0.01449 | $MnCO_3$ | 57.474 | 0.01740 |
| $NaNO_3$ | 84.9946 | 0.01177 | $Mn(HCO_3)_2$ | 88.486 | 0.01130 |
| $NaBO_2$ | 65.80 | 0.01520 | $FeSO_4$ | 75.95 | 0.01317 |
| $Na_3AsO_4$ | 69.2961 | 0.01443 | $Fe_2(SO_4)_3$ | 66.64 | 0.01501 |
| NaF | 41.9881 | 0.02382 | $FeCO_3$ | 57.928 | 0.01726 |
| NaHS | 56.06 | 0.01784 | $Fe(HCO_3)_2$ | 88.941 | 0.01124 |
| $Na_3PO_4$ | 54.6488 | 0.01830 | $Fe_2O_3$ | 26.615 | 0.03757 |
| $Na_2S$ | 39.02 | 0.02563 | $Al_2(SO_4)_3$ | 57.02 | 0.01754 |
| $Na_2SiO_3$ | 61.032 | 0.01638 | $Al_2O_3$ | 16.9935 | 0.05885 |

Operating conditions: 25 cm × 4.6 mm (5 μm) LC8 reverse phase column (Supelco), operated at room temp. Mobile phase $H_2O$–MeOH–$CH_3CN$ (50 + 38 + 12, v/v), prepd in graduates, not vol. flask. Prep. 750–1000 mL, vac.-filter thru solv.-washed Whatman glass microfiber filter to remove particulate matter, and degas. Prep. fresh solv. daily. Flow rate 1.5 mL/min. Set integrator threshold low enough to avoid neg. intercept in working curve and high enough to avoid pos. intercept. Set chart speed at 0.2 in./min.

(**b**) *LC syringe*.—Liq.-tight syringe of 0.5–1.0 mL capacity (Hamilton 750, or equiv.).

(**c**) *Filters*.—*(1)* Nuclepore syringe filter, 25 mm diam. *(2)* 0.4 μm Nuclepore polycarbonate, 25 mm diam., or Millex-SR 0.5 μm filter unit.

(**d**) *Filtration syringe*.—25 mL, glass or polyethylene (e.g., Plastipak, Becton, Dickinson and Co., Stanley St, Rutherford, NJ 07070, or equiv.)

(**e**) *Scintillation vials*.—20 mL glass with polyethylene, not Al, cap insert. Use as received.

Soak all vol. glassware overnight in detergent, scrub briefly, rinse well with hot tap $H_2O$, rinse with acetone, rinse with deionized $H_2O$, oven-dry at 105°, and rinse with appropriate soln before filling.

### C. Reagents

(**a**) *LC solvents*.—$H_2O$, MeOH, $CH_3CN$, all LC grade.

(**b**) *Methanol-acetonitrile diluent*.—MeOH–$CH_3CN$ (76 + 24). Use throughout as diluent for all $H_2O$ samples. Prep. using graduates, not vol. flasks. (Diln with this mixt., rather than MeOH alone, eliminates neg. peak which elutes just before HMX and may affect integration.)

### D. Calibration Standards

(**a**) *Reference stds*.—DNT, TNT, RDX, and HMX ref stds available from U.S. Army Toxic and Hazardous Materials Agency, Aberdeen Proving Ground, MD 21010, for federal contract work only. Reference stds for TNT and DNT available from Chem Service, Inc., 660 Tower La, PO Box 3108, West Chester, PA 19381-3108.

(**b**) *Individual stock std solns*.—Vac.-dry anal. ref. stds of DNT, TNT, RDX, and HMX at room temp. to const wt (±1 mg). Use vac. desiccator or vac. oven attached to $H_2O$ aspirator or vac. pump. For RDX and HMX, remove most of isopropanol by Pasteur pipet, air-dry for 3–4 h, then vac.-dry. Store std anal. ref. materials in desiccator over dry $CaCl_2$ or Drierite and place in dark when not in use.

Accurately weigh ca 0.1 g dried std onto weighing paper, transfer carefully to 250 mL vol. flask, and reweigh weighing paper. Record mass to 0.1 mg.

For DNT and TNT, dissolve and dil. to vol. with MeOH. For HMX and RDX, add 100 mL $CH_3CN$ to dissolve, then dil. to vol. with MeOH.

Wrap stoppered joint with Parafilm to protect against evapn. Calc. concn exactly in mg/L and label flasks. Store at ca 4° (not <0°).

(**c**) *Combined analyte stock soln.*—Remove stock std solns from refrigerator and let warm to room temp. (>30 min but not overnight). Invert flasks several times to mix. Into 1 L vol. flask, pipet 10.0 mL each of DNT and TNT stock std solns and 25.0 mL each of RDX and HMX stock std solns. Dil. to vol. with MeOH. This std soln contains ca 4.0 μg DNT and TNT/mL and ca 10.0 μg RDX and HMX/mL.

Calc. concns exactly in μg/mL; label and date flask. Wrap stoppered joint with Parafilm and store flask in refrigerator when not in use. Combined stock std soln can be used 1 week.

(**d**) *Working std solns.*—Prep. fresh each day as needed. Remove combined analyte stock std soln from refrigerator and let warm to room temp. (>30 min but not overnight). Invert flask several times to mix. Transfer 2.00, 5.00, 10.00, and 20.00 mL by pipet to four 250 mL vol. flasks, resp. Fill to mark with MeOH–$CH_3CN$ diluent. Stopper and invert 10 times to mix. Calc. concns exactly in μg/L; label and date flasks.

(**e**) *Injection std solns.*—For each working std soln, pipet 10.0 mL into scintillation vial. Add 10.0 mL $H_2O$ by pipet, cap, and shake to mix. Prep. blank by combining 10.0 mL MeOH–$CH_3CN$ diluent with 10.0 mL LC grade $H_2O$ in vial. Cap and mix. Label all vials appropriately.

Solns contain following concns in 10 mL aliquot:

| Aliquot vol. combined std, mL | Approx. concns, μg/L For DNT and TNT | For RDX and HMX |
|---|---|---|
| 2 | 32 | 80 |
| 5 | 80 | 200 |
| 10 | 160 | 400 |
| 20 | 320 | 800 |

These values represent concns before addn of $H_2O$. Actual concns are half as large. Solns are treated similarly: 1-to-1 diln is made by adding 10.0 mL MeOH–$CH_3CN$ diluent to 10.0 mL aq. sample. Thus, anal. results derived from working curve do not need to be corrected for this extra diln.

Make 10.0 mL MeOH–$CH_3CN$/$H_2O$ mixts in scintillation vials rather than in vol. flasks because of slight vol. contraction which would cause systematic error. Because std solns would be dild. with $H_2O$ to vol., and samples would be dild with org. solv. to vol., vol. contraction would lead to samples richer in org. solv. than are std solns. Take care at this step to pipet these 10.0 mL vols accurately because significant error at this stage is compounded when peak areas are measured.

### E. Liquid Chromatography

*Initial conditioning*—Follow procedure below for instrument warmup, except pass ≥30 void vols (ca 60 mL) mobile phase thru column. Continue until UV detector baseline is level when set to greatest sensitivity.

Calc. plate no. as follows:

Take 1 mL aliquot from combined working stock std soln and dil. to 100 mL in vol. flask with MeOH–$CH_3CN$. Use proper sample prepn and injection procedure described to obtain chromatogram. All 4 analytes should elute within 10 min. Use conditions described in *Apparatus*, but select chart speed that spreads peaks abnormally wide (such that widths at half ht are ≥2.0 cm). Est. peak width at half ht to nearest 0.1 mm. Calc. no. of theoretical plates *(N)* on column from each peak.

$$N = 5.54 \times (t_r/t_{0.5})^2$$

where $t_r$ = retention time and $t_{0.5}$ = width of peak at half ht, both in min.

Average results for all 4 analytes. If av. value is <3000 plates, carefully recheck calcn. If no error is apparent, let another 30 void vols of mobile phase wash thru column and repeat experiment. If calcd value of $N$ still does not exceed 3000, column is not performing to specification and should be replaced.

*Warmup procedure.*—Turn on all electronic equipment and let warm up >30 min. Pass >15 void vols of mobile phase thru column (20 min at 1.5 mL/min) and continue until UV detector baseline is level when set to greatest sensitivity. Make sure pumps are not experiencing vapor lock as indicated by large pressure fluctuations. Check system thoroly for leaks.

*Sample injection procedure.*—Fill analytical syringe with MeOH–$CH_3CN$ and discharge to waste. Repeat twice more to remove traces of previous sample. Rinse syringe 3 times with sample. Fill syringe with sample to >500 μL and pass most of this thru sample loop, avoiding introducing air bubbles. Overfilling loop in this manner assures that sample injected is not dild by solv. in loop.

### F. Preparation of Working Curve

Obtain chromatograms of 4 working stds and blank in duplicate (10 injections total). Sequence injections randomly. Plot peak area vs concn for each of the 4 analytes. Do not average duplicates before plotting. Inspect plot for gross deviations from linearity. Analytical response should be linear from 10 μg/L to 20 mg/L for DNT and TNT and from 25 μg/L to 50 mg/L for RDX and HMX. Significant deviation from linearity is evidence for systematic bias. Calc. regression line for each analyte.

### G. Analysis of Water Samples

Remove combined analyte working stock std solns from refrigerator and let warm to room temp. (>30 min but not overnight). Warm up instrument and condition LC column. Pipet 10.0 mL sample into scintillation vial. Add 10.0 mL MeOH–$CH_3CN$ diluent by pipet. Attach cap tightly. Shake vigorously. Let stand >15 min before filtration.

Load new Nuclepore (or Millex-SR) filter into filter holder. Rinse 25 mL filtration syringe with MeOH–$CH_3CN$ diluent, then fill to ca 10 mL with sample. Filter sample and discard filtrate. Fill syringe with remaining sample. Filter into new scintillation vial. Label vial appropriately.

Using proper procedure, inject these solns into liq. chromatograph. Typical retention times for HMX, RDX, TNT, and 2,4-DNT are 3.2, 4.1, 7.0, and 7.8 min, resp.

### H. Calculations

Prep. working curve for each analyte in the form, $y = b_o + b_1x$, where $x$ = analyte concn in μg/L and $y$ = peak area. Det. concn of each analyte in $H_2O$ sample by substituting measured peak area into calcn.

Refs.: U.S. Army Cold Regions Research and Engineering Laboratory (1984) CRREL Report 84-29, National Technical Information Service, Springfield, VA. Anal. Chem. **58,** 170(1986); **58,** 176(1986).

## 925.55 SALT

### A. Preparation of Sample—Procedure

If sample is coarser than "20 mesh," grind so that all will pass No. 20 sieve, but avoid undue grinding so that as much as possible will be retained on No. 80 sieve. Mix sample by quartering and weigh all needed portions as nearly at same time as possible.

Ref.: JAOAC **5,** 384(1922).

#### B. Moisture—First Action

Place ca 10 g sample in dry, weighed 200 mL erlenmeyer. Weigh flask and sample. Spread sample evenly over bottom of flask by shaking gently and insert small funnel in neck. Heat flask and sample for periods of 1 hr each at ca 250° until 2 consecutive weighings agree within 5 mg. Occasionally shake flask so that sample will dry evenly. Report loss of wt as $H_2O$.

#### C. Matters Insoluble in Water—First Action

Place 10 g sample in 250 mL beaker, add 200 mL $H_2O$ at room temp., and let stand 30 min, stirring frequently. Filter thru weighed gooch with asbestos mat dried at 110°. Transfer residue to gooch with aid of policeman, using total of ≤50 mL $H_2O$. Wash residue with ca ten 10 mL portions $H_2O$, until 10 mL filtrate shows only faint opalescence upon addn of few drops $AgNO_3$ soln. Dry crucible and contents to const wt at 110°. Report increase in wt gooch as "matters insol. in $H_2O$" and report results in % on $H_2O$-free basis. If matters insol. in $H_2O$ are >0.1%, det. their nature.

#### D. Matters Insoluble in Acid—First Action

Treat 10 g sample with 200 mL HCl (1 + 19), boil 2–3 min, and let stand 30 min, stirring frequently. Filter thru gooch with mat dried at 110°. Wash, dry at 110°, cool, and weigh. Express results in %.

Refs.: JAOAC **5,** 385(1922); **6,** 129(1923).

#### E. Preparation of Solution for Sulfate, Calcium, and Magnesium—Procedure

Weigh ca 20 g sample, transfer to 400 mL beaker, and dissolve in 200 mL HCl (1 + 3). Cover beaker, heat to bp, and continue boiling gently 10 min. Filter thru paper and wash residue with small amts of hot $H_2O$ until filtrate is Cl-free. Unite filtrate and washings, cool, and dil. to 500 mL (*Soln X*).

#### F. Sulfate—First Action

Place 250 mL *Soln X*, **925.55E**, in 400 mL beaker, heat to bp, and add slight excess hot 10% *$BaCl_2$ soln* dropwise while stirring. Conc. by heating gently and finally evap. to dryness on steam bath. Facilitate removal of free acid by stirring partly dried residue. Wash ppt by decantation with small amts of hot $H_2O$, finally transferring ppt to close-grain filter paper with aid of policeman and stream of hot $H_2O$. Test filtrate for presence of Ba. Wash ppt on paper until filtrate is Cl-free. Dry and ignite paper contg ppt over Bunsen flame. Report % $SO_4$ in sample on $H_2O$-free basis.

#### G. Calcium—First Action

Place remainder of *Soln X* in 400 mL beaker. Add excess of 10% *$H_2C_2O_4.2H_2O$ soln* (10 mL usually is enough). Add few drops Me orange; neutze while hot by adding $NH_4OH$ dropwise, stirring constantly. Add ca 1 mL excess $NH_4OH$, stir, and let stand in warm place 3 hr. Decant supernate thru filter, reserving filtrate for detn of Mg. Test filtrate for Ca with $(NH_4)_2C_2O_4$ soln. Wash ppt in beaker once with 10 mL 1% *$(NH_4)_2C_2O_4$ soln*, decanting thru filter paper. Combine filtrate and washings. Dissolve ppt on paper with hot HCl (1 + 1), using same beaker; dil. to 100 mL, add little more $H_2C_2O_4$ soln, and ppt as before. Let stand 3 hr, filter, and wash with 1% $(NH_4)_2C_2O_4$ soln as before, reserving filtrate and washings. Transfer ppt to crucible, dry, ignite, and heat over blast lamp to const wt (CaO). Report as % Ca on $H_2O$-free basis.

#### H. Magnesium—First Action

Combine filtrates and washings from Ca detn, conc. if necessary by boiling gently to ca 150 mL, and proceed as in **920.200**. Report as % Mg on $H_2O$-free basis.

#### I. Lead

*See* **934.07A** and **B**.

## 925.56 Iodine in Iodized Salt

### Titrimetric Method

### Final Action

#### A. Reagents

(**a**) *Bromine water*.—(*Caution:* See safety notes on bromine.) For alternative method, **925.56C(b)**, det. approx. concn (mg Br/mL) by adding measured vol. from buret to flask contg 50 mL $H_2O$, 5 mL 10% KI soln, and 5 mL $H_2SO_4$ (1 + 9), and titrg liberated I with 0.1$N$ $Na_2S_2O_3$.

(**b**) *Sodium thiosulfate*.—0.005$N$. Prep. daily by dilg 0.1$N$ soln, **942.27**.

(**c**) *Starch soln*.—1% (freshly prepd). Mix ca 1 g sol. starch with enough cold $H_2O$ to make thin paste, add 100 mL boiling $H_2O$, and boil ca 1 min with stirring.

(**d**) *Potassium iodide control soln*.—0.3270 g KI/250 mL. Dil. 50 mL to 250 mL, and use 5 mL (= 1.0 mg I and 1.308 mg KI) for control.

#### B. Preparation of Sample

Dissolve 50 g sample in $H_2O$ and dil. to 250 mL in vol. flask. Take 25, for **925.56C(a)**, or 50 mL, for **925.56C(b)**, aliquot for analysis.

#### C. Determination

(**a**) *Application when $Na_2S_2O_3$ content is ≤0.5%*.— Place sample aliquot in 600 mL beaker and dil. to ca 300 mL. Neutze to Me orange with $H_3PO_4$ and add 1 mL excess. Proceed as in **935.14**, third par.

(**b**) *Alternative method. Not applicable in presence of $Na_2S_2O_3$*.—Pipet 50 mL sample soln into 200 mL erlenmeyer. Neutze to Me orange with 2$N$ $H_2SO_4$. Add Br-$H_2O$ dropwise from buret in amt equiv. to 20 mg Br. After few min destroy greater portion of remaining free Br by adding 1% $Na_2SO_3$ soln *dropwise while mixing*. Wash down neck and sides of flask with $H_2O$ and complete removal of Br by adding 1 or 2 drops 5% phenol soln. Add 1 mL 2$N$ $H_2SO_4$ and 5 mL 10% KI soln, and titr. liberated I with $Na_2S_2O_3$ soln, adding 1 mL starch indicator near end of titrn. Correct detn for blank on reagents and make 1 or more control detns, using 50 mL 20% reagent grade NaCl soln to which has been added appropriate amts of dil. control KI soln. 1 mL 0.005$N$ $Na_2S_2O_3$ = 0.1058 mg I and 0.1384 mg KI.

Refs.: Biochem. Z. **138,** 383(1923); **174,** 364(1926). JAOAC **26,** 440(1943).

CAS-7553-56-2 (iodine)

## 925.57 Constituents in Salt

### Method of Reporting Results

### Procedure

(In absence of added drying agents such as $MgCO_3$, Ca phosphate, etc.)

Convert sulfate to $CaSO_4$ and unused Ca to $CaCl_2$, unless sulfate in sample exceeds amt necessary to combine with Ca, in which case convert Ca to $CaSO_4$ and unused sulfate first to $MgSO_4$ and remaining sulfate, if any, to $Na_2SO_4$. Convert unused Mg to $MgCl_2$. Add percentages of $CaCl_2$ and $MgCl_2$. Report on $H_2O$-free basis % of matter insol. in $H_2O$, of $SO_4$, of Ca, of Mg, of $CaSO_4$, of $CaCl_2$, and of $MgCl_2$. Also report results of qual. examination of matters insol. in $H_2O$, if amt is >0.1% on $H_2O$-free basis.

# 12. Microchemical Methods

## 969.47 Molecular Weight (MW)
### Thermoelectric-Vapor Pressure Method
**First Action 1969**
**Final Action 1975**

(Applicable to materials with MW <500)

### A. Apparatus and Reagents

(**a**) *Molecular weight apparatus.*—Vapor pressure osmometer, Hewlett-Packard; isothermal distn app., Thomas Scientific; or equiv. equipment using vapor pressure equilibrium technic. Instrument must use sensitive bridge system to measure temp. difference between solv. and test soln drops suspended on thermistors in const temp. cell whose atm. is satd with solv. vapor.

(**b**) *Standards.*—Benzil, MW 210.23, mp 94.5–95.5°, and C and H analyses within 0.2% of theoretical values (C, 79.99; H, 4.79). Recrystallize from EtOAc, acetone, or $CHCl_3$, if necessary.

If sample is ionizable salt sol. in $H_2O$, use reagent grade KCl as std. If sample is not a salt and sol. only in $H_2O$, use sucrose as std.

(**c**) *Solvents.*—Use reagent grade solv. from same lot and preferably from same bottle to sat. cell and to prep. sample and std solns. Solv. must completely dissolve sample, preferably without heating. (Proper choice of solv. and std is critical.) Preferred solvs are (number indicates order of choice):

| | Nature of Sample | | | | |
|---|---|---|---|---|---|
| Solvent | Unknown | Neutral | Acidic | Basic | Salt |
| Acetone | 2 | 1 | 1 | – | – |
| Ethyl acetate | 1 | 2 | 2 | 1 | – |
| Chloroform | 3 | 3 | – | 2 | – |
| Water | – | – | – | – | 1 |

For samples not sol. in solvs listed, test solubility in $H_2O$ (if thermistor wiring is completely encased in glass or plastic), *n*-heptane, and benzene. Other solvs that may be used are: alcohol, $CCl_4$, methylethyl ketone, dioxane, cyclohexane, $CH_2Cl_2$, dimethyl formamide, toluene, and $CH_3CN$. Use solvs such as esters, ketones, or alcohols for samples which tend to form dimers thru H bonding, e.g., org. acids.

### B. Determination

Follow manufacturer's instructions, including recommended concn range for solns, instrument operation, and reading of $\Delta R$ response.

Adjust cell temp. so vapor pressure of solv. is 150–350 mm, preferably 200–300 mm. If instrument is not equipped to cool cell, cell temp. must be enough above ambient (ca 5°) so thermostatic control maintains const cell temp.

Construct calibration curve with std and solv. to be used in analysis. Det. $\Delta R$ response at 4 std concns in recommended range and plot $\Delta R$ against mole fraction (MF). Prep. sample soln in recommended range and obtain 3 $\Delta R$ readings. Use median $\Delta R$ value to calc. MW. If calibration curve is straight line, calc. MW of sample by:

$$MW = (\text{g solute})(\text{MW solv.})(K - \Delta R)/(\Delta R)(\text{g solv.})$$

where $K = (\Delta R \text{ std})/(\text{MF std})$ and

$$\text{MF std} = [(\text{g std})/(\text{MW std})]/[(\text{g std}/\text{MW std}) + (\text{g solv.}/\text{MW solv.})]$$

If $\Delta R$–MF plot yields curved line, interpolate MF of sample from calibration curve and calc. MW by:

$$MW = (\text{g solute})(\text{MW solv.})(K - \Delta R)/(\Delta R)(\text{g solv.})$$

where $K = (\Delta R \text{ std})/(\text{MF std})$ and

$$\text{MF std} = [(\text{g std})/(\text{MW std})]/[(\text{g std}/\text{MW std}) + (\text{g solv.}/\text{MW solv.})]$$

If $\Delta R$–MF plot yields curved line, interpolate MF of sample from calibration curve and calc. MW by:

$$MW = (\text{g solute})(\text{MW solv.})(1 - \text{MF})/(\text{MF})(\text{g solv.})$$

Refs.: JAOAC **51,** 992, 1231(1968).

## 952.24 Microchemical Determination of Bromine, Chlorine, or Iodine
### Carius Combustion Method
**Final Action**

(Do not alter combustion conditions such as temp., size of sample, vol. of acid, etc. Variations from specified conditions present dangerous explosion hazard.)

### A. Reagents

(**a**) *Fuming nitric acid.*—Reagent grade, halogen-free, sp gr 1.50.

(**b**) *Silver nitrate.*—Reagent grade, powd.

### B. Apparatus

(**a**) *Combustion tubes.*—Fig. **952.24**. Use clean, heavy- or std-wall Pyrex tubes, free from flaws, with round seal at bottom, and with following specifications. (Vol. $HNO_3$ and temp. depend on combustion tube used.)

| Dimensions | Heavy-Wall | Std-Wall |
|---|---|---|
| Wall thickness, mm | 2.3±0.3 | 1.2±0.2 |
| Outside diam., mm | 13±0.8 | 13±0.7 |
| Length, mm | 210±10 | 240±10 |
| Length of sealed tube between bottom and start of taper at shoulder, mm | 150–175 | 180–210 |
| Vol. $HNO_3$ (sp gr 60°F, ca 1.5), mL | 0.5 | 0.3 |
| Temp., °C | 250 | 300 |

(**b**) *Furnace.*—Elec., to hold ≥4 tubes at ca 45° angle. Must maintain temp. of 250 ± 10° or 300 ± 10° for ≥5 hr, with ≤5° difference between any 2 points on a tube or ≤5° difference between similar points on any 2 tubes. Must have variable resistor or other device to adjust furnace to desired temp. Open end of furnace wells must have safety device to retain glass in furnace in case tube explodes, and device must be provided for removing individual tubes from wells.

(**c**) *Filter tubes.*—Micro 3 mL filter tube with medium-coarse porosity fritted disk (av. pore diam. 15–25 μm).

(**d**) *Siphon.*—Make from 3 mm od glass tubing, with parallel arms, one 50 and other 250 mm long, and with 110 mm connecting section rising with 13° slope to longer arm.

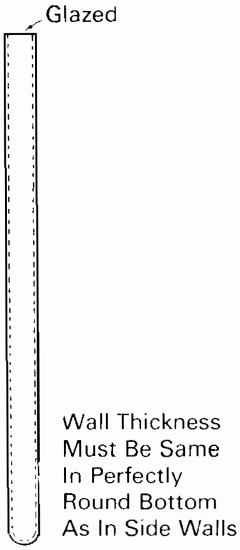


**FIG. 952.24—Combustion tube**

### *C. Sample*

Using microchem. balance, weigh 5–20 mg sample contg min. of 1.5 mg Cl, 2.5 mg Br, or 3.2 mg I; or using semimicrochem. balance, weigh 10–20 mg sample contg min. of 2.5 mg Cl, 4.5 mg Br, or 5.7 mg I.

(**a**) *Solid samples.*—Weigh by difference in weighing tube.

(**b**) *Viscous liquids or gummy solids.*—Weigh in porcelain boat.

(**c**) *Volatile liquids.*—Weigh in 5 cm sealed glass tube, 1–2 mm id with capillary tip. Break off tip of capillary before placing in combustion tube, sealed end down.

### *D. Determination*

Place weighed sample in combustion tube, add powd $AgNO_3$ 100% in excess of amt estd to be necessary, and add 0.5 ± 0.05 or 0.3 ± 0.03 mL fuming $HNO_3$, depending on type of combustion tube, **952.24B(a)**. Using blast lamp and holding at 30–40° angle, slowly rotate tube in flame until wall thickens, pull out, and seal off narrow neck of tube. Wall of seal should be $\geq ^3/_4$ of thickness of tube wall and sealed tube should have length shown in table. (If sample and $HNO_3$ react at room temp., immediately cool bottom of tube in ice-$H_2O$ or solid $CO_2$-acetone bath, remove, and seal at once.) Immediately place tube in furnace and heat 5 hr at 250 or 300 ± 10°, according to tube size.

Observe following precautions before and during opening of combustion tubes: (*a*) Place asbestos glove on hand used to hold small burner or hand torch; (*b*) protect face by transparent face mask or work behind safety shield; (*c*) be certain tube has cooled to room temp.; (*d*) force tip of tube 2–5 cm out of furnace well; (*e*) gently flame end to drive all acid from tip and upper walls; and (*f*) soften tip with small hot flame until pressure in tube is released by blowing out softened glass.

Remove vented tube from furnace and cut off constricted end by scratching tube with file ca 1 cm from shoulder of open end, moistening scratch, and touching with tip of very hot glass rod. Remove end of tube with care and fire polish to avoid contaminating ppt with glass splinters.

Rinse walls of tube with $H_2O$ until tube is ca $^3/_4$ full, place in steam or boiling $H_2O$ bath, protected from light, and digest until ppt coagulates (ca 30 min). Longer digestion is required for I than for Br or Cl since eutectic mixt. of $AgNO_3$ and AgI is formed, which melts below temp. of steam bath and persists as heavy yellow oil on bottom of tube. Stirring with glass rod speeds up soln of $AgNO_3$ and greatly reduces digestion time, which *must* be continued until ppt is in form of *fine powder*. If excessive amts of $AgNO_3$ have been used, greater dilns than specified are required for complete pptn. Therefore, after digestion appears complete, pipet few drops of clear supernate aq. soln into test tube contg several mL $H_2O$. If turbidity occurs, entire supernate must be dild with $H_2O$ until pptn stops, and digestion to coagulate ppt must be repeated. If no turbidity occurs on diln, pipetted portion may be discarded.

Place previously washed, dried, and weighed filter tube in 1-hole stopper in suction flask, connect short arm of siphon tube to filter tube thru small rubber stopper, and adjust tube so that long arm of siphon almost touches ppt. Transfer ppt to filter tube by suction. Rinse tube and ppt alternately with 1% $HNO_3$ and alcohol, using 2 or 3 mL portions for each rinse.

Remove siphon, rinse tip and stopper with alcohol, and rinse filter tube and ppt first with the acid, then with alcohol. Wipe outside of filter tube with moist chamois (or cheesecloth) and dry 30 min at 125° in air oven or 30 min at 80° in vac. oven; cool to room temp. (ca 30 min) and weigh. Handle dry tube with chamois finger cots or tweezers. Make blank detn and subtract any correction from wt sample ppt.

$$\% \text{ Cl} = (\text{wt ppt} - \text{blank}) \times \frac{\text{Cl}}{\text{AgCl}} \times \frac{100}{\text{wt sample}}$$

$$\% \text{ Br} = (\text{wt ppt} - \text{blank}) \times \frac{\text{Br}}{\text{AgBr}} \times \frac{100}{\text{wt sample}}$$

$$\% \text{ I} = (\text{wt ppt} - \text{blank}) \times \frac{\text{I}}{\text{AgI}} \times \frac{100}{\text{wt sample}}$$

Refs.: JAOAC **35**, 291(1952); **36**, 91, 319(1953); **40**, 381 (1957); **41**, 297(1958). Anal. Chem. **21**, 1555(1949); **23**, 1689(1951).

CAS-7726-95-6 (bromine)
CAS-7782-50-5 (chlorine)
CAS-7553-56-2 (iodine)

## 974.36 Microchemical Determination of Bromine, Chlorine, or Iodine

**Oxygen Flask Combustion Method**

**First Action 1974**
**Final Action 1975**

### *A. Apparatus and Reagents*

(**a**) *s-Diphenylcarbazone indicator.*—1.5% alc. soln. Heat to dissolve, if necessary. Prep. fresh daily.

(**b**) *Bromophenol blue indicator.*—0.05%. Dil. 5 mL 1% alc. soln to 100 mL with alcohol.

(**c**) *Mercuric nitrate std soln.*—Dissolve 1.7 g $Hg(NO_3)_2.H_2O$ in 500 mL $H_2O$ contg 2 mL $HNO_3$ and dil. to 1 L. Adjust to pH 1.7, using pH meter, by adding $HNO_3$ dropwise. Stdze as follows: Accurately weigh 4–6 mg KCl and transfer to 250 mL erlenmeyer. Add 20 mL $H_2O$ and 80 mL alcohol, and stir mag. at moderate speed. Add 5 drops bromophenol blue indicator and 0.5*N* $HNO_3$ to yellow end point; then add 3 drops excess. Add 5 drops *s*-diphenylcarbazone indicator and titr. at $\leq$5 mL/min with Hg-$(NO_3)_2$ std soln to orchid-pink. Subtract reagent blank. Repeat stdzn $\geq$3 times.

$$\text{Normality} = \text{mg KCl}/[74.551 \times \text{mL Hg(NO}_3)_2]$$

(**d**) *Hydrogen peroxide soln.*—30%.

(**e**) *Hydrazine sulfate soln.*—Satd aq. soln.

(**f**) *Buret.*—Graduated to 0.01 mL. Tip should be fine enough that 1 drop is ca 0.015 mL.

### *B. Determination*

Accurately weigh sample contg 1.5–3 mg Cl, 3–6 mg Br, or 6–9 mg I and fold in paper carrier. Insert carrier in Pt holder in stopper of 500 mL Schöniger flask, **975.53B(b)**. Add 2.0 mL 0.5*N* KOH, 4 drops satd aq. hydrazine sulfate soln, and 10 mL $H_2O$ to flask. Flush flask ≥3 min with rapid stream of O. Add 1 drop long-chain alcohol (e.g., dodecanol) to carrier in basket (not on tail) just before combustion. Ignite carrier and immediately insert into flask. (*Caution:* Use safety barrier and reinforced gloves. Remote control igniting device is available.) After combustion is complete, shake stoppered flask 10 min or until all visible cloudiness disappears. Let stoppered flask stand 5 min at room temp. Add ca 3 mL $H_2O$ at funnel portion of stoppered flask as $H_2O$ seal and stopper wash. Remove stopper, and rinse stopper, Pt holder, and flask walls with ca 15 mL $H_2O$. Add 8 drops 30% $H_2O_2$ to flask and boil until small bubbles no longer evolve (ca 10 min). (Do not let contents go to dryness. Add $H_2O$ if necessary.) Cool to *room temp.* and proceed within 5 min.

Rinse walls of flask with enough $H_2O$ to bring to ca 75 mL; then add 150 mL alcohol. Stir mag. at moderate speed. Add 15 drops bromophenol blue indicator, and proceed as in stdzn, (**c**), beginning ". . . and 0.5*N* $HNO_3$ . . .", taking as end point change in color from faint yellow to orchid-pink. Subtract paper blank from vol. used. Typical blank is 0.15 mL $Hg(NO_3)_2$.

$$\% \text{ Halogen} = [\text{mL } Hg(NO_3)_2 \times \text{atomic wt of halogen} \times \text{normality} \times 100]/\text{mg sample}$$

Refs.: JAOAC **56,** 888(1973); **57,** 26(1974).

CAS-7726-95-6 (bromine)
CAS-7782-50-5 (chlorine)
CAS-7553-56-2 (iodine)

## 949.12 Microchemical Determination of Carbon and Hydrogen
**Combustion Method**
**Final Action**

### *A. Reagents*

(**a**) *Copper oxide.*—Wire form, ca 1 mm diam. and 3–4 mm long; discard material finer than "20-mesh." Ignite 1 hr at 800–900° before placing in combustion tube.

(**b**) *Platinum gauze, 52 mesh.*—From three 3 × 5 cm sections, make 3 rolls, each 30 mm long × 7 mm od. Boil 15 min in $HNO_3$ (1 + 1) and ignite in nonluminous Bunsen flame.

(**c**) *Asbestos.*—Gooch asbestos; ignite 30 min at 800–900° and store in wide-mouth bottle. (*Caution:* See safety notes on asbestos.)

(**d**) *Silver.*—Fine wire or ribbon; if tarnished, reduce in stream of H at 350–450°. (*Caution:* H ignites explosively in O. Flush reduction app. with $CO_2$ or N before and after H use. Vent exhaust gas into effective fume removal device. Perform reduction behind safety barrier.)

(**e**) *Lead dioxide.*—Pellets, 1–2 mm diam., special grade for microanalysis; or prep. by digesting com. grade powder 2 hr in $HNO_3$, let stand 1 hr, decant, wash with $H_2O$ until acid-free, evap. to dryness, and cut into 2 mm cubes. Roll cubes in jar to round corners and sieve out powder.

(**f**) *Glass wool.*—Pyrex, pliable.

(**g**) *Dehydrite or Anhydrone.*—($Mg(ClO_4)_2$, anhyd.) Break pieces to <3 mm long; discard portion passing thru No. 40 sieve.

(**h**) *Ascarite.*—(NaOH on asbestos.) Use com. prepn of "8–20 mesh."

### *B. Apparatus (See Fig. 949.12)*

(**a**) *Oxygen.*—Cylinder with pressure regulator adjustable from 0 to 10 lb pressure (69 kPa) on low-pressure side and with needle-valve control.

(**b**) *Preheater.*—Specifications as recommended by Committee on Microchemical Apparatus, Div. Anal. Chem. (Anal. Chem. **21**, 1555(1949)), except with 12/2 ball joint. Rubber connectors may be used.

(**c**) *Bubble counter and U-tube.*—According to recommended specifications, except with ball joints. Rubber connectors may be used.

(**d**) *Combustion tube.*—Fused quartz (or Vycor), dimensions according to recommended specifications but with 12/2 ball joint on side arm and 5/12 or 7/15 inner joint on exit end. Rubber connectors may be used. Pyrex glass tubes may be used, but furnace temps should be ≤725°.

(**e**) *Absorption tubes.*—Pregl-type, according to recommended specifications but with 5/12 joints; alternatively, Prater-type, semimicro size with 7/15 joints. Rubber connectors may be used.

(**f**) *Bubble counter or flowmeter.*—Any convenient arrangement to measure 10–30 mL/min gas flow from exit end of second absorption tube.

(**g**) *Preheater furnace.*—Elec., 12–14 mm id × 13 cm (5″) long, maintained at 600 ± 25°. Gas heaters may be used for all furnaces but specified temps should be maintained. Temps of furnaces are measured at center of furnace inside empty combustion tube with one end stoppered.

(**h**) *Burning furnace.*—Elec., 13–14 mm id × 10 cm (4″) long. Furnace should reach 600–700° in 5 min, ca 800° in 15 min, with max. of 850° in 30 min. *See* (**g**).

(**i**) *Long furnace.*—Elec., 13–14 mm id × 20 cm (8″) long; maintained at 775–800°. *See* (**g**).

(**j**) *Constant temperature mortar.*—Elec., 13–14 mm id × 8 cm (3″) long, thermostatically controlled at 177 ± 2°. *See* (**g**).

### *C. Preparation of Apparatus*

(**a**) *Preheater.*—Place CuO in preheater tube, connect spiral cooling coil, immerse coil in beaker of $H_2O$, and support assembly by suitable clamps and stand. Place furnace over preheater tube and maintain at ca 600°. Connect side arm of combustion unit to needle valve of O pressure regulator by suitable tubing, rubber or Tygon.

(**b**) *Bubble counter-U-tube.*—Fill bubble counter and U-tube by placing glass wool plug at bottom of U, fill side next to bubble counter with Dehydrite to within 12 mm of side arm, and cap with another glass wool plug. Place Ascarite layer in other side to within 38 mm of side arm; then insert glass wool plug, ca 25 mm of Dehydrite, and finally second plug. Cement in stoppers with glass cement or paraffin; then with medicine dropper add $H_2SO_4$ to bubbler until level is 3–4 mm above bubbler tip. Connect to preheater with pressure clamp.

(**c**) *Combustion tube.*—Clean and dry combustion tube. Place 10 mm roll of Ag in exit end with 1 or 2 strands reaching to open end of ground joint. Insert loose asbestos plug (not choking plug), 40 mm $PbO_2$, asbestos plug, and second Ag roll 25 mm long, which should extend into long furnace ca 12 mm. Insert asbestos plug, 60 mm CuO, asbestos plug, 30 mm Pt gauze roll, asbestos plug, 60 mm CuO, asbestos plug, and finally 30 mm Pt gauze, which should extend about 10 mm beyond end of long furnace. Place prepd tube in furnaces with exit end protruding far enough beyond const temp. mortar to permit connecting absorption tubes. Connect side arm to bubble counter-U-tube.

(**d**) *Absorption tubes.*—Place glass wool plug in end of $H_2O$ absorption tube, fill tube to within 12 mm of other end with

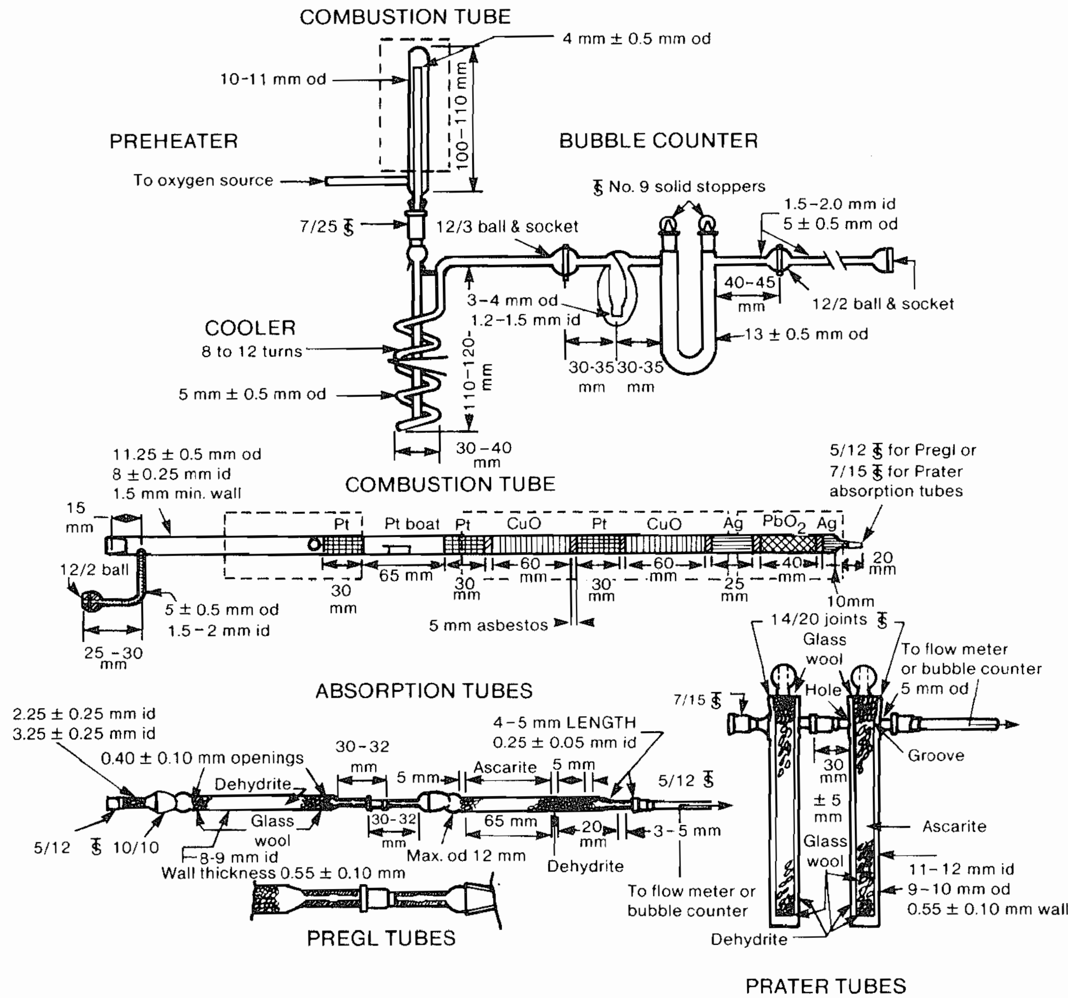


**FIG. 949.12—Carbon and hydrogen apparatus**

Dehydrite or Anhydrone, and cap with second glass wool plug. If Pregl tubes are used, seal ground-glass joint with enough glass cement to give clear seal, and remove any excess on outer surface of tube with cotton dipped in benzene or other solv. If Prater tubes are used, lubricate lower $^2/_3$ of inner joint with min. of light stopcock grease and insert in outer tube.

Prep. $CO_2$ absorption tube by placing glass wool plug in end and fill tube to within ca 38 mm of other end with Ascarite. Insert 6 mm glass wool plug, add 20 mm layer of Dehydrite, and cap filling with another glass wool plug. Complete assembly of absorption tube as for $H_2O$ absorption tube. Connect absorption tubes to combustion tube with ground joints (use no lubricant) or with special impregnated rubber tubing.

Attach calibrated bubble counter or flowmeter to exit end of $CO_2$ absorption tube.

### D. Determination

(**a**) *Conditioning apparatus.*—Condition prepd and assembled app. by heating combustion tube 3–4 hr with long furnace at 775–800° and with O flowing thru app. at rate of 15–20 mL/min. Use 3–4 lb (21–28 kPa) O pressure on low pressure side of regulator. At the same time, make 2 simulated sample burnings, without sample, with burning furnace at 825–850°. (Temp. must be ca 100° lower if Pyrex combustion tubes are used.)

Burn unweighed 10–15 mg sample to condition combustion and absorption tubes. With absorption tubes connected, adjust needle valve on regulator so that O flow is 15–20 mL/min and place burning furnace ca 75 mm from long furnace. Place micro Pt boat contg sample in combustion tube ca 50 mm from long furnace. Insert third Pt roll 25 mm from boat, and stopper tube. Turn on burning furnace and let it reach ca 600° before starting sample combustion by moving furnace over sample at rate of 25 mm in 6–8 min. Move burning furnace across sample only once, taking 18–24 min for full travel of furnace. Turn off burning furnace 5 min after it reaches long furnace but continue to sweep O thru tube for addnl 15 min before disconnecting absorption tubes.

Remove absorption tubes and place by balance to equilibrate. Handle tubes only with clean chamois finger cots. If Prater tubes are used, turn joints $^1/_4$ turn to seal. If rubber connections are used, wipe only tips of tubes with moist, then dry, chamois before placing them by balance. Wait 10 min if ground joints were used or 15 min if rubber connections were made; then weigh $CO_2$ absorption tube first and $H_2O$ absorption tube next, using glass tare with vol. and surface approx. equal to that of absorption tubes. Record wts of tubes and reconnect to combustion tube for subsequent analysis.

(**b**) *Proving the apparatus.*—Replace boat with one contg 10–15 mg sample of std compd such as NIST microchem. std, weighed to nearest 0.01 mg. Repeat combustion and weighing as in (**a**). Calc. % C and H in std sample from increase in wt of $CO_2$ and $H_2O$ absorption tubes. Repeat analysis until results from 2 consecutive runs are within 0.30% of theoretical values and means of C and H results are within 0.20% of theoretical value for the std compd. (Humidity conditions of room may make it necessary to correct apparent wt of $H_2O$ by subtracting a blank value.)

When app. meets this test, analyze samples as above.

$$\%C = \text{wt } CO_2 \times 0.2729 \times 100/\text{wt sample}$$
$$\%H = \text{wt } H_2O \times 0.1119 \times 100/\text{wt sample}$$

Refs.: JAOAC **32,** 561(1949); **34,** 94, 607(1951). Anal. Chem. **23,** 911(1951).

CAS-7440-44-0 (carbon)
CAS-1333-74-0 (hydrogen)

## 972.43 Microchemical Determination of Carbon, Hydrogen, and Nitrogen
### Automated Method
**First Action 1972**
**Final Action 1975**

#### A. Apparatus

(See instrument instruction manuals)

(**a**) *Automatic carbon-hydrogen-nitrogen (C-H-N) analyzer.*—Model 185 (FM) (current model 185B, Hewlett-Packard), Perkin-Elmer 240 (PE) (current model 240B, or 240C 2400 CHN Elemental Analyzer, Perkin-Elmer Corp.), or equiv.

(**b**) *Helium.*—Cylinder with pressure regulator and needle valve control. Preheater and purifier optional.

(**c**) *Oxygen.*—For PE only. Cylinder with pressure regulator and needle valve control.

(**d**) *Line voltage regulator.*—Optional; 50 amp, output 115 v ± 0.25%.

(**e**) *On-line computer or integrator.*—Mandatory for FM app. but optional for PE app.

#### B. Reagents

(*See* instrument instruction manuals.)

(**a**) *Catalyst.*—Solid oxidn catalyst (oxides of Co, W, Mn, or Ag) required for FM; optional for PE if time and temp. meet conditions specified.

(**b**) *Std compounds.*—NIST Acetanilide, or equiv.

#### C. Preparation of Apparatus

Prep. and assemble app. as in manual. Adjust preliminary settings and regulate He flow (He and O for PE). Set and let temp. systems equilibrate until const. Use combustion temp. >1080° for FM and 980–1000° for PE. Use specified 500° and 650° reduction temps, resp., for FM and PE. Maintain detector column suboven within 5–15° of main oven. Adjust bridge current to value specified. After sweeping air "slug" from combustion chamber, use 20–50 sec range combustion period (gas flow diverted) for FM (40–50 sec for samples difficult to burn). Use extended "Hold 30 sec" combustion period for PE. Add $Co_3O_4$ + $Ag_2WO_4$, $Ag_2O$ + $Ag_2WO_4$, or CoO + $WO_3$ to combustion tube filling of PE. However, if PE is in optimum condition, only 2 of 3 required conditions (temp., time, and catalyst) need be adhered to.

#### D. Determination

Burn 2 unweighed samples ca 2 mg (PE) or 0.6 mg (FM) to condition app. Make ≥2 blank runs (simulated sample runs without sample) to check and adjust timing of each phase where necessary, to check pattern of final measurements, and to obtain blank factors if required in calcns. Then run std and sample compds, weighed to nearest 0.001 mg or better for PE and 0.0001 mg for FM. Calc. factors as suggested in manual. Rerun std to check factors. Different type std may be used for this rerun. Initially check factors until 2 of 3 detns are within 0.3% of theoretical value. Calc. % C, H, and N, using factors obtained from std compds.

#### E. Special Precautions for Volatile Samples

Weigh volatile samples in capillaries, Al capsules, or Al weighing pans. During sweeping period, volatile samples must be in cooler portion of combustion tube, as near orifice as possible.

Refs.: JAOAC **54,** 808(1971); **55,** 676(1972).

CAS-7440-44-0 (carbon)
CAS-1333-74-0 (hydrogen)
CAS-7727-37-9 (nitrogen)

## 961.16 Microchemical Determination of Fluorine
### Titrimetric Method
**First Action 1961**
**Final Action 1969**

#### A. Reagents

(**a**) *Sodium alizarin sulfonate indicator.*—(Alizarin Red S) 0.035% aq. soln.

(**b**) *Sodium fluoride std soln.*—0.01*N*. Dissolve 0.4200 g NaF in $H_2O$ and dil. to 1 L.

(**c**) *Thorium nitrate std soln.*—0.01*N*. Dissolve 1.38 g $Th(NO_3)_4.4H_2O$ in $H_2O$ and dil. to 1 L. Stdze by titrg against 0.01*N* NaF, using 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mL portions and plotting curve.

#### B. Apparatus

(**a**) *Schöniger combustion flask.*—500 mL with filter paper carriers.

(**b**) *Distillation apparatus.*—*See* Fig. **961.16**. Attach to steam generator. Steam enters thru joint, $J_1$, passes thru 2 concentric tubes, $IT_1$ and $ET_1$, and enters distn flask, *D*, thru 2 openings. Vapors enter condenser, *C*, which consists of 3 concentric tubes. In $IT_2$ and $ET_2$, vapors are condensed; in $ET_3$, cooling $H_2O$ is circulated. Distillate drains off on right thru descending tube. Ground joint, $J_2$, serves as opening for addn of soln and as seat for thermometer which records temp. of liq., *L*. Elec. heating jacket, *H*, surrounds section of distn flask contg liq. and is prepd from 600 cm Nichrome wire, *W*, of 2.120 ohms/foot, 420 cm of which is wound on 48 mm diam. glass cylinder covered with Al foil and asbestos, then covered with insulating cement, asbestos, and another layer of cement. Jacket is held in place with ring, *R*, and temp. is controlled with 7.5 amp variable transformer.

#### C. Determination

(**a**) *In absence of arsenic, mercury, and phosphorus.*—Place sample contg 0.5–0.7 mg F on filter paper carrier. (Weigh liq. samples in gelatin or Me cellulose capsules and place closed capsule on paper carrier.) Add ca 15–20 mg $Na_2O_2$, wrap mixt. in filter paper, and place in Pt basket carrier in stopper of Schöniger flask. Place 20 mL $H_2O$ in flask, introduce O several min, ignite sample, and immediately insert into flask. (*Caution:* Use safety barrier and reinforced gloves. Remote control igniting device is available.) After combustion is complete, shake vigorously until cloudiness disappears, and let flask stand undisturbed ca 15 min to ensure complete absorption of oxidn products. (If enough I is present to give yellow soln, warm on steam bath to dispel color.) Wash soln into titrg vessel, adjust to pH 3.0 ± 0.05 with 1*N* and 0.1*N* HCl and 0.1*N* NaOH, using pH meter, and add 2 mL Na alizarin sulfonate indicator. (pH adjustment is critical, since alizarin sulfonate is also acid-base indicator.) Titr. with std $Th(NO_3)_4$ soln to pink end point, preferably using photoelec. photometer with 520 nm filter. Use entire soln rather than aliquot. If visual titrn is used, compare color with controls in fluorescent light. Det. mg F from std curve.

$$\% \text{ F} = \text{mg F} \times 100/\text{mg sample}$$

**(b)** *In presence of arsenic, mercury, and phosphorus.*—Burn sample as in **(a)** and transfer soln to distn app. thru joint $J_2$ with as little $H_2O$ as possible. Add 20 mL 70–72% $HClO_4$, 1 mL *25% $AgClO_4$ soln,* and ca 12 glass beads. Heat mixt. by means of jacket, and as temp. rises, start steam generation. Maintain temp. of mixt. at 135 ± 2° after raising temp. to this point as quickly as possible by adjusting transformer. Collect distillate in 250 mL vol. flask. (Practice is required for successful manipulation of distn. To avoid sucking back of soln, keep vol. in flask at min. and keep steam generation const. Addn of phthln and small amt of 0.1*N* NaOH to generator provides means of detg if suck-back has occurred.) (Clean distn app. between detns by replacing steam generator with bottle or flask connected to suction and immersing distillate delivery tube in F-free $H_2O$, which is sucked thru entire system.)

Transfer distillate to titrg vessel, adjust to pH 3.0 ± 0.05, add 2 mL Na alizarin sulfonate indicator, and titr. as in **(a)**.

Ref.: JAOAC **44,** 258(1961).

CAS-7782-41-4 (fluorine)

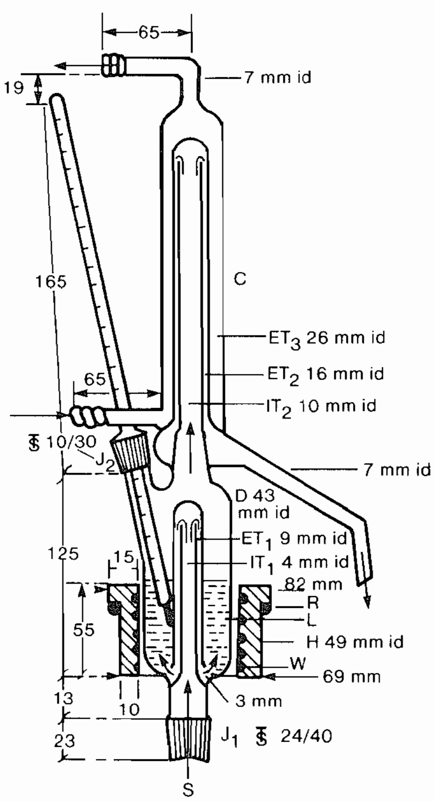


**FIG. 961.16—Upper section of distilling apparatus. From Anal. Chem. 29, 141(1957)**

## 960.52 Microchemical Determination of Nitrogen

### Micro-Kjeldahl Method

**First Action 1960**
**Final Action 1961**

(Not applicable to material contg N–N or N–O linkages)

### A. Reagents

**(a)** *Sulfuric acid.*—Sp gr 1.84, N-free.
**(b)** *Mercuric oxide.*—N-free.
**(c)** *Potassium sulfate.*—N-free.
**(d)** *Sodium hydroxide-sodium thiosulfate soln.*—Dissolve 60 g NaOH and 5 g $Na_2S_2O_3.5H_2O$ in $H_2O$ and dil. to 100 mL or add 25 mL 25% $Na_2S_2O_3.5H_2O$ to 100 mL 50% NaOH soln.
**(e)** *Boric acid soln.*—Satd soln.
**(f)** *Indicator soln.*—(*1*) *Methyl red-methylene blue.*—Mix 2 parts 0.2% alc. Me red soln with 1 part 0.2% alc. methylene blue soln; or (*2*) *Methyl red-bromocresol green soln.*—Mix 1 part 0.2% alc. Me red soln with 5 parts 0.2% alc. bromocresol green soln.
**(g)** *Hydrochloric acid.*—0.02*N*. Prep. as in **936.15A** and stdze as in **936.15E–G**.

### B. Apparatus

**(a)** *Digestion rack.*—With either gas or elec. heaters which will supply enough heat to 30 mL flask to cause 15 mL $H_2O$ at 25° to come to rolling boil in ≥2 but <3 min.
**(b)** *Distillation apparatus.*—One-piece or Parnas-Wagner distn app. recommended by Committee on Microchemical Apparatus, ACS.
**(c)** *Digestion flasks.*—Use 30 mL regular Kjeldahl or Soltys-type flasks (Ref.: Anal. Chem. **23,** 523(1951)). For small samples, 10 mL Kjeldahl flasks may be used.

### C. Determination

Weigh sample requiring 3–10 mL 0.01 or 0.02*N* HCl and transfer to 30 mL digestion flask. If sample wt is <10 mg, use microchem. balance (max. wt 100 mg dry org. matter). Use charging tube for dry solids, porcelain boat for sticky solids or nonvolatile liqs, and capillary or capsule for volatile liqs. Add 1.9 ± 0.1 g $K_2SO_4$, 40 ± 10 mg HgO, and 2.0 ± 0.1 mL $H_2SO_4$. If sample wt is >15 mg, add addnl 0.1 mL $H_2SO_4$ for each 10 mg dry org. matter >15 mg. Make certain that acid has sp gr ≥1.84 if sample contains nitriles. (10 mL flasks and $^1/_2$ quantities of reagents may be used for samples <7 mg.) Add boiling chips which pass No. 10 sieve. If boiling time for digestion rack heaters is 2–2.5 min, digest 1 hr after all $H_2O$ is distilled and acid comes to true boil; if boiling time is 2.5–3 min, digest 1.5 hr. (Digest 0.5 hr if sample is known to contain no refractory ring N.)

Cool, add min. vol. of $H_2O$ to dissolve solids, cool, and place thin film of Vaseline on rim of flask. Transfer digest and boiling chips to distn app. and rinse flask 5 or 6 times with 1–2 mL portions $H_2O$. Place 125 mL Phillips beaker or erlenmeyer contg 5 mL satd $H_3BO_3$ soln and 2–4 drops indicator under condenser with tip extending below surface of soln. Add 8–10 mL NaOH-$Na_2S_2O_3$ soln to still, collect ca 15 mL distillate, and dil. to ca 50 mL. (Use 2.5 mL $H_3BO_3$ and 1–2 drops indicator, and dil. to ca 25 mL if 0.01*N* HCl is to be used.) Titr to end point. Make blank detn and calc.

$$\%\text{N} = [(\text{mL HCl} - \text{mL blank}) \times \text{normality} \times 14.007 \times 100]/\text{mg sample}$$

Refs.: JAOAC **32,** 561(1949); **33,** 179(1950); **43,** 689(1960).

CAS-7727-37-9 (nitrogen)

## 963.29 Microchemical Determination of Oxygen

**Gravimetric Method**

**First Action 1963**
**Final Action 1965**

### A. Principle

Org. O compds are thermally decomposed in inert atm. to $H_2O$, CO, and $CO_2$. At 1120°, following reactions are complete: $C + CO_2 \rightarrow 2CO$; $H_2O + C \rightarrow H_2 + CO$. The CO is converted to $CO_2$ by reaction with CuO and $CO_2$ is detd gravimetrically.

### B. Reagents

(**a**) *Copper oxide*.—See **949.12A(a)**.
(**b**) *Ascarite*.—*See* **949.12A(h)**.
(**c**) *Dehydrite or Anhydrone*.—*See* **949.12A(g)**.
(**d**) *Nitrogen*.—Purify high purity N by passing thru series of scrubbing bottles contg different solid desiccants ($CaCl_2$, anhyd. $Mg(ClO_4)_2$, and $P_2O_5$), then thru tube contg closely packed reduced Cu turnings at 600°, thru Anhydrone, and finally thru bubble counter and U-tube.
(**e**) *Carbon*.—Fisher, C-198, lampblack, or equiv. Pelletize by swirling constantly while adding $CCl_4$ dropwise; dry in oven at 100–120°. Purify by digesting with HCl. Add large vol. $H_2O$, stir mech., let C settle, and decant $H_2O$. Repeat until $H_2O$ wash is Cl-free. Dry C, place in quartz tube, and heat in slow stream of N, (**d**), several hr, increasing temp. gradually to 550°.
(**f**) *Quartz chips*.—Clean chips with HF, rinse with $H_2O$, and dry in oven.

### C. Apparatus (See Figs. 963.29A and B)

(**a**) *Long stationary furnace, 675°*.—**949.12B(i)**, except maintained at 675 ± 5°; (*A*).
(**b**) *Long stationary furnace, 1120°*.—*See* (**c**); (*B*).
(**c**) *Short movable furnace, 1120°*.—This furnace (*C*) and that in (**b**) may be available as unit.
(**d**) *Nitrogen purification train*.—Preheater furnace (*D*), **949.12B(g)**, with section of combustion tubing, quartz, Vycor, or Pyrex glass No. 1720, or equiv., packed with ca 10 cm reduced Cu turnings.
(**e**) *Bubble counter and U-tube*.—*See* **949.12B(c)** and **949.12C(b)**.
(**f**) *Cap*.—Ŧ 14/35 cap with 2 mm stopcock; (*F*).
(**g**) *Thermal decomposition tube*.—Clear fused quartz, solar radiation grade, 7.5–8 mm id, 10–11 mm od, and 60 cm long, exclusive of capillary tube; (*G*). At one end is Ŧ 14/35 male joint. About 1 cm from joint is bent side arm, 6–7 mm od and 2–4 mm id. At other end is 15 cm capillary tube 6–7 mm od and 1–2 mm id. Wash tube with HF and $H_2O$, and dry.

Fill tube as shown in Fig. **963.29B**, with repeated tapping to avoid channeling. The C must *at all times* be in section of furnace that is at 1120°.
(**h**) *By-pass stopcocks*.—3 way T-type Ş ball joint on side arm; (*H*) and (*H'*).
(**i**) *Spiral by-pass tube*.—Glass tube (*I*), contg spiral, for flexibility, and Ş socket joints on each end. Length should be sufficient to connect to Ş ball joints on Ş stopcocks, (*H*) and (*H'*).
(**j**) *Quartz tube*.—Filled with reduced Cu; (*J*).
(**k**) *Short stationary furnace, 900°*.—**949.12B(h)**, except capable of maintaining 900°; (*K*).

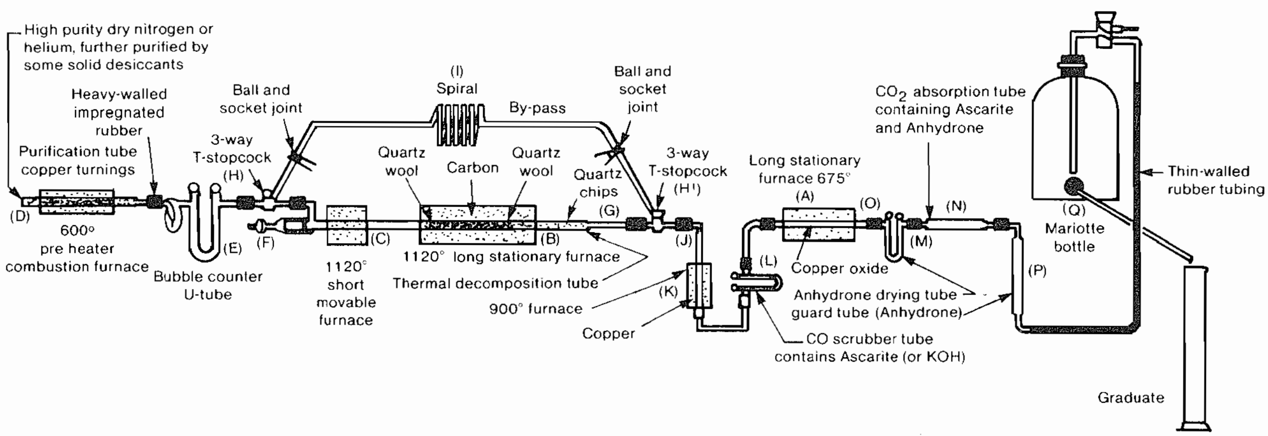


**FIG. 963.29A—Gravimetric setup for oxygen determination. (Note: (1) All rubber connections made of heavy-walled impregnated rubber except Mariotte bottle. (2) Tubes marked (M) and (L) may be replaced with regulation absorption tubes)**

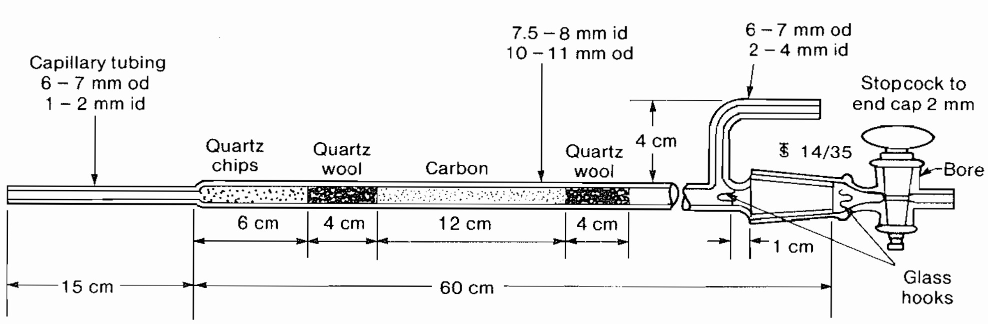


**FIG. 963.29B—Quartz reaction tube and filling for oxygen determination**

(l) *Scrubber tube.*—Any type of drying tube fitted with crushed KOH pellets or Ascarite, (*L*), for removing halogens and S.

(m) *Drying tube.*—*See* **949.12B(e)**, or use U-tube. Fill with Anhydrone only, as in **949.12C(d)**; (*M*).

(n) *Carbon dioxide absorption tube.*—Fill with Ascarite and Anhydrone as in **949.12C(d)**; (*N*).

(o) *Oxidation tube.*—Tube, 30–40 cm, exclusive of tip, similar to that in (**d**) except packed with ca 25 cm CuO wire. Let 5 cm extend beyond furnace. Maintain at 675 ± 5° to oxidize CO to $CO_2$; (*O*).

(p) *Guard tube.*—Glass tube 110–120 mm × 10–12 mm od, 1 mm wall, contg Anhydrone; (*P*).

(q) *Mariotte bottle.*—Glass 2 L bottle. Place 1-hole rubber stopper in top of bottle. Insert glass tube to 7–10 cm of bottom of bottle. Attach 3-way stopcock to top of tube. In bottom opening insert 1-hole rubber stopper contg drain tube to 1 L graduate. Fill bottle with $H_2O$; (*Q*).

(r) *Safety trap.*—Gas washing bottle, 125 mL, Drechsel tall-form, Kimble Glass, No. 15060 or equiv. Connect T-tube to top of center tube. Fill bottle ca $^1/_3$ with Hg and connect by T-tube between preheater and N supply. (Gas pressure valve, Friedrich, or other regulator may also be used.)

(s) *Nitrogen tank.*—Use tank of high purity N equipped with pressure reducing valve, and safety valve to blow off at set pressure.

### D. Assembling Apparatus

Assemble as in Fig. **963.29A**, starting with N tank (**s**), not shown, and connecting as follows, using ground glass joints and paraffin-impregnated heavy-wall tubing: Safety trap (**r**), not shown; purification train (*D*); bubble counter and U-tube (*E*); thermal decomposition tube (*G*); spiral by-pass tube (*I*); quartz tube (*J*); U-tube (*L*); oxidn tube (*O*); drying tube (*M*); $CO_2$ absorption tube (*N*); guard tube (*P*); and Mariotte bottle (*Q*).

### E. Conditioning Apparatus

Assemble app. up to $CO_2$ absorption tube; set various furnaces in place. Pass slow stream of N (10 mL/ min) thru system 1–2 hr at *room temp*. Heat all units to specified temps and continue N stream 2 days. Attach remainder of app. with arm of Mariotte bottle slightly below horizontal, and adjust ht of Hg in safety trap (**r**) to obtain 10 mL N/min thru system with all parts at operating temp. Record rates of bubble flow thru bubble counter and Mariotte bottle.

C in thermal decomposition tube (**g**) must be at 1120°. Best results are obtained by keeping furnaces at specified temp. at all times, even when not in use.

### F. Determination

Adjust N flow to 10 mL/min with all furnaces heated to specified temp. Cool short movable furnace (*C*) to room temp. Weigh enough sample to produce 1.0–1.3 mg O; if solid, weigh directly in Pt boat; if liq., weigh in capillary tube, preferably quartz, and insert in Coombs-Alber Pt sleeve or long Pt boat. Turn stopcocks (*H*) and (*H'*) and open stopcock on cap (*F*) to let N flow in reverse direction thru tube (*G*). Remove cap (*F*), and insert Pt boat contg sample to within ca 7 cm of long furnace (*B*) with aid of Pt hook on end of glass rod. Immediately replace cap (*F*) with its stopcock open and let reverse flow of N continue ca 20 min to expel all air.

Weigh $CO_2$ absorption tube (*N*) as in **949.12D(a)** and attach in position. Close stopcock on cap (*F*) and turn stopcocks (*H*) and (*H'*) so N flows forward thru decomposition tube (*G*). Open stopcock on Mariotte bottle (*Q*) and let N flow thru entire system at 10 mL/min. Heat movable furnace (*C*) to ca 1120°, move to within ca 3 cm of sample, and turn on automatic drive to pyrolyze sample slowly. About 25–30 min is required for furnace (*C*) to reach furnace (*B*). Move furnace forward and heat parts of tube insulated by walls of furnaces 5–10 min to pyrolyze any material condensed in cooler portions of tube. Return furnaces to original positions. Let furnace (*C*) cool. Continue flow of N until ca 700 mL has passed thru from beginning of pyrolysis, using exactly same amt in detn and blank. Remove $CO_2$ absorption tube *N*, and reweigh as in **949.12D(a)**.

Perform blank detn with empty Pt boat and subtract wt $CO_2$ of blank from that of detn. (Well-functioning app. gives zero blank.)

$$\% \text{ O} = \text{Wt } CO_2 \times 0.3635 \times 100/\text{wt sample}$$

### G. Cleaning Reaction Tube

When visibility becomes poor on thermal decomposition tube from deposited C, remove as follows: With entire app. assembled (with absorption tube and Mariotte bottle attached), close stopcock (*H*) to both (*G*) and (*I*). Turn stopcock (*H'*) to connect (*G*) to (*J*). Open cap stopcock (*F*), lower arm of Mariotte bottle, and suck air thru reaction tube. Heat movable furnace to 1120° and move it over against long furnace; C will be burned off in few min. Close stopcock of cap (*F*), and turn stopcock (*H*) to connect reaction tube (*G*) with N supply. Pass N thru system overnight to remove air.

Refs.: Steyermark, A., "Quantitative Organic Microanalysis," 2nd Ed., Academic Press, New York (1961). JAOAC **46**, 559(1963).

CAS-7782-44-7 (oxygen)

## 957.18 Microchemical Determination of Phosphorus

### Kjeldahl Digestion Method

### Final Action

### A. Reagents

(a) *Nitric-sulfuric acid mixture.*—Slowly pour 420 mL $HNO_3$ into 580 mL $H_2O$; then slowly add 30 mL $H_2SO_4$.

(b) *Ammonium nitrate soln.*—2%. Prep. 2% soln of $NH_4NO_3$ in $H_2O$, add 2 drops $HNO_3$, and store in g-s bottle. Filter immediately before use.

(c) *Molybdate reagent.*—Dissolve 150 g powd $NH_4$ molybdate in 400 mL $H_2O$ and cool under tap. Place 50 g $(NH_4)_2SO_4$ in 1 L vol. flask, dissolve in mixt. of 105 mL $H_2O$ and 395 mL $HNO_3$, and cool under tap. Pour cooled molybdate soln slowly into $(NH_4)_2SO_4$ soln with const stirring and cooling under tap. Dil. soln to 1 L, store in refrigerator 3 days, filter, and store in paraffin-lined, g-s, brown bottle in refrigerator. Filter reagent immediately before use and check by periodically analyzing std sample.

### B. Apparatus

(a) *Kjeldahl digestion flasks (30 mL), rack, and manifold.*—*See* **960.52B(a)** and (**c**).

(b) *Filter tubes and filtration assembly.*—*See* **952.24B(c)** and (**d**).

(c) *Rubber stoppers.*—Two or three small, solid rubber stoppers to loosen ppt from walls of flask.

### C. Determination

Weigh 3–20 mg sample, depending on P content and whether microchem. or semimicrochem. balance is used (max. wt ppt = 50 mg). Weigh in charging tube, if possible, and transfer to Kjeldahl flask. Use porcelain boat for sticky solids and viscous liqs, and glass capillary for volatile liqs.

Add 0.5 mL $H_2SO_4$ followed by 4–5 drops $HNO_3$. Heat on digestion rack to white $SO_3$ fumes and cool under tap. Add 4–5 drops $HNO_3$, repeat digestion, and cool under tap. Add 4–5 drops $HNO_3$ and again digest to $SO_3$ fumes. Cool to room temp.; add 2 mL acid mixt., (**a**), and 12.5 mL $H_2O$, rinsing down neck of flask. (If porcelain boat was used to add sample, remove boat with Pt wire; if glass capillary was used, filter digestion mixt. to remove capillary. Rinse filter and boat or capillary with 12.5 mL $H_2O$ used to dil. sample.)

Place flask on steam bath 15 min to convert P to $H_3PO_4$. Remove from steam bath and pipet 15 mL molybdate reagent, (**c**), into center of digest, not down walls of flask. Let stand 2–3 min; then gently swirl to mix contents, being careful to prevent reagents from splashing on neck of flask. Cover flask and set in dark place overnight.

Condition filter tube as described below and weigh empty tube. Connect tared filter tube to filtration assembly and transfer ppt to filter thru siphon tube. Wash flask alternately with 1–2 mL portions of the $NH_4NO_3$ soln and alcohol. Add 2–3 small rubber stoppers to digestion flask, shake to loosen any ppt, and transfer with the $NH_4NO_3$ soln and alcohol. Disconnect siphon tube; rinse ppt from tip and stopper into filter tube with the $NH_4NO_3$ soln and alcohol. Wash ppt with more $NH_4NO_3$ soln, alcohol, and finally with acetone, and suck dry. Wipe filter tube with chamois skin, place in vertical position in vac. desiccator contg no desiccant, and evacuate to 1 mm for 30 min with mech. vac. pump in continuous operation. Release vac. and weigh *immediately* to nearest 0.1 mg. (Rapid weighing is essential because of hygroscopic nature of ppt.)

$$\% \text{ P} = \text{mg ppt} \times 0.014524 \times 100/\text{mg sample}$$

Ref.: JAOAC **40**, 386(1957).

CAS-7723-14-0 (phosphorus)

## 952.25 Microchemical Determination of Sulfur

### Titrimetric Carius Combustion Method

### Final Action

(Not applicable in presence of P)

#### *A. Reagents*

(**a**) *Fuming nitric acid.*—Reagent grade, sp gr 1.50.

(**b**) *Sodium chloride.*—Reagent grade, fine crystals.

(**c**) *Barium chloride soln.*—Approx. 0.02*N*. Stdze by titrg 5–7 mg freshly dried $K_2SO_4$, ACS (weighed to nearest 0.01 mg), by method used for sample titrn, **952.25D**. Correct titrn for indicator error by blank detn.

$$\text{Normality} = \text{mg } K_2SO_4/174.258\ (\text{mL BaCl}_2 \text{ soln} - \text{mL blank})$$

(**d**) *Potassium sulfate.*—ACS, powd and dried.

(**e**) *Phenolphthalein soln.*—0.5% soln in 50% alcohol.

(**f**) *Sulfate indicator.*—Tetrahydroxyquinone (THQ) sulfate indicator (No. 215, Betz Laboratories, Inc., 4636 Somerton Rd, Trevose, PA 19047-6783) or mix 0.1 g K rhodizonate with 15 g sucrose by grinding in mortar.

#### *B. Apparatus*

(**a**) *Combustion tubes and furnace.*—*See* **952.24B(a)** and (**b**).

(**b**) *Titration assembly.*—5 mL buret graduated in 0.01 mL; rectangular titrn cell ca 2 × 4 × 5 cm with min. capacity of 50 mL; and std orange-red glass color filter (Corning Glass Works No. 3482, CS 3-67) selected to have 37% *T* at 550 nm. (Alternatively, use ref. titrn cell contg 30 mL of soln of 20 g $Na_2Cr_2O_7$/L $H_2O$.) Place cell and filter side by side on milk glass window illuminated from below, preferably by fluorescent light. Mask light source so that only cells and filter are illuminated. For best results use no overhead illumination. Place microscope slides (1–3) with ground glass surface (prepd by grinding with $H_2O$ suspension of fine SiC) over glass filter to compensate for increased turbidity.

#### *C. Sample*

Using microchem. balance, weigh 5–20 mg sample contg ≥0.75 mg S, or using semimicrochem. balance, weigh 10–20 mg sample contg ≥0.75 mg S (1.5 mg for gravimetric detn). Weigh samples as in **952.24C**.

#### *D. Determination*

Place weighed sample in combustion tube, add NaCl 100% in excess of amt equiv. to S in sample, and proceed as in **952.24D**, beginning ". . . and add . . . fuming $HNO_3$, . . ." thru end of third par. ". . . with glass splinters."

Transfer contents of tube to 50 mL beaker, rinsing tube 4–6 times with 3–5 mL portions $H_2O$. Evap. to dryness on steam bath.

Dissolve residue in 10 mL $H_2O$, pour soln into titrn cell, add 1 drop phthln, and make just alk. with ca 0.1*N* NaOH, then acid with ca 0.02*N* HCl, adding 1 drop excess. Add ca 0.15 g of the sulfate indicator, stir to dissolve, and rinse beaker 2 or 3 times, using enough alcohol so that final soln contains ca 50%. Titr. with std $BaCl_2$ soln from 5 mL buret until stable color of soln immediately after stirring matches std glass color filter. Make certain end point taken is real and not pseudo end point which fades on standing 1–2 min. (Addn of 1–2 drops $BaCl_2$ soln develops definite red.) Det. blank on reagents and correct titrn.

$$\% \text{ S} = (\text{mL BaCl}_2 - \text{mL blank}) \times \text{normality} \times 16.032 \times 100/\text{mg sample}$$

Refs.: JAOAC **35**, 305(1952); **36**, 94, 335(1953).

CAS-7704-34-9 (sulfur)

## 955.48 Microchemical Determination of Sulfur

### Gravimetric Carius Combustion Method

### Final Action

(Applicable in presence of P)

#### *A. Apparatus*

*Crucible and filter stick.*—Porcelain crucible, ca 15 mL capacity, with black inside glaze, wt ca 10 g; with porcelain filter stick, with unglazed bottom, wt ca 2 g. (Altho filter stick is weighed with crucible, it is removed before addn of soln of residue.) (Anal. Chem. **21**, 1555(1949).)

#### *B. Determination*

Dissolve residue, **952.25D**, in 3 mL $H_2O$, pour into previously ignited and weighed (with filter stick) porcelain crucible, and rinse beaker with four 2 mL portions $H_2O$. Place crucible on steam bath until soln is near bp. If vol. exceeds 10–11 mL, evap. to this vol. Add dropwise 0.5 mL *10% $BaCl_2$ soln* (1 mL for samples contg >5 mg S), digest ≥15 min, and cool 15 min.

Connect porcelain filter, previously ignited and weighed with crucible, to arm of siphon with rubber tubing. Connect other arm of siphon to suction flask thru rubber stopper. Lower filter into crucible, slowly draw off soln, and rinse ppt, walls of crucible, and filter with five or six 3 mL portions HCl (1 +

300), drawing off as much liq. as possible. Carefully detach filter, place in crucible, wipe outside of crucible and end of filter with moist chamois or cheesecloth, and handle thereafter with crucible tongs. Place crucible and filter in larger crucible and dry in oven 10 min at ca 110°. Ignite in furnace 10 min at 700–750° (ppt may also be ignited by heating larger crucible contg crucible and filter to dull red heat with Meker burner), cool on metal block 30 min or in desiccator 1 hr, and weigh. Det. blank on reagents.

$$\% \text{ S} = (\text{wt } BaSO_4 - \text{blank}) \times 0.1374 \times 100/\text{wt sample}$$

Ref.: JAOAC **38,** 377(1955).

CAS-7704-34-9 (sulfur)

## 975.53 Microchemical Determination of Sulfur
### Oxygen Flask Combustion Method
### First Action 1975

(Not applicable in presence of P)

### *A. Reagents*

(**a**) *Barium chloride soln.*—Approx. 0.02*N*. Stdze as follows: Accurately weigh 3.5–5.5 mg $K_2SO_4$ into titrn cell, dissolve in 15 mL $H_2O$, add 0.15 g sulfate indicator, (**f**), and dissolve; dil. soln to 30 mL with alcohol. Titr. to end point (same color as ref. glass) with $BaCl_2$ soln, making certain end point taken is real; *see* **975.53C**. Titr. blank. Calc. normality as in **952.25A(c)**.

(**b**) *Hydrogen peroxide soln.*—30% (Fisher Scientific Co. No. H-325, or equiv. purity). (*Caution:* See safety notes on peroxides.)

(**c**) *Oxygen cylinder.*—With regulator and connections for filling combustion flask.

(**d**) *Potassium sulfate.*—ACS, powd and dried.

(**e**) *Phenolphthalein soln.*—0.5% soln in 50% alcohol.

(**f**) *Sulfate indicator.*—Tetrahydroxyquinone (THQ) sulfate indicator (Betz Laboratories, Inc., 4636 Somerton Rd, Trevose, PA 19047-6783) or mix 0.1 g K rhodizonate with 15 g sucrose by grinding in mortar. Vac.-dry overnight at room temp.

### *B. Apparatus*

(**a**) *Mechanical shaker.*—Optional.

(**b**) *Oxygen flask combustion apparatus.*—Thomas-Ogg infrared igniter (Thomas Scientific No. 6516-G10) and 500 mL thick wall combustion flask (No. 6514-F10), black sample wrappers (No. 6514-F70), Pt sample carrier, stopper, and clamp to avoid loss of sample during pressure changes which occur during combustion. App. is completely shielded within hinged cabinet. Precautions used when employing other manually operated elec. units should include proper safety shielding and reinforced gloves. Flasks must be free of org. solvs to avoid explosion.

(**c**) *Titration assembly.*—*See* **952.25B(b)**.

### *C. Determination*

Weigh sample contg ca 0.75 mg S and fold into paper carrier. Add 5 mL 0.1*N* NaOH and 3 drops $H_2O_2$ to 500 mL combustion flask. Flush flask with O ≥2 min. Place paper carrier contg sample in Pt basket, hang on hook of stopper, and insert stopper in flask. Ignite. Shake 30 min. (If gas phase has not cleared, let stand 10 min.) Open flask, and rinse stopper and Pt sample basket with $H_2O$. Transfer soln to 100 mL beaker, rinsing flask with min. vol. $H_2O$. Acidify with 2 mL 0.5*N* $HNO_3$ (≥1 mL in excess of base) and evap. to dryness on steam bath. Dissolve acid-free residue in ca 5 mL $H_2O$ and transfer to titrn cell. Rinse beaker with ca 5 mL $H_2O$. Add 1 drop phthln and make just alk. with ca 0.1*N* NaOH; then acidify with ca 0.02*N* $HNO_3$. Add ca 0.15 g THQ indicator, stir to dissolve, and rinse beaker 2–3 times with enough alcohol so that final soln in cell contains ca 50% alcohol. Titr. with std $BaCl_2$ soln from 5 mL buret until stable color of soln immediately after stirring matches std glass color filter as in **952.25D**.

$$\% \text{ S} = [(\text{mL } BaCl_2 - \text{mL blank}) \times \text{normality} \times 16.032 \times 100]/\text{mg sample}$$

Ref.: JAOAC **58,** 146(1975).

CAS-7704-34-9 (sulfur)

## 976.29 Microchemical Determination of Sulfur
### Alternative Oxygen Flask Combustion Method
### First Action 1976

(Not applicable in presence of P. All reagents must be as pure as possible, since high concns of Cl, F, $NO_3$, $PO_4$, K, and Na interfere. Useful in absence of titrn assembly necessary for THQ titrn.)

### *A. Reagents*

(**a**) *Barium perchlorate std soln.*—Approx. 0.01*N*. Accurately weigh ca 6 g $Ba(ClO_4)_2.3H_2O$ and dissolve in 200 mL $H_2O$. Add 2 drops HCl and dil. to 1 L. Stdze as follows: Accurately weigh ca 3.8 mg *S*-benzylisothiourea.HCl, (**e**), and proceed as in **976.29C**, beginning ". . . and fold into paper carrier." and continuing to ". . . and correct titrn values." Calc. factor *F*, mg S/mL $Ba(ClO_4)_2$.

$$F = (\text{mg } S\text{-benzylisothiourea.HCl} \times 0.1582)/[\text{mL } Ba(ClO_4)_2 - \text{mL blank}]$$

where 0.1582 is fraction S in *S*-benzylisothiourea.HCl.

(**b**) *Hydrogen peroxide soln.*—6%. Dil. 20 mL 30% $H_2O_2$ (Fisher Scientific Co., No. H-325, or equiv. purity) to 100 mL with $H_2O$. (*Caution:* See safety notes on peroxides.)

(**c**) *Methylene blue indicator soln.*—Approx. 0.0125%. Dissolve 12.5 mg methylene blue (J. T. Baker Chemical Co., or equiv.) in 100 mL $H_2O$.

(**d**) *Oxygen cylinder.*—*See* **975.53A(c)**.

(**e**) *S-Benzylisothiourea.HCl.*—Purity equiv. to NIST specifications.

(**f**) *Thorin indicator soln.*—Approx. 0.2%. Dissolve 200 mg thorin (J. T. Baker Chemical Co., or equiv.) in 100 mL $H_2O$.

### *B. Apparatus*

(**a**) *Mechanical shaker.*—Optional.

(**b**) *Oxygen flask combustion apparatus.*—*See* **975.53B(b)**.

### *C. Determination*

Weigh sample contg ca 0.60 mg S and fold into paper carrier. Add 10 mL 6% $H_2O_2$ soln to 500 mL combustion flask. Flush flask with O ≥2 min. Place paper carrier contg sample in Pt basket, hang on hook of stopper, and insert stopper in flask. Ignite. Shake 30 min. (If gas phase has not cleared, let stand addnl 10 min.) Open flask, and rinse stopper and Pt sample basket with alcohol. Transfer soln to 200 mL beaker, rinsing flask with alcohol. Place mag. stirring bar in beaker. Add alcohol to 100 mL mark. Add 2 drops thorin indicator soln and 2 drops methylene blue indicator soln, and titr. with

$Ba(ClO_4)_2$ soln to faint pink end point, stirring mag. Det. blank on reagents and correct titrn values. Calc. % S, using factor $F$ from **976.29A(a)**.

$$\% \text{ S} = [(\text{mL } Ba(ClO_4)_2 - \text{mL blank}) \times F \times 100]/\text{mg sample}$$

Ref.: JAOAC **59,** 1135(1976).

CAS-7704-34-9 (sulfur)

## 952.26★ Microchemical Determination of Sulfur

### Titrimetric Catalytic Combustion Method

**Final Action**
**Surplus 1970**

*See* **38.031–38.034**, 10th ed.

## 955.49★ Microchemical Determination of Sulfur

### Gravimetric Catalytic Combustion Method

**Final Action**
**Surplus 1970**

*See* **38.035**, 10th ed.

## 956.07 Microchemical Determination of Alkoxyl Groups

### Titrimetric Method

**Final Action**

### *A. Reagents*

(**a**) *Acetic acid-potassium acetate-bromine soln.*— Dissolve 10 g KOAc in enough HOAc to make 100 mL, and add 3 mL Br. Prep. fresh.

(**b**) *Sodium acetate soln.*—Dissolve 25 g $NaOAc.3H_2O$ in enough $H_2O$ to make 100 mL.

(**c**) *Starch indicator.*—Mix ca 2 g finely powd potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring constantly. Add ca 1 mL Hg, shake, and let soln stand over the Hg.

(**d**) *Sodium thiosulfate std soln.*—0.02$N$. Prep. daily by dilg 0.1$N$ soln, **942.27**.

(**e**) *Hydriodic acid.*—Place 250 mL constant boiling HI (57%, sp gr 1.7) in 500 mL r-b flask connected by ₮ joint to air condenser, and reflux 2 hr while stream of $CO_2$ or N bubbles thru from glass tube extending to bottom. Do not let acid vapors come in contact with org. material. As soon as refluxing stops, discontinue gas flow. Cool, and store in g-s bottle.

### *B. Apparatus*

Use modified Clark app., Figs. **956.07A** and **B**.

### *C. Determination*

Fill scrubber halfway with NaOAc soln, and fill receiver $^2/_3$ full with freshly prepd KOAc-Br soln. Weigh enough sample

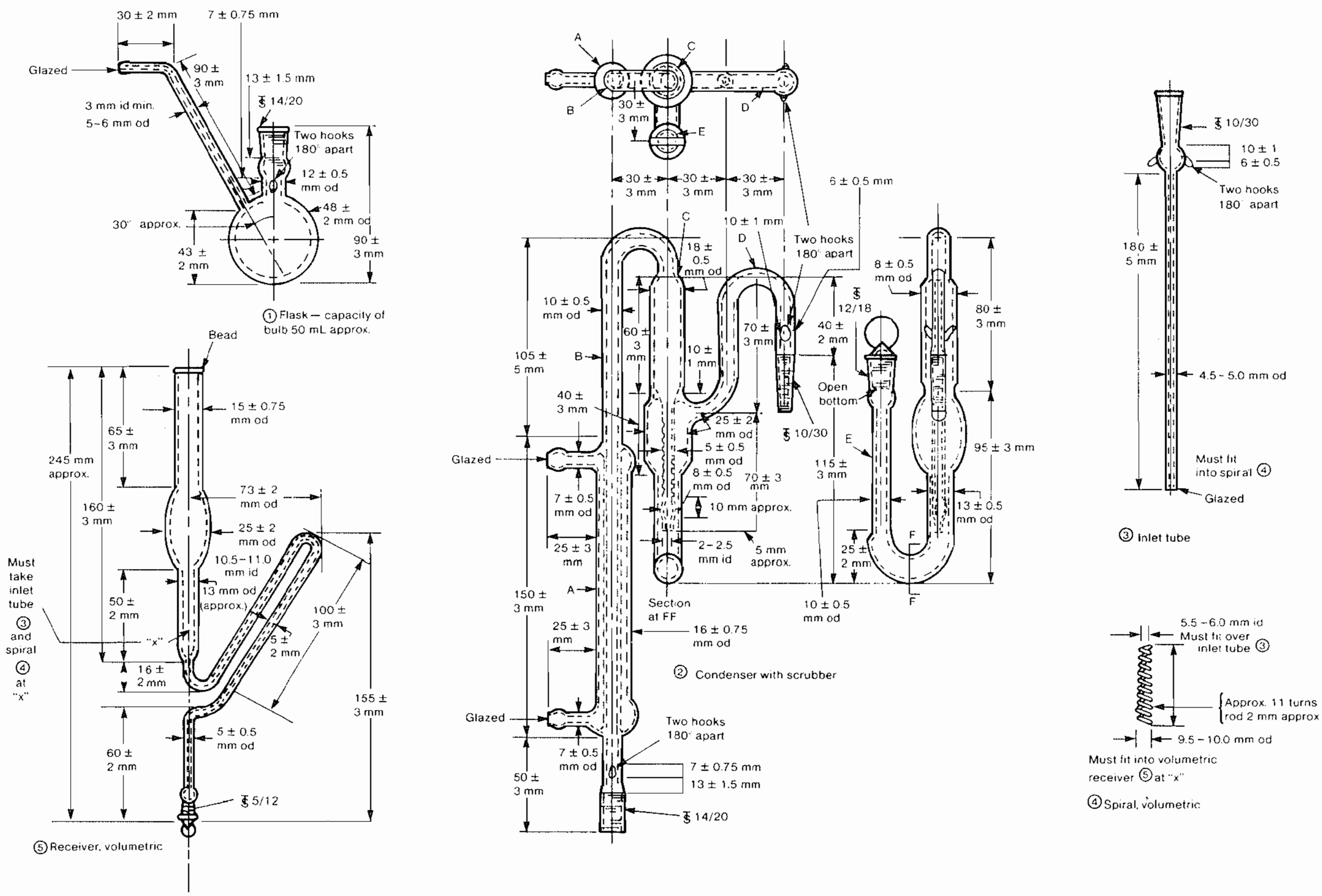


FIG. 956.07A—Details of modified Clark apparatus

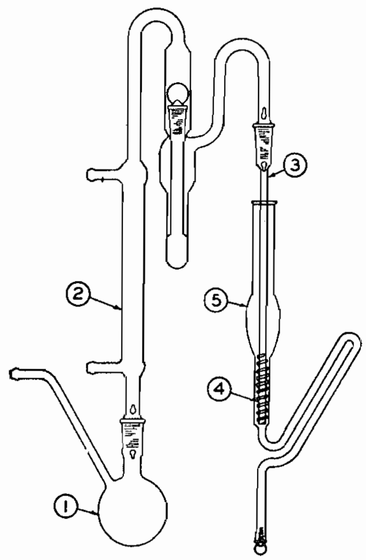


**FIG. 956.07B—Modified Clark apparatus**

in Pt boat to require ca 8 mL $Na_2S_2O_3$ soln in detn, and place in bottom of boiling flask. Add 2.5 mL melted *phenol* from wide-tip pipet and 5 mL of the HI, and connect boiling flask. Pass $CO_2$ thru app. from side arm of flask at uniform rate of 15 mL/min. Let reaction mixt. remain at room temp. 30 min. With manteled microburner, boil liq. at such rate that vapors of boiling liq. rise into condenser, but not more than halfway; continue boiling 60 min (first 30 min with $H_2O$ circulating thru condenser and last 30 min with $H_2O$ drained from condenser). Disconnect flask, remove receiver, and rinse delivery tube and contents of receiver into 125 mL erlenmeyer contg 5 mL NaOAc soln. Adjust vol. to ca 50 mL and add *formic acid* dropwise until excess Br is destroyed.

Remove any Br vapors by blowing air over liq.; then add 0.5 g KI and 5 mL 10% $H_2SO_4$. Swirl soln to dissolve KI and mix contents; then titr. liberated I with the $Na_2S_2O_3$ soln, using starch indicator as in stdzn.

Det. blank on all reagents by making detn without sample.

$$\% \text{ Alkoxyl group} = (\text{mL in detn} - \text{mL in blank}) \times \text{normality} \times \text{equiv. wt} \times 100/\text{mg sample}$$

Equiv. wt: methoxyl = 5.173; ethoxyl = 7.510.

Ref.: JAOAC **39,** 108, 401(1956).

# 13. Radioactivity

**Edmond J. Baratta, *Associate Chapter Editor***
*Food and Drug Administration*

## 930.39★ Radioactivity of Substances

**Qualitative Method**
**Final Action**
**Surplus 1965**

(Applicable to solids)

*See* **40.001**, 10th ed.

## 933.09★ Radioactivity of Substances

**Quantitative Methods**
**Final Action**
**Surplus 1965**

### *A. Emanation or Radon Method*

(Applicable only to Ra in amts $<10^{-9}$ g. Limit is arbitrary, depending on particular equipment used and accuracy required.)

*See* **40.002–40.005**, 10th ed.

### *B. Gamma Ray Method Using Electroscope*

(Applicable only to Ra in amts $>10^{-5}$ g. Limit is arbitrary, depending on particular equipment used and accuracy required.)

*See* **40.006–40.010**, 10th ed.

### *C. Gamma Ray Method Using Geiger-Muller Counter*

(Applicable only to Ra in amts $>10^{-7}$ g. Limit is arbitrary, depending on particular equipment used and accuracy required.)

*See* **40.011–40.015**, 10th ed.

## 969.48 Tritium in Water

**Scintillation Spectrometric Method**
**First Action 1965**
**Final Action 1969**

### *A. Principle*

Sample is distd to remove quenching materials and nonvolatile radioactive materials. Distn is to dryness to ensure complete transfer of $^3$H to distillate. Aliquot of distillate is mixed with scintillation soln and counted in liq. scintillation spectrometer (coincidence-type). Std $^3$H and background samples are prepd and counted alternately to nullify errors produced by aging of scintillation medium or instrument drift.

### *B. Apparatus*

(**a**) *Liquid scintillation spectrometer*.—Coincidence-type. Available from Packard Instrument Co., 2200 Warrenville Rd, Downers Grove, IL 60515; and others.

(**b**) *Liquid scintillation vial*.—20 mL; low-K glass, polyethylene, nylon, or equiv. bottles, available from manufacturers under (**a**).

### *C. Reagents*

(**a**) *Scintillation soln*.—Thoroly mix 4 g PPO (2,5-diphenyloxazole), 0.05 g POPOP (1,4-bis(5-phenyloxazol-2-yl) benzene), and 120 g solid naphthalene in 1 L spectral grade 1,4-dioxane. (Available from Aldrich Chemical Co., Inc.). Store in dark bottles. Soln is stable 2 months.

(**b**) *Tritium std soln*.—Pipet 4 mL $H_2O$ of known $^3$H activity and 16 mL scintillation soln into scintillation vial, tightly cover vial with screw cap, and mix thoroly by shaking.

(**c**) *Background soln*.—Mix 4 mL distd $H_2O$ (free of $^3$H activity to be measured in samples) with 16 mL scintillation soln as in (**b**).

### *D. Preparation of Sample*

Distil 20–30 mL sample to dryness. Mix 4 mL sample distillate with 16 mL scintillation soln as in **969.48C(b)**.

### *E. Determination*

Prior to counting, dark-adapt and cool sample, background, and std solns ca 3 hr in instrument freezer at >2° (to prevent solidification of soln with time), or at ambient temp. if ambient temp. liq. scintillation spectrometer is used. Count solns for total of 200,000 counts or 100 min, whichever is sooner.

### *F. Calculation*

$$\text{Counting efficiency, } E = (S - B)/D$$

$$^3\text{H, pCi (picoCuries)/mL} = (C - B)/(E \times 4 \times 2.22)$$

where $S$ = gross cpm (counts/min) of std, $B$ = cpm background, $D$ = dpm (disintegrations/min) of $^3$H activity in std, and $C$ = gross cpm for sample.

Ref.: JAOAC **52,** 90(1969).

CAS-10028-17-8 (tritium)

## 973.66 Strontium-90 in Water

**Beta Particle Counting Method**
**First Action 1973**
**Final Action 1974**

### *A. Principle*

Applicable to $H_2O$, and to sewage and industrial waste if org. matter is destroyed and interfering ions are eliminated.

Added carrier Sr along with radionuclides are sepd from other radioactive elements and inactive sample solids by pptn as $Sr(NO_3)_2$ from fuming $HNO_3$. Sr is finally pptd as $SrCO_3$, which is dried, weighed, and set aside ca 2 weeks for ingrowth of $^{90}$Y. Ppt is then dissolved and $^{90}$Y is prepd for counting by (*a*) extn by tributyl phosphate and evapg on planchet, or (*b*) addn of Y carrier and pptg as oxalate.

Radioactive Ba and Ra which interfere are removed by addn of Ba carrier. Ca interferes with Sr pptn, but is removed by $HNO_3$ pptn and acetone treatment.

### *B. Apparatus*

(**a**) *Counting pans*.—Stainless steel, ca 50 mm diam. and 7 mm deep.

(**b**) *Filtration assembly*.—For mounting ppts for counting. Consists of (*1*) 2-piece filtering app. for 2.4 cm filter such as stainless steel filter holder (Interex Corp., 3 Strathmore Rd, Natick, MA 01760, No. 12-103; ICN Pharmaceuticals, Inc., Life Sciences Group, No. 83012), Teflon filter holder, or equiv. (*2*) Nylon (Zytel 101) disk with ring for mounting ppt.

(**c**) *Film, Mylar*.—To cover ppts during counting and storage, ca 1 mil (0.025") thick. Available in rolls 1.5" (3.8 cm) wide as manufacturer's No. 92A, E. I. DuPont Co., Inc., Electronics Dept, Barley Mill Plaza, Kirk Mill: Mylar Product Information, Wilmington, DE 19805.

(**d**) *Glass fiber filter paper*.—No. 934-AH, 2.4 cm diam., available from Whatman, Inc.

(**e**) *Centrifuge tubes*.—40 mL, heavy duty with short cone bottom and pour-out lip.

(**f**) *Beta particle counter*.—Low background, shielded, anticoincidence counter. Det. counter efficiency for $^{90}Y$ as oxalate and $^{89}Sr$ as carbonate for specific counter and geometry.

### C. Reagents

(**a**) *Dilute acetic acid*.—6*N*. Add 345 mL HOAc to $H_2O$ and dil. to 1 L.

(**b**) *Ammonium acetate buffer*.—pH 5.5. Dissolve 154 g $NH_4OAc$ in 700 mL $H_2O$, add 57 mL HOAc, adjust pH to 5.5 with dropwise addn of either HOAc or 6*N* $NH_4OH$, as necessary, and dil. to 1 L.

(**c**) *Dilute ammonium hydroxide*.—6*N*. Dil. 400 mL $NH_4OH$ to 1 L with $H_2O$.

(**d**) *Barium carrier soln*.—10 mg Ba/mL. Dissolve 19.0 g $Ba(NO_3)_2$ in $H_2O$ and dil. to 1 L.

(**e**) *Dilute hydrochloric acid*.—6*N*. Add 500 mL HCl to $H_2O$ and dil. to 1 L.

(**f**) *Fuming nitric acid*.—21*N*. Sp gr 1.48, 90% $HNO_3$.

(**g**) *Dilute nitric acid*.—(*1*) *14N*.—Add 875 mL $HNO_3$ to $H_2O$ and dil. to 1 L. (*2*) *6N*.—Add 384 mL $HNO_3$ to $H_2O$ and dil. to 1 L. (*3*) *0.1N*.—Add 6.25 mL $HNO_3$ to $H_2O$ and dil. to 1 L.

(**h**) *Oxalic acid soln*.—Satd. Approx. 11 g $H_2C_2O_4.2H_2O$ in 100 mL $H_2O$.

(**i**) *Mixed rare earth carrier soln*.—Dissolve 12.8 g $Ce(NO_3)_3.6H_2O$, 14 g $ZrOCl_2.8H_2O$, and 25 g $FeCl_3.6H_2O$ in 600 mL $H_2O$ contg 10 mL HCl, and dil. to 1 L. (*Caution:* $Ce(NO_3)_3$ is toxic. Wear resistant rubber or plastic gloves.)

(**j**) *Sodium carbonate soln*.—2*N*. Dissolve 142 g $Na_2CO_3.H_2O$ in $H_2O$ and dil. to 1 L.

(**k**) *Sodium chromate soln*.—0.5*M*. Dissolve 117 g $Na_2CrO_4.4H_2O$ in $H_2O$ and dil. to 1 L.

(**l**) *Sodium hydroxide soln*.—6*N*. Dissolve 240 g NaOH in $H_2O$ and dil. to 1 L.

(**m**) *Strontium carrier soln*.—10 mg Sr/mL. Dissolve 24.16 g $Sr(NO_3)_2$ in $H_2O$ and dil. to 1 L. Stdze by pipetting (in triplicate) 10 mL soln into 40 mL centrf. tubes and adding 15 mL 2*N* $Na_2CO_3$. Stir and heat in boiling $H_2O$ bath 15 min. Filter thru weighed, fine porosity, fritted glass, 15 mL crucible. Wash with three 5 mL portions $H_2O$ and three 5 mL portions absolute alcohol or acetone, wipe crucible with absorbent tissue, and dry to const wt at 110° (ca 20 min). Cool in desiccator and weigh.

$$\text{mg Sr/mL} = \text{mg } SrCO_3 \times 0.5935/10$$

(**n**) *Tributyl phosphate* (*TBP*), *equilibrated*.— Shake TBP with equal vol. 14*N* $HNO_3$. Sep. and discard lower acid phase.

(**o**) *Yttrium carrier soln*.—10 mg Y/mL. Dissolve 12.7 g $Y_2O_3$ in 30 mL $HNO_3$ by stirring and heating. Add addnl 20 mL $HNO_3$ and dil. to 1 L with $H_2O$. 1 mL = 34 mg $Y_2(C_2O_4)_3.9H_2O$. Det. exact equivalence as in **973.66D(f)** or **(g)**.

### D. Determination

(*Caution:* See safety notes on nitric acid and fuming acids.)

(**a**) *Precipitation as carbonate*.—To 1 L drinking $H_2O$ (or less, but contg ≥25 pCi $^{90}Sr$) or filtered raw $H_2O$ sample in beaker, add 2.0 mL $HNO_3$ and mix. Add 2.0 mL each of Ba and Sr carrier solns and mix well. (Ppt of $BaSO_4$ will not cause difficulty.) Heat to bp, and add 20 mL 6*N* NaOH and 20 mL 2*N* $Na_2CO_3$. Stir, and let simmer ca 1 hr at 90–95°. Let cool until ppt has settled (1–3 hr). Decant and discard supernate. Transfer ppt to 40 mL centrf. tube, centrf., and discard supernate.

(**b**) *Purification as nitrate*.—Cautiously add 4 mL $HNO_3$ dropwise to ppt. Heat to bp, stir, and cool under running $H_2O$. Add 20 mL fuming $HNO_3$, cool 5–10 min in ice bath, stir, and centrf. Discard supernate. Add 4 mL $H_2O$ to residue, stir, and heat to bp to dissolve $Sr(NO_3)_2$. Centrf. while hot and decant supernate into clean centrf. tube. Add 2 mL 6*N* $HNO_3$ to residue, heat to bp, centrf. while hot, and combine supernate with aq. supernate. Discard insol. residue of $BaSO_4$, $SiO_2$, etc.

Cool combined supernates, add 20 mL fuming $HNO_3$, cool 5–10 min in ice bath, stir, centrf., and discard supernate. Add 4 mL $H_2O$ to ppt and dissolve by heating, cool, add 20 mL fuming $HNO_3$, cool 5–10 min in ice bath, stir, centrf., and discard supernate. If >200 mg Ca is present in sample, repeat $H_2O$ soln and fuming $HNO_3$ pptn.

(**c**) *Removal of rare earths*.—After last $HNO_3$ pptn, invert tube in beaker ca 10 min to drain off most of excess $HNO_3$. Add 20 mL acetone to ppt. Stir thoroly, cool, centrf., and discard supernate. Dissolve ppt of Sr and Ba nitrates in 10 mL $H_2O$ and boil 30 sec to remove any remaining acetone.

Add 0.25 mL mixed rare earth carrier soln and ppt rare earth hydroxides by making soln basic with 6*N* $NH_4OH$. Digest in boiling $H_2O$ bath 10 min. Cool in ice bath, centrf., decant supernate to clean tube, and discard ppt. Repeat addn of rare earth carrier soln, pptn, and decantation. Note time as beginning of $^{90}Y$ ingrowth period.

(**d**) *Removal of barium*.—Add 2 drops Me red indicator and then 6*N* HOAc, dropwise with stirring, until soln is red. Add 5 mL acetate buffer soln, heat to bp, and add 2 mL $Na_2CrO_4$ soln dropwise with stirring. Digest in boiling $H_2O$ bath 5 min. Cool in ice bath, centrf., decant supernate into clean tube, and discard residue.

(**e**) *Precipitation as strontium carbonate*.—Add 2 mL 6*N* NaOH to supernate; then add 5 mL 2*N* $Na_2CO_3$ and heat to bp. If pH is <9, add addnl NaOH soln. Cool in ice bath ca 5 min, centrf., and discard supernate. Add 15 mL $H_2O$ to ppt, stir, centrf., and discard wash $H_2O$. Repeat washing and weigh $SrCO_3$ as in (*1*) or (*2*):

(*1*) Slurry ppt with small vol. $H_2O$, and transfer to weighed stainless steel pan. Dry under IR lamp, cool, and weigh. (*2*) Transfer ppt to weighed paper or glass filter mounted in 2 piece funnel. Let settle by gravity for uniform deposition; then apply suction. Wash ppt with three 5 mL portions $H_2O$, three 5 mL portions alcohol, and three 5 mL portions ether or acetone. Dry 15–30 min in 110–125° oven, cool, and weigh.

Store ppt ≥2 weeks to permit ingrowth of $^{90}Y$. Sep. and count $^{90}Y$ by (**f**) or (**g**).

(**f**) *Separation by TBP extraction*.—If $SrCO_3$ is weighed in pan, place pan in small funnel in mouth of 60 mL separator and carefully add 1 mL 6*N* $HNO_3$ dropwise. Tilt pan to empty, and rinse with two 2 mL portions 6*N* $HNO_3$.

If $SrCO_3$ is weighed on filter, dislodge bulk of ppt into small funnel in mouth of 60 mL separator. Cautiously add 1 mL 6*N* $HNO_3$ dropwise to dissolve remaining ppt. Rinse filter and funnel with two 2 mL portions of 6*N* $HNO_3$.

Remove pan or filter and add 10 mL fuming $HNO_3$ to separator thru funnel. Remove funnel and add 1 mL Y carrier soln to separator. Add 5.0 mL TBP, shake thoroly 3–5 min, let sep., and transfer aq. layer to second 60 mL separator. Add 5.0 mL TBP to second separator, shake 5 min, let sep., and transfer aq. layer to third 60 mL separator. Combine TBP exts and wash with two 5 mL portions 14*N* $HNO_3$. Record time as beginning of $^{90}Y$ decay. (Combine acid washings with aq. phase in third separator if second ingrowth of $^{90}Y$ is desired.)

Back-ext $^{90}Y$ from combined org. phase with 10 mL 0.1*N* $HNO_3$ 5 min. Either (*1*) repeat TBP extn as above, beginning "Add 5.0 mL TBP, . . ." and finally back-extg $^{90}Y$ into 10 mL 0.1*N* $HNO_3$ and continue as in (**g**), line 10 beginning "Gradually . . ."; or (*2*) transfer aq. phase to 50 mL beaker and evap. on hot plate to 5–10 mL. Transfer residual soln to weighed stainless steel counting pan and evap. Rinse with two 2 mL portions 0.1*N* $HNO_3$, add rinsings to counting pan, evap. to dryness, and weigh. Count in internal proportional or end window counter and calc. $^{90}Sr$ as in **973.66E**.

(**g**) *Separation by yttrium oxalate precipitation*.— Dissolve $SrCO_3$ by cautiously adding 2 mL 6*N* $HNO_3$ dropwise, and transfer to 40 mL centrf. tube, rinsing with 0.1*N* $HNO_3$. Add 1 mL Y carrier soln, 2 drops Me red, and $NH_4OH$ dropwise to Me red end point. Add addnl 5 mL $NH_4OH$ and record time as end of $^{90}Y$ ingrowth and beginning of decay. Centrf. and decant supernate into beaker. (Save supernate and washings for second ingrowth, if desired.) Wash ppt with two 20 mL portions hot $H_2O$. Add 5–10 drops 6*N* $HNO_3$, stir to dissolve ppt, add 25 mL $H_2O$, and heat in $H_2O$ bath at 90°. Gradually add 15–20 drops satd oxalic acid soln with stirring, and adjust pH to 1.5–2.0 (pH meter or indicator paper) with dropwise addn of $NH_4OH$. Digest ppt 5 min; then cool in ice bath with occasional stirring.

Transfer ppt to weighed glass fiber filter in 2-piece funnel. Let ppt settle by gravity for uniform deposition and then apply suction. Wash ppt with 10–15 mL $H_2O$, three 5 mL portions alcohol, and then three 5 mL portions ether. Air dry ppt 2 min with suction, weigh, mount on nylon disk and ring with Mylar cover, count, and calc. $^{90}Sr$ as in **973.66E**.

### E. Calculations

(**a**) *Strontium-90 calculation.*—

$$^{90}\text{Sr, pCi/L} = \text{net cpm}/(\text{abcdfg} \times 2.22)$$

where a = counting efficiency for $^{90}Y$; b = chem. yield (fraction) of extd or pptd $^{90}Y$; c = mg final Y oxalate ppt/mg Y oxalate in 1 mL carrier; d = chem. yield (fraction) of Sr detd as in **973.66D(e)** (20 mg Sr equiv. to 33.6 mg $SrCO_3$) or by flame photometry; f = vol., L, original sample; g = $^{90}Y$ decay factor = $e^{-\lambda t}$; e = base of natural logarithms; $\lambda = 0.693/T_{1/2}$; $T_{1/2}$ = 64.2 hr for $^{90}Y$; and t = time, hr, between sepn and counting.

(**b**) *Counting efficiency*.—Prep. curve from various wts Y oxalate ppt spiked with $^{90}Sr/^{90}Y$, pptd as in **973.66D(g)**.

(**c**) *Correction for carrier recovery*.—If sample contains more than trace stable Sr, it will act as carrier and will result in >100% yield. In such case det. Sr by flame photometry.

Ref.: JAOAC **56,** 208(1973).

CAS-37380-96-4 (strontium-90)

## 974.37 Strontium-89 and Strontium-90 in Milk
### Ion Exchange Method
### First Action 1974
### Final Action 1976

### A. Principle

Fresh milk samples are preserved with HCHO and stored to obtain $^{90}Y$ ingrowth. After storage, Y, Sr, and Ba carriers and citrate soln are added. Citrate forms Y complex which is adsorbed on anion exchange resin. Y is desorbed and sepd from radionuclides by tributyl phosphate extn. Y is re-extd into dil. $HNO_3$ and pptd as oxalate, which is weighed and counted for $^{90}Y$ activity to calc. $^{90}Sr$.

Radio-Sr is desorbed along with Ca and radio-Ba; Ca, radio-Ba, and rare earth radionuclides are sepd by repetitive pptns; Sr is pptd as $SrCO_3$ and counted. Total radio-Sr minus $^{90}Sr$ by $^{90}Y$ measurement yields value for $^{89}Sr$.

Milk contg known increments of $^{89}Sr$ and $^{90}Sr$ detd in triplicate by 11 laboratories showed following results (av. of triplicates):

| | Amt Present, pCi/L | Std Dev. | | Bias | |
|---|---|---|---|---|---|
| | | % | pCi/L | % | pCi/L |
| $^{89}Sr$ | 29.0 | 10.0 | 2.9 | +7.0 | +2.0 |
| | 197.0 | 3.4 | 7.2 | +1.5 | +3.0 |
| $^{90}Sr$ | 32.4 | 0.9 | 0.3 | +0.3 | +0.1 |
| | 151.2 | 2.8 | 4.2 | −0.9 | −1.3 |

### B. Operating Notes

Radio-Ba and La radionuclides will interfere without purification. Purification from Ca is important for recovery tests but need not be as thoro if Sr recovery is detd by $^{85}Sr$ tracer or flame photometry. Thoroly desorb columns before re-use and test periodically to assure complete desorption.

### C. Apparatus

*See* **973.66B(b)–(f)**, plus following:

*Ion exchange system*.—Consists of 1 L graduated separator, 250 mL separator with fritted glass disk as cation exchange column, and 30 mL separator with fritted glass disk as anion exchange column (Kontes Glass Co., No. K-427530).

### D. Reagents

*See* **973.66C(c), (e)–(g)**, plus following:

(**a**) *Ammonium acetate buffer*.—pH 5.0. Dissolve 153 g $NH_4OAc$ in 900 mL $H_2O$. Adjust pH to 5.0 with HOAc, using pH meter, and dil. to 1 L.

(**b**) *Anion exchange resin*.—Dowex 1-X8 (Cl form), anal. grade, 50–100 mesh size, available from Bio-Rad Laboratories.

(**c**) *Barium carrier soln*.—20 mg Ba/mL. Dissolve 38.1 g $Ba(NO_3)_2$ in $H_2O$, add 1 mL $HNO_3$, and dil. to 1 L.

(**d**) *Cation exchange resin*.—Dowex 50W-X8 (Na form), anal. grade, 50–100 mesh size, available from Bio-Rad Laboratories. Convert com. available H form into Na form by passing 1.5 L 4*N* NaCl thru 170 mL resin placed in column and rinsing with ca 500 mL $H_2O$ until wash $H_2O$ is Cl-free when tested with 1% $AgNO_3$.

(**e**) *Citrate soln*.—2*M*. Dissolve 384 g anhyd. citric acid (420 g monohydrate) in $H_2O$, adjust to pH 6.5 with dil. NaOH soln, and dil. to 1 L.

(**f**) *Oxalic acid soln*.—2*N*. Dissolve 126 g $H_2C_2O_4.H_2O$ in warm $H_2O$, cool, and dil. to 1 L.

(**g**) *Silver nitrate soln.*—1%. Dissolve 1 g $AgNO_3$ in $H_2O$ and dil. to 100 mL. Store in brown bottle.

(**h**) *Sodium chloride soln.*—4*N*. Dissolve 236 g NaCl in $H_2O$ and dil. to 1 L.

(**i**) *Sodium carbonate soln.*—3*N*. Dissolve 159 g $Na_2CO_3$ in $H_2O$ and dil. to 1 L.

(**j**) *Sodium chromate soln.*—1*N*. Dissolve 81 g $Na_2CrO_4$ in $H_2O$ and dil. to 1 L.

(**k**) *Strontium carrier soln.*—20 mg Sr/mL. Dissolve 48.3 g $Sr(NO_3)_2$ in $H_2O$, add 1 mL $HNO_3$, and dil. to 1 L. Stdze by pipetting 1 mL portions into six sep. 40 mL centrf. tubes contg 15 mL $H_2O$. Adjust pH (indicator paper or meter) to 8.5–9.0 with 6*N* $NH_4OH$. Add, with stirring, 3–5 mL 3*N* $Na_2CO_3$ and digest 5 min in near boiling $H_2O$ bath. Cool to room temp. and process ppt as in **974.37G(d)** or (**e**).

(**l**) *Tributyl phosphate (TBP), pre-equilibrated.*— Add 150 mL $H_2O$ and 30 mL 3*N* $Na_2CO_3$ to 300 mL TBP in 1 L separator. Shake 2–3 min and let sep. Discard lower aq. phase. Add 150 mL $H_2O$ to separator, shake 2–3 min, and let sep. Discard lower aq. phase. Add 150 mL 14*N* $HNO_3$ and shake 5 min. Let sep. and discard lower aq. phase. Repeat 14*N* $HNO_3$ treatment twice.

(**m**) *Yttrium carrier soln.*—10 mg Y/mL. Dissolve 12.7 g $Y_2O_3$ in 50 mL $HNO_3$ by heating (avoid boiling). Dil. to 900 mL with $H_2O$, adjust pH to 2.0 with $NH_4OH$, and dil. to 1 L with $H_2O$. Stdze by pipetting 1 mL portions into each of six 40 mL centrf. tubes contg 15 mL $H_2O$. Add 5 mL 2*N* oxalic acid and adjust pH to 1.5 with 6*N* $NH_4OH$, using pH meter. Digest in hot $H_2O$ bath 10 min, and cool to below room temp. Centrf. and discard supernate. Process ppt as in **974.37G(d)** or (**e**).

Use $Y_2O_3$ of 99.999% purity (Morton-Thiokol Inc., 152 Andover St, Danvers, MA 01923). Material of lower purity may require purification because of radioactive contaminants.

### *E. Preparation of Sample*

Preserve freshly drawn sample with ca 3 mL HCHO soln for each L milk and refrigerate for known period of time up to 2 weeks to allow $^{90}Y$ ingrowth. Thoroly mix preserved, stored sample. If homogeneous, transfer 1 L to separator, **974.37C**. If nonhomogeneous, before transfer, filter thru loose bed of Pyrex glass wool to prevent clogging of resin columns.

### *F. Removal of Radioelements by Ion Exchange*

Combine 1.00 mL each of Y, Sr, and Ba carriers with 10 mL citrate soln, (**e**) in small beaker or vial. Using distd $H_2O$, transfer quant. to 1 L sample in separator, and mix well.

Add 170 mL Dowex 50W-X8, (**d**), to 250 mL separator filled with $H_2O$. Add 15 mL Dowex 1-X8, (**b**), to 30 mL separator filled with $H_2O$. Connect all separators together in order sample (top), anion column, cation column (bottom), and place beaker to collect effluent. Open stopcocks of sample, anion, and cation separators, in that order, and note time. Control effluent rate at 10 mL/min with anion column stopcock. Check and adjust effluent flow periodically.

Stop flow when milk level reaches top of each resin bed and note time. Record as mean time the av. period of effluent flow. This time is taken as beginning of $^{90}Y$ decay. Do not permit unnecessary delay during elution. Discard eluate.

Connect separator contg 300 mL warm $H_2O$, continue elution at 10 mL/min as above, and discard. Sep. columns.

### *G. Yttrium-90 Separation, Purification, and Determination*

Connect separator contg 100 mL 2*N* HCl to top of anion separator. Open upper stopcock and then lower stopcock, and control effluent flow at 2 mL/min. Collect 15 mL eluate. Close both stopcocks and remove top separator. Stir resin thoroly with glass stirring rod, and rinse into resin column with small vol. 2*N* HCl. Reconnect separator, and continue 2*N* HCl elution, collecting total of 70 mL Y eluate. Retain eluate.

Adjust flow rate to 10 mL/min for remaining 30 mL acid to recharge separator. Discard this eluate. Wash resin with ≥100 mL $H_2O$ until Cl-free by $AgNO_3$ test. Separator is ready for next detn.

Add 5 mL 2*N* oxalic acid to retained eluate and adjust pH to 1.5 with 6*N* $NH_4OH$, using pH meter. Stir, heat to near bp in $H_2O$ bath, cool in ice bath, centrf., decant, and discard supernate. Proceed as in (**a**) or (**b**), depending on whether $^{140}Ba$-$^{140}La$ is absent or present from gamma analysis of sample.

(**a**) *If fresh fission products are absent.*—Dissolve ppt in 1 mL 6*N* HCl, add 15 mL $H_2O$, and filter thru Whatman No. 541 paper into 40 mL centrf. tube. Wash paper, collecting washings in tube, discard paper, and continue as in (**c**).

(**b**) *If fresh fission products are present.*—Dissolve ppt in 10 mL $HNO_3$; transfer soln to 60 mL separator, washing centrf. tube with addnl 10 mL $HNO_3$. Add 10 mL equilibrated TBP, (**l**), shake 2–3 min, let sep., and drain and discard lower acid phase. Add 15 mL 14*N* $HNO_3$ to separator, shake 2–3 min, let sep., and drain and discard lower acid phase. Repeat 14*N* $HNO_3$ treatment to remove light lanthanide elements, particularly $^{140}La$. Add 15 mL $H_2O$ to separator and shake 2–3 min. Let sep., and drain aq. phase contg most of Y into 40 mL centrf. tube. Repeat wash, using 15 mL 0.1*N* $HNO_3$, adding it to centrf. tube.

(**c**) *Preparation of yttrium oxalate.*—Add 5 mL 2*N* oxalic acid to purified Y soln from (**a**) or (**b**), and adjust to pH 1.5 with $NH_4OH$, using pH meter. Digest soln in hot $H_2O$ bath 10 min with occasional mixing. Cool in ice bath, centrf., and discard supernate. Sep. and count $^{90}Y$ oxalate as in (**d**) or (**e**), stdze carrier by the same technic used for sample, and calc. $^{90}Sr$ activity from $^{90}Y$ count as in **974.37I(a)**.

(**d**) *Filtration method.*—Place 2.8 cm glass fiber filter on stainless steel planchet and weigh together. Transfer tared filter to filter holder, **973.66B(b)**(*1*), and assemble.

With $H_2O$ spray, quant. transfer Y oxalate ppt to filter funnel, using min. of suction so that ppt is distributed uniformly over filter area. Increase suction as necessary after most of ppt is on filter. Wash ppt with three 10 mL portions warm $H_2O$, three 5 mL portions alcohol, and three 5 mL portions ether. Continue suction ca 2–3 min. Carefully remove filter, place on original planchet, and let stand at room temp. 10–15 min. Weigh and calc. yield Y oxalate (likely $Y_2(C_2O_4)_3.9H_2O$) by dividing this wt by wt obtained on stdzn of carrier, **974.37D(m)**.

Remove filter from planchet, place on top of nylon disk, cover with piece of Mylar film, place nylon ring over Mylar film, and press ring onto nylon disk. Cut off excess film. Count $^{90}Y$ activity, without undue delay, in low background anticoincidence beta counter. Repeat counting after 3 days to confirm purity of $^{90}Y$ by its rate of decay. Record dates and time of counting.

(**e**) *Direct dispersion method.*—Wash ppt twice with 20 mL portions warm $H_2O$, cool to below room temp., centrf., and discard supernate. Quant. transfer ppt to tared stainless steel dish. Uniformly disperse ppt over dish bottom and dry under IR lamp to const wt. Count in β particle counter.

### *H. Strontium-89 Separation, Purification, and Determination*

(*Caution:* See safety notes on nitric acid and fuming acids.)

Connect 1 L separator contg 1 L 4*N* NaCl to cation separator. Open upper stopcock and then lower stopcock, and control effluent flow at 10 mL/min. Collect ca 1 L eluate in 2 L beaker, but leave resin covered with 2–3 mL soln. Retain eluate.

Wash cation separator with 500 mL $H_2O$ from top separator at rate of 10 mL/min. Discard wash $H_2O$. If resin becomes clogged with milk solids, back-wash separator or transfer resin to beaker, agitate with $H_2O$, and decant.

Dil. retained eluate to 1.5 L with $H_2O$, heat to 85–90° on hot plate, and add 100 mL 3*N* $Na_2CO_3$ with gentle stirring. Remove from heat and cool to room temp. Decant bulk of clear supernate. Quant. transfer ppt to 250 mL centrf. bottle with $H_2O$ and centrf.; discard supernate. Add 50 mL $H_2O$ and disperse ppt. Centrf., discard supernate, and repeat. Dry ppt 4 hr in oven at 110°.

Dissolve ppt with vigorous stirring by adding ca 4 mL 6*N* $HNO_3$ in small amts (mag. stirrer is helpful). Filter thru Whatman No. 541 paper into 40 mL graduated centrf. tube. Rinse bottle with 4 mL 6*N* $HNO_3$ and pour washing thru paper. Discard paper. Add 20 mL 21*N* $HNO_3$ to filtrate. Stir and cool in ice bath; centrf. and discard supernate. ($Sr(NO_3)_2$ pptn is critical in obtaining good recovery of Sr adequately sepd from Ca.) Recoveries from single pptn are as follows:

| [$HNO_3$] | Sr Rec., % | Ca Rec.,% |
|---|---|---|
| 14*N* | 81±4 | 2.6±0.9 |
| 16*N* | 98±1.4 | 11 ±2 |
| 18*N* | 100±1.7 | 51 ±3 |

Dissolve ppt in 5 mL $H_2O$ and adjust to pH 5.0 with $NH_4OH$, using pH meter. Add 5 mL $NH_4OAc$ buffer. Heat in $H_2O$ bath, add 1 mL 1*N* $Na_2CrO_4$, and mix well. Digest in bath 5 min. Centrf. and decant supernate into small beaker. Evap. to ca 2 mL, add 2 mL 6*N* $HNO_3$, and transfer to 40 mL centrf. tube, using one 3 mL $H_2O$ rinse. Add 20 mL 21*N* $HNO_3$, stir, cool in ice bath, centrf., and discard supernate. Add 3 mL $H_2O$ and 5 mL 6*N* $HNO_3$ to dissolve ppt. Add 20 mL 21*N* $HNO_3$, stir, cool in ice bath, centrf., and discard supernate. Record time as beginning of $^{90}Y$ ingrowth.

Dissolve ppt in few mL $H_2O$ and adjust pH to 8.5–9.0 with 6*N* $NH_4OH$. Add 3–5 mL 3*N* $Na_2CO_3$ to ppt $SrCO_3$. Centrf., and discard supernate. Disperse ppt in 10 mL $H_2O$, centrf., and discard supernate. Sep. and count $SrCO_3$ as in (**a**) or (**b**):

(**a**) *Filtration method.*—Proceed as in **974.37G(d)**, but wash ppt with three 5–10 mL portions $H_2O$, transfer to original planchet, and dry 30 min in oven at 110°. Cool in desiccator and weigh. Count as in **974.37G(d)**, record time of counting, and calc. $^{89}Sr$ as in **974.37I(b)**.

(**b**) *Direct dispersion method.*—Wash ppt twice with ca 10 mL portions $H_2O$, dispersing ppt, centrf., and decant and discard supernate. Quant. transfer ppt to tared stainless steel dish. Uniformly disperse ppt over dish bottom, dry 30 min in 110° oven. Cool in desiccator and weigh. Count in β particle counter. Record time of counting and calc. $^{89}Sr$ as in **974.37I(b)**

### *I. Calculations*

(**a**) *For strontium-90 activity.*—

$$^{90}\text{Sr activity, pCi/L} = (\text{cpm} \pm \sigma)/R_SR_YE_YD_YI_YV$$

where cpm = net beta count rate of $^{90}Y$

$$\sigma = \sqrt{\frac{N_s}{t_s} + \frac{N_b}{t_b}}$$

$N_s$ = sample count rate
$N_b$ = background count rate
$t_s$ = sample counting time
$t_b$ = background counting time
$R_S$ = fraction Sr carrier recovered
$R_Y$ = fraction Y carrier recovered
$E_Y$ = counter efficiency for $^{90}Y$ as Y oxalate, cpm/pCi
$D_Y$ = decay correction factor (= $e^{-\lambda t}$, defined in **973.66E(a)**) for $^{90}Y$, where t is time of sepg $^{90}Y$ from $^{90}Sr$ to time of counting, **974.37G(d)** or (**e**)
$I_Y$ = ingrowth correction factor (= $1 - e^{-\lambda t}$) for degree of equilibrium attained during $^{90}Y$ ingrowth period, where t is time from start of ingrowth period to time of sepg $^{90}Y$ from $^{90}Sr$
V = sample vol., L

(**b**) *For strontium-89 activity.*—

$$^{89}\text{Sr activity, pCi/L} = \frac{1}{E_SD_S}\left[\frac{N_S + \sigma}{R_SV} - C_S(a_SE'_S + E_YI_Y)\right]$$

where $E_S$ = counter efficiency for $^{89}Sr$ as $SrCO_3$, cpm/pCi
$D_S$ = decay correction factor (= $e^{-\lambda t}$) for $^{89}Sr$, where t is time from sample collection to time of counting

$$\sigma = \sqrt{\frac{N_s}{t_s} + \frac{N_b}{t_b}}$$

$R_S$ = fraction Sr carrier recovered
$N_S$ = net counts/min of observed radio-Sr
V = sample vol., L
$C_S$ = $^{90}Sr$ activity, pCi/L
$a_S$ = absorption factor for $^{90}Sr$ as $SrCO_3$ obtained from self-absorption calibration curve. (Self-absorption curves for $^{89}Sr$ and $^{90}Sr$ derived by pptg series of carrier $SrCO_3$ concns over expected recovery range in presence of const amt of $^{89}Sr$ and $^{90}Y$-free $^{90}Sr$, resp. Ordinate is ratio of count rate for each thickness to count rate at 0 sample thickness and abcissa is sample wt for given type of sample mount.)
$E'_S$ = counter efficiency for $^{90}Sr$ as $SrCO_3$, cpm/pCi
$E_Y$ = counter efficiency for $^{90}Y$ as Y oxalate, cpm/pCi
$I_Y$ = correction factor (= $1 - e^{-\lambda t}$) for degree of equilibrium attained during $^{90}Y$ ingrowth period, where t is time $^{90}Y$ was sepd from $SrCO_3$ to time of counting, **974.37H(a)** or (**b**)

Refs.: JAOAC **56,** 213(1973); **57,** 37(1974).

CAS-31083-24-6 (strontium-89)
CAS-37380-96-4 (strontium-90)
CAS-10098-91-6 (yttrium-90)

## 973.67 Iodine-131, Barium-140, and Cesium-137 in Milk and Other Foods
### Gamma-Ray Spectroscopic Method

($^{137}Cs$ in milk: first action 1973, final action 1974. $^{137}Cs$ in other foods: first action 1986. $^{140}Ba$ in milk: first action 1982, final action 1983. $^{131}I$ in milk: first action 1982, final action 1983. $^{131}I$ in other foods: first action 1986.)

### *A. Principle*

Applicable to $^{131}I$, $^{140}Ba$, and $^{137}Cs$ in fluid milk preserved with HCHO, and I and Cs in foods. Because of the nature of gamma-emitting radionuclides, attenuation of gamma-rays in food slurries or mixt. would be similar to that of milk or $H_2O$. Unlike in milk samples, other radionuclides might be present in foods. Therefore, before performing calcn, gamma-ray energy spectrum should be inspected for any radionuclides besides $^{40}K$, $^{131}I$, $^{137}Cs$, and $^{140}Ba$. Since cessation of above-ground weapons testing in 1960s, no other gamma-ray emitters have been regularly observed or detected in food. (Should any be detected, matrix technic should be expanded using std source for suspected radionuclide to det. matrix coefficient.)

Known vol. is placed in counting vessel positioned over and around right cylinder scintillation crystal detector NaI (Tl) of multichannel gamma spectrometer. Gamma radiation is counted for given time. Accumulated pulses from selected photon energy range are sepd from other gamma-emitting radionuclides and background radiation by simultaneous equations. $^{40}K$ is always present as natural contaminant and may contribute counts in 1 or more of photopeak ranges. Mutual interferences among these 4 photopeaks are eliminated by applying matrix technic to sep. activities of the 4 nuclides. Measurement of one std source of each nuclide provides the matrix coefficients.

In special cases, newly formed fission products may be present, e.g., $^{133}I$ and $^{135}I$, which may interfere either thru direct overlapping of photopeaks or by contributing Compton-continuum counts. Such interference may be minimized by waiting for decay of short-lived radionuclides, by addnl counting following decay, or by chem. sepn.

Milk contg known increments of $^{131}I$, $^{137}Cs$, and $^{140}Ba$, detd in triplicate by 25 laboratories, and 2nd milk contg known increment of $^{131}I$, detd in triplicate by 40 laboratories, showed following results (av. of triplicates);

| Amt Nuclide Present, pCi/L | Std Dev. (CV, %) Within Labs | Between Labs | Bias ± 95% Uncertainty pCi/L | % |
|---|---|---|---|---|
| $^{131}I$ | | | | |
| 98 | 6.1 (6.2) | 8.2 (8.3) | +0.9 ± 3.7 | +0.9 ± 3.8 |
| 633 | 29.0 (4.6) | 30.1 (4.8) | +2.3 ± 14.3 | +0.4 ± 2.3 |
| $^{140}Ba$ | | | | |
| 72 | 6.5 (9.1) | 11.2 (15.6) | +4.0 ± 4.8 | +5.5 ± 6.7 |
| 515 | 19.5 (3.8) | 35.8 (7.0) | +7.9 ± 15.8 | +1.5 ± 3.1 |
| $^{137}Cs$ | | | | |
| 52 | 4.7 (9.1) | 4.1 (8.0) | +1.3 ± 2.0 | +2.4 ± 3.8 |
| 305 | 11.4 (3.7) | 13.5 (4.4) | −9.8 ± 6.1 | −3.2 ± 2.0 |
| $^{131}I$ | | | | |
| 82 | 5.6 (6.8) | 6.8 (8.3) | −0.4 ± 2.4 | −0.5 ± 2.9 |

### B. Apparatus

(**a**) *Alignment sources*.—Gamma ray energies, at least 1 near $^{137}Cs$ spectrum, with well known energies and abundance of gamma rays in photopeaks, for alignment. Solid sources, ca 0.1 μCi, are preferred over liq. sources. $^{207}Bi$ is satisfactory single source with several photopeaks; $^{137}Cs$ and $^{60}Co$ are good pair.

(**b**) *Counter*.—Low level gamma spectrometer consisting of shielded Tl-activated NaI scintillation detector, 4 × 4 in., coupled to multichannel pulse-ht analyzer and readout system.

(**c**) *Counting vessel* (*Marinelli beaker*).—Use 3.5 L beaker, Fig. **973.67**, for 4 × 4 in. detector. Beaker and lid available from plastic laboratory-ware suppliers such as Bel-Art Products, Industrial Rd, Pequannock, NJ 07440-1992, No. H26852 for beaker and No. H26587 for lid.

### C. Reagents

(*Caution:* See safety notes on radioactive chemicals.)

(**a**) *Carrier solns*.—10 mg/mL. Prep. solns of CsCl (1.267 g/100 mL), NaI (1.181 g/100 mL), and $BaCl_2.2H_2O$ (1.779 g/100 mL). Store in polyethylene or glass bottles.

(**b**) *Stock std solns*.—10 000 pCi/mL. Dil. calibrated solns of $^{131}I$, $^{140}Ba$, and $^{137}Cs$ to approx. indicated strength.

(**c**) *Potassium-40 stock std soln*.—1.89 dpm (disintegrations/min) $^{40}K$/mg K. Dissolve 240 g KCl (equiv. to 126 g K) in 3 L $H_2O$ in Marinelli beaker and dil. to 3.5 L.

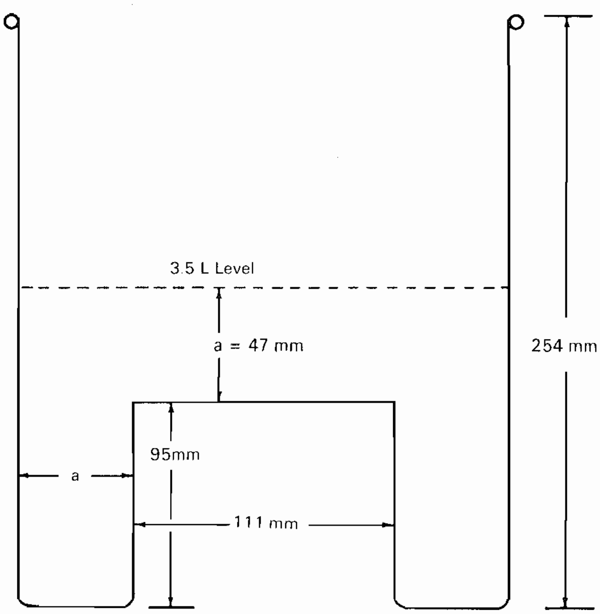


**FIG. 973.67—Cross-section of Marinelli beaker**

(**d**) *Calibrating solns*.—For $^{137}Cs$ and $^{140}Ba$, add 3–5 mL carrier soln, (**a**), to 3 L $H_2O$ in Marinelli beaker, mix, add convenient amt of stock std soln, (**b**), sufficient to reduce counting error to ca 1% when counted within 10–100 min, mix, adjust pH to 3.5–4.5, and dil. to 3.5 L. Prep. $^{131}I$ soln similarly, but adjust pH to 8.5.

### D. Preparation of Sample

No special preparation is needed for milk samples. For other foods, do not include inedible material such as bone, apple cores, nut shells, and egg shells as part of sample. Homogenize sample in blender or mech. homogenizer. Dietary samples prepd for consumption do not require blending, but must be sufficiently mixed to ensure representative sample.

### E. Determination

Using alignment sources centered on detector, adjust spectrometer to cover range at least between 0 and 2 meV, in intervals (channels) of 10 or 20 keV. Adjust voltage or gain control so that the 2 gamma photopeaks of std fall in their appropriate channels. Check and adjust alignment daily.

Place Marinelli beaker contg 3.5 L calibrating soln, (**d**), over detector, and count std for time (10–100 min) sufficient to reduce counting error to ca 1%. Repeat with each calibrating soln and with $H_2O$. Recalibrate spectrometer yearly or more frequently if gamma ray resolution changes.

Transfer 3.5 L well mixed sample at room temp. into Marinelli beaker, place over detector, and count 100 min or time sufficient to give desired counting statistics.

### F. Calculations

(**a**) *Counter efficiency*.—Total individual counts observed in channels of photopeak range for each calibrating soln. Subtract total background count for same photopeak range. Divide net count by counting time in min and amt of radionuclide in pCi, and record cpm/pCi for each.

(**b**) *Interference coefficients*.—When counting std soln of each radionuclide, $^{131}I$, $^{137}Cs$, $^{140}Ba$, $^{40}K$, ratio of net counting rate in energy range of each of the other radionuclides to net counting rate in its own photon energy range gives its fractional interfering coefficient for each of the other energy ranges, e.g., $^{131}I$ ratio of net counting rate in $^{137}Cs$ energy range to net counting rate in $^{131}I$ photon energy range gives its fractional interfering coefficient for $^{137}Cs$ energy range.

Designate counting rate for $^{131}I$, $^{140}Ba$, $^{137}Cs$, and $^{40}K$ with symbols I, B, C, and K, resp. Designate net counting rates (observed—background) in their resp. photon energy ranges as $N_i$, $N_b$, $N_c$, and $N_k$, resp. Then, f, fractional coefficients or contributions of nuclide in particular range, is designated by 2 lower case subscripts; first one indicates nuclide contributing counts to energy range (column) and second, nuclide photon energy range (row). The following 4 equations:

$$N_i = I + f_{bi} B + f_{ci} C + f_{ki} K \quad (1)$$

$$N_b = f_{ib} I + B + f_{cb} C + f_{kb} K \quad (2)$$

$$N_c = f_{ic} I + f_{bc} B + C + f_{kc} K \quad (3)$$

$$N_k = f_{ik} I + f_{bk} B + f_{ck} C + K \quad (4)$$

can be solved simultaneously by matrix algebra, using inversions to provide numerical consts W, X, Y, and Z in equations 5, 6, 7, and 8. These consts are used to solve for concn of each of these 4 nuclides in sample. Net counting rate for each nuclide is:

$$^{131}I = I = W_1N_i + W_2N_b + W_3N_c + W_4N_k \quad (5)$$

$$^{140}Ba = B = X_1N_i + X_2N_b + X_3N_c + X_4N_k \quad (6)$$

$$^{137}Cs = C = Y_1N_i + Y_2N_b + Y_3N_c + Y_4N_k \quad (7)$$

$$^{40}K = K = Z_1N_i + Z_2N_b + Z_3N_c + Z_4N_k \quad (8)$$

Calibration to derive values for consts in equations 5, 6, 7, and 8 is applicable as long as instrument alignment and mode of operation remain const and gamma-emitting nuclides are limited to the 4 elements in matrix. Long-hand inversion of 4 × 4 matrix is tedious and subject to mistakes. Use of computer is recommended to provide numerical consts for equations 5–8. Thereafter, desk calcns can det. concns of $^{131}I$, $^{140}Ba$, $^{137}Cs$, and $^{40}K$ in samples in absence of computer by summing counts in each photopeak, subtracting background, and applying equations 5–8.

(**c**) *Iodine-131, barium-140, cesium-137, potassium-40 activities.*—From spectral gamma counts of sample, substitute net value from equations 5 thru 8 and convert net counts/min for each nuclide to pCi/L sample at time of counting:

$$^{131}I(pCi/L) = (\text{net cpm})_i / (E_i \times V)$$

$$^{140}Ba(pCi/L) = (\text{net cpm})_b / (E_b \times V)$$

$$^{137}Cs(pCi/L) = (\text{net cpm})_c / (E_c \times V)$$

$$^{40}K(pCi/L) = (\text{net cpm})_k / (E_k \times V),$$

where $E_i$, $E_b$, $E_c$, $E_k$ = counting efficiency/pCi from std solns for $^{131}I$, $^{140}Ba$, $^{137}Cs$, $^{40}K$, resp., and V = sample vol., L.

Refs.: JAOAC **56,** 204(1973); **65,** 1039(1982); **69,** 270, 301(1986); **72,** 667(1989).

CAS-10045-97-3 (cesium-137)
CAS-10043-66-0 (iodine-131)

## 955.50 Radioactive Contamination
### Emergency Level Measures
### Procedure

***A. Apparatus***

(**a**) *Portable count-rate meter.*—Consists of: (*1*) *Self-quenching glass Geiger-Müller tube,* side wall ≤32 mg/cm², mounted in slide opening metal shield; threshold ca 800 v, operated at ca midpoint of voltage plateau, slope of which is ≤10%, connected with coaxial cable to (*2*) *Suitable power supply and electronic amplifier unit* with meter calibrated in milliroentgens (mr)/hr, connected thru sensitivity switch providing 3 ranges of scale reading, *e.g.*, 0–20, 0–2, and 0–0.2 mr/hr; linear response within each range.

(**b**) *Comparison std.*—Induces meter response identical to that from surface of $H_2O$ contaminated with fission products decaying at rate of $2 \times 10^5$ dpm/mL (emergency tolerance level for $H_2O$ to be consumed for ≤10 day period). Construct such std as follows: Uniformly suspend suitable amt of "60-mesh" $UO_2(OAc)_2.2H_2O$ (ca 3 g, adjusted by trial) (*Caution:* See safety notes on uranyl acetate and toxic dusts.) in 5 g liq. casting plastic, level, and solidify in shallow container, such as lid of ointment tin, ca 80 mm diam. and side wall 15 mm deeper than layer of plastic. Base of ointment tin, fitted with indented ring 15 mm below its edge, serves as container for liqs and finely divided solids to be tested, and to protect comparison std when not in use. Supplementary std of ½ this activity may be prepd similarly for monitoring supplies to be consumed over 30 day period.

***B. Determination***

With selectivity switch set for highest range (*e.g.*, 0–20 mr/hr), and with shield open, place G-M tube diametrically across std in contact with edge of container at 2 points. Adjust meter pointer to convenient value ca midway of scale with calibration screw and record reading as av. of fluctuations over 1–2 min. Duplicate reading should check within ±5%. Avoid extraneous radiation, such as that from luminous dial watch.

Fill sample container with liq. or finely divided solid to level of indented ring and obtain duplicate readings. Sample readings within ±100% of std reading are of practical quant. significance for monitoring under emergency conditions.

Ref.: JAOAC **38,** 678(1955).

# 14. Veterinary Analytical Toxicology

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Methods have been included in this chapter because they provide data which may be used to support a clinical diagnosis. Other methods in this book may also be used for this purpose provided the method has been adopted for the analyte/substrate combination involved.

## Arsenic in Feeds

*See* **957.22**.

## 986.30 Arsenic in Liver Tissue
### Spectrophotometric Method
**First Action 1986**
**Final Action 1989**

### *A. Principle*

Liver tissue is dry-ashed overnight at 500°, ash is dissolved, and portion is reacted with Zn metal to evolve arsine gas. Arsine is trapped and As is detd spectrophtric.

### *B. Apparatus and Reagents*

(**a**) *Hydrochloric acid.*—3N.

(**b**) *Cupric sulfate.*—Anhyd., powd (J.T. Baker, Inc., No. 1850, or equiv.).

(**c**) *Magnesium oxide-magnesium nitrate slurry.*—Suspend 7.5 g MgO and 10.5 g $Mg(NO_3)_2.6H_2O$ in enough $H_2O$ to make 100 mL. Agitate vigorously before adding to sample.

(**d**) *Stannous chloride soln, 20% (w/v).*—Dissolve 20 g As-free $SnCl_2.2H_2O$ in HCl and dil. to 100 mL with HCl.

(**e**) *Silver diethyldithiocarbamate (AgDDC) soln.*—Dissolve 0.50 g AgDDC salt in pyridine and dil. to 100 mL with pyridine. Mix and store in amber bottle. Reagent is stable several months at room temp. (Fisher Scientific Co., Cat. No. S-666, or equiv.).

(**f**) *Arsenic std solns.—(1) Stock soln.*—500 μg/mL. Accurately weigh 0.660 mg NIST Ref. Std $As_2O_3$, or equiv., dissolve in 25 mL 2N NaOH, and dil. to 1 L with $H_2O$. *(2) Intermediate soln.*—10 μg/mL. Transfer 2 mL stock soln to 100 mL vol. flask, and dil. to vol. with $H_2O$. *(3) Working soln.*—2 μg/mL. Transfer 10 mL intermediate soln to 50 mL vol. flask and dil. to vol. with $H_2O$.

(**g**) *External control.*—NIST Std Ref. Material (SRM) 1566 Oyster Tissue (13.4 ± 1.9 mg As/kg or equiv.

(**h**) *Potassium iodide soln, 15% (w/v).*—Dissolve 15 g KI in $H_2O$ and dil. to 100 mL.

(**i**) *Zinc.*—Shot contg <0.00001% As (Fisher Scientific Co., No. Z-12).

(**j**) *Distillation apparatus.*—*See* **973.78A**(**e**). Use 125 mL erlenmeyer instead of 250 mL. Use narrow test tube as receiver and submerge delivery tube in AgDDC soln.

### *C. Preparation of Standard Curve*

Transfer 0.5, 1.0, 3.0, 6.0, and 10.0 mL aliquots of working soln corresponding to 1, 2, 6, 12, and 20 μg As to sep. 125 mL erlenmeyers. Dil. to 50 mL with 3N HCl. Carry these solns thru distn procedure. Plot *A* at 540 nm on ordinate vs μg As on abscissa. Det. best fitting straight line, using all 5 points, by method of least squares.

### *D. Preparation of Sample*

Blend tissue in high-speed blender until completely homogeneous. Accurately weigh 2.00 g tissue into 30 mL Coors crucible. Analyze one external control with each set of 10 samples or fraction thereof. Add 5mL well mixed MgO/$Mg(NO_3)_2.H_2O$ slurry and mix thoroly with stirring rod. Prep. blank by adding 5 mL well mixed slurry to sep. crucible and carrying it thru subsequent steps in procedure. Dry samples, controls, and blank to apparent dryness on hot plate or in drying oven at <100°. Cover each crucible with watch glass and place in cold muffle furnace. Set furnace temp. at 250° for 3 h; then gradually increase temp. to 500° and leave overnight.

Cool crucibles to room temp., moisten residue with 5 mL $H_2O$, and transfer quant. to 50 mL vol. flask with 3N HCl. Dil. to vol. with 3N HCl and mix well. Transfer 25 mL aliquot to 125 mL erlenmeyer and dil. to 50 mL with 3N HCl.

### *E. Distillation*

Add 2 mL 15% KI soln and swirl. Add 1 mL $SnCl_2$ soln and swirl. Cool flasks in freezer or ice bath 45 min or until samples reach 4°. Pipet 6 mL AgDDC soln into narrow receiver test tube, one for each std, external control, sample, and blank. Have all parts of distn app. ready for immediate assembly. Quickly add 10 g Zn shot and pinch of $Cu_2SO_4$ to erlenmeyer, assemble app., and distil 1 h at room temp. Det. *A* at 540 nm for blank, external control, sample, and std AgDDC solns in suitable spectrphtr. Subtract blank reading from sample and control, and det. mg As/kg directly from std curve. External control results must fall within accepted range (95% confidence limit) for all results to be valid.

Ref.: JAOAC **69,** 493(1986).

## 985.40 Copper in Liver
### Atomic Absorption Spectrophotometric Method
**First Action 1985**
**Final Action 1987**

### *A. Principle*

One g liver tissue is digested overnight at 60° in 5 mL $HNO_3$, then dild to 25 mL with $H_2O$ and analyzed by AAS.

### *B. Apparatus and Reagents*

(**a**) *Atomic absorption spectrophotometer (AAS).*—Equipped with nebulizer and 10 cm, air-$C_2H_2$ burner head. Monitor performance by assuring that 4.0 mg Cu/L std produces ≥0.200 absorbance unit.

(**b**) *External control.*—Standard Reference Material (SRM 1577) Bovine Liver (193 ± 10 mg Cu/kg; NIST, Washington, DC 20243) or equiv.

(**c**) *Nitric acid.*—Concd and dild (1 + 4).

(**d**) *Teflon screw-cap bottles.*—30 mL wide mouth (Cole-Parmer, K-6103-30) or equiv.

(**e**) *Copper std solns.—(1) Stock std soln.*—1000 mg Cu/L. Dissolve 1.000 g Cu metal in 10 mL $HNO_3$-$H_2O$ (1 + 1).

Dil. to 1000 mL with 1% $HNO_3$. *(2) Intermediate std soln.*—100 mg Cu/L. Dil. 10 mL stock std soln to 100 mL with $H_2O$. *(3) Working std solns.*—Dil. 0.0, 0.25, 0.5, 1.0, 2.0, and 4.0 mL intermediate std soln to 100 mL with $HNO_3$-$H_2O$ (1 + 4) to give Cu stds contg 0.0, 0.25, 0.5, 1.0, 2.0, and 4.0 mg Cu/L, resp.

### C. Sample Preparation

Rinse all glassware with 2N HCl. Mix samples thoroly before weighing. Into sep. Teflon screw-cap bottles, accurately weigh 1.0 g liver tissue and 0.25 g external control for each 10 samples or fraction thereof. (*Note:* Complete digestion will not occur for >0.5 g dry wt of samples or controls.) Add 5 mL concd $HNO_3$ to each bottle, tighten cap, and place bottles overnight in 60°, ventilated oven.

Remove bottles from oven and cool to room temp. Using $H_2O$ to rinse bottles, transfer sample digests to 25 mL vol. flasks, allowing any fat to remain adhering to digestion bottles. Dil. flasks to vol. with $H_2O$.

### D Determination

Analyze by AAS using following conditions: wavelength 324.7 nm; slit 0.7 nm; flame air-$C_2H_2$ (lean-blue). Aspirate series of working std solns, external control solns, and sample dilns. Prep. std curve of concn (mg Cu/L) vs $A$ and det. sample soln concns. Dil. with $HNO_3$ (1 + 4) any samples above range of working stds. Repeat analysis if external control Cu value is not within accepted range. Calc. mg Cu/kg tissue ($X$):

$$X = (C \times 25 \times D)/W$$

where $C$ = sample soln concn (mg Cu/L); $D$ = addnl sample diln, if necessary; and $W$ = tissue wt (g).

Ref.: JAOAC **68,** 44(1985).

CAS-7440-50-8 (copper)

## 983.24 Copper in Serum

### Atomic Absorption Spectrophotometric Method

**First Action 1983**
**Final Action 1986**

### A. Principle

Samples are dild 1 + 1 with $H_2O$, and Cu is detd by AAS using std solns prepd in 10% glycerol.

### B. Apparatus and Reagents

(**a**) *Atomic absorption spectrophotometer.*—Equipped with nebulizer and air–$C_2H_2$ burner head. Monitor performance by assuring that 4.0 mg/L std produces response ≥0.200 absorbance unit.

(**b**) *External control.*—Precilip, Cat. No. 125067 (Bio-Dynamics/bmc, 9115 Hague Rd, Indianapolis, IN 46250), or equiv. with established value for Cu. Dil. according to label.

(**c**) *Glycerol USP.*—10% (v/v) aq. soln.

(**d**) *Copper std solns.*—*(1) Stock std soln.*—1000 mg/L. Dissolve 1.000 g Cu metal in min. vol. of $HNO_3$–$H_2O$ (1 + 1). Dil. to 1000 mL with 1% $HNO_3$. *(2) Intermediate std soln.*—100 mg/L. Dil. 10 mL stock std soln to 100 mL with $H_2O$. *(3) Working std solns.*—Dil. 0.0, 0.25, 0.5, 1.0, 2.0, and 4.0 mL intermediate std soln to 100 mL with 10% glycerol to give std solns contg 0.0, 0.25, 0.5, 1.0, 2.0, and 4.0 mg Cu/L.

### C. Sample Preparation

Rinse all glassware used with 2N HCl. Mix samples thoroly before pipetting. Using Mohr pipet, transfer 1.0 mL serum and 1.0 mL Precilip (external control) to sep. test tubes. Add 1.0 mL $H_2O$ to each and mix 5 s on vortex mixer or cap tubes and shake 10 s. Use 1 external control for each 10 samples or fraction thereof.

### D. Determination

Analyze by AAS using the following conditions: wavelength 324.7 nm; slit 0.7 nm; flame air–$C_2H_2$ (lean-blue). Aspirate series of working std solns, external control soln, and sample dilns. Repeat analysis if Cu value in external control soln is not within accepted range. Prep. std curve of concn, mg Cu/L, vs $A$, and det. concn of sample. Multiply result by 200 to account for sample diln and to convert result to μg Cu/100 mL.

Ref.: JAOAC **66,** 1140(1983).

CAS-7440-50-8 (copper)

## Drugs in Feeds

*See* chapter on drugs in feeds.

## Drug Residues in Animal Tissues

*See* chapter on drug residues in animal tissues.

## Metals

*See* chapter on metals and other elements at trace levels.

## Mycotoxins

*See* chapter on natural poisons.

## 984.32 Pesticide Residues

### A. General Methods

*See* **970.52**.

### B. Specific Methods

*See* chapter on pesticide residues.

## 984.33 Urea in Feeds

*See* **941.04** and **967.07**.

## 986.31 Nitrate in Forages

### Potentiometric Method

**First Action 1986**
**Final Action 1989**

### A. Principle

Nitrate is extd from sample into aq. $Al_2(SO_4)_3$ soln and detd potentiometrically. Lower limit of detection is 0.50% $KNO_3$.

### B. Apparatus

(**a**) *Drying oven.*—Forced air, capable of heating to 100°.

(**b**) *Nitrate-specific ion electrode.*—Model 93-07, Orion Research, Inc., or equiv. Monitor performance by assuring absolute value of std curve per decade slope ≥54 mV.

(**c**) *Reference electrode.*—Double junction (Model 90-02, Orion Research, Inc., or equiv.). Use extg soln as outer filling soln.

**Table 986.31 Conversion Factors for Units of Nitrate and Nitrite Concentrations**[a]

| To \ From | $NO_3$–N (14.01) | $NO_3$ (62.01) | $NaNO_3$ (85.01) | $KNO_3$ (101.11) | $NO_2$–N (14.01) | $NO_2$ (46.01) | $NaNO_2$ (69.01) | $KNO_2$ (85.11) |
|---|---|---|---|---|---|---|---|---|
| $NO_3$–N (14.01) | 1.000 | 0.2259 | 0.1648 | 0.1386 | 1.000 | 0.3045 | 0.2030 | 0.1646 |
| $NO_3$ (62.01) | 4.426 | 1.000 | 0.7294 | 0.6133 | 4.426 | 1.348 | 0.8986 | 0.7286 |
| $NaNO_3$ (85.01) | 6.068 | 1.371 | 1.000 | 0.8408 | 6.068 | 1.848 | 1.232 | 0.9988 |
| $KNO_3$ (101.11) | 7.217 | 1.631 | 1.189 | 1.000 | 7.217 | 2.198 | 1.465 | 1.188 |
| $NO_2$–N (14.01) | 1.000 | 0.2259 | 0.1648 | 0.1386 | 1.000 | 0.3045 | 0.2030 | 0.1646 |
| $NO_2$ (46.01) | 3.284 | 0.7420 | 0.5412 | 0.4550 | 3.284 | 1.000 | 0.6667 | 0.5406 |
| $NaNO_2$ (69.01) | 4.926 | 1.113 | 0.8118 | 0.6825 | 4.926 | 1.500 | 1.000 | 0.8108 |
| $KNO_2$ (85.11) | 6.075 | 1.373 | 1.001 | 0.8418 | 6.075 | 1.850 | 1.233 | 1.000 |

[a] Find current unit of concentration on top row. Find desired unit of concentration in left column. Obtain conversion factor at intersection of row and column. Desired concentration = current concentration × conversion factor. Numbers in parentheses below units of concentration are formula masses. Atomic masses: N: 14.01; O: 16.00; Na: 23.00; K: 39.10. 1% = 10 000 ppm.

(**d**) *pH meter.*—Capable of measuring electrode potentials to nearest mV.

### C. Reagents

(**a**) *Preservation soln.*—Dissolve 0.1 g phenylmercuric acetate in 20 mL dioxane (*Caution:* May form dangerous peroxides; see safety notes on peroxides.) and dil. to 100 mL with $H_2O$.

(**b**) *Extracting soln.*—Dissolve 15.76 g $Al_2(SO_4)_3.18\ H_2O$, 70.0 mg oven-dried $KNO_3$ (dry 2 h at 100°), and 1.0 mL preservation soln, (**a**), in 500 mL $H_2O$. Dil. to 1 L with $H_2O$.

(**c**) *Nitrate std solns.—(1) Stock std soln.*—100 000 mg $KNO_3$/L. Weigh 20.00 g $KNO_3$ (dried 2 h at 100°) into 200 mL vol. flask, dissolve in 100 mL $H_2O$, and dil. to vol. with $H_2O$. *(2) Intermediate std soln.*—10 000 mg $KNO_3$/L. Dil. 20.0 mL soln *(1)* to 200 mL with extg soln. *(3) Working std solns.*—Dil. 1.00, 2.00, 4.00, and 10.0 mL soln *(2)* to 200 mL with extg soln to make 120, 170, 270, and 570 mg $KNO_3$/L solns. (*Note:* Prep. all std solns from same lot of extg soln.)

### D. Preparation of Standard Curve

Det. potential of blank (use extg soln as blank, equiv. to 70 mg $KNO_3$/L) and each working std soln while mag. stirring. Plot potential against nitrate concn (mg $KNO_3$/L) on semilog paper with concn on log scale. Det. std curve per decade slope. Per decade slope specification of electrode manuf. should be met or exceeded. (*Note:* Measure potential only after sample and stds are at same temp.)

### E. Preparation of Sample

Dry sample in 60° forced air oven to const wt. Grind dried sample to pass 2 mm screen and thoroly mix. Ext 1.00 g mixed ground sample with 100 mL extg soln by shaking 15 min. Keep ext sealed in container until potential is measured.

### F. Quality Control

Analyze external control samples of known nitrate concn or spiked samples (at least one for every 10 samples or fraction thereof). Results should indicate acceptable accuracy.

Hold suspect any analyses with unacceptable external control results and unacceptable std curve per decade slopes.

### G. Determination

Det. potential of unfiltered ext at same temp. that std potentials are measured, stirring ext at same rate used for stds. Record potential after reading becomes stable or 1 min after insertion, whichever comes first.

### H. Calculation

Calc. forage nitrate concn (% $KNO_3$ on dry wt basis):

$$\%\ KNO_3 = C_u = (C_g - 70) \times 0.010$$

where $C_u$ = forage nitrate concn and $C_g$ = nitrate concn (mg $KNO_3$/L) of ext obtained from std curve. If desired, convert nitrate concn expressed as % $KNO_3$ to other units as shown in table **986.31**.

Ref.: JAOAC **69,** 196,283(1986).

# 15. Cosmetics

**Anthony D. Hitchins, *Associate Chapter Editor***
*Food and Drug Administration*

## GENERAL METHODS

### 966.22 Water and Ethyl Alcohol in Cosmetics

**Gas Chromatographic Method**

**First Action 1966**

***A. Principle***

Sample is dissolved or dispersed in ethylene glycol monomethyl ether, which also serves as internal std, and $H_2O$ and alcohol are detd by GC, using relatively inert column, perfluorocarbon substrate coated with high MW polyethylene glycol, to minimize tailing.

***B. Apparatus***

*Gas chromatograph.*—With thermal conductivity detector operated at following temps (°): Detector ca 250, injection port ca 260, oven, ca 100; He flow rate, 50 mL/min; bridge current, as directed by manufacturer.

***C. Standard Solutions***

Prep. 3 std solns in 125 mL g-s flasks contg $H_2O$ and absolute alcohol, weighed to 0.1 mg, in ethylene glycol monomethyl ether weighed to 10 mg, as follows:

| $H_2O$, mg | Alcohol, mg | Ethylene Glycol Monomethyl Ether, g |
|---|---|---|
| 125 | 375 | 24.50 |
| 250 | 250 | 24.50 |
| 375 | 125 | 24.50 |

$H_2O$ content of ethylene glycol monomethyl ether should be <0.05%. Use same batch for stds and samples. Com. product is usually satisfactory, but because of hygroscopicity, *expose solns to air as little as possible.*

***D. Preparation of Column***

Weigh 10 g polyethylene glycol 20000 (Carbowax 20M) into 800 mL beaker, dissolve completely in ca 400 mL warm $CH_2Cl_2$, and cool to ca 0°. Slurry cold soln with 190 g precooled (ca 0°) Fluoropak 80, 40–60 mesh (Applied Science). Transfer to 15 cm crystg dish, place in hood, and evap. to dryness at room temp. with occasional stirring. Recool to ca 0° and screen thru No. 40 on No. 60 precooled screen. Pack fraction remaining on No. 60 in 4.6 m (15′) × $^1/_4$″ precooled Cu column, using vibrator. (Use of cooled column packer (Press-Pak, available from Alltech Associates, 2051 Waukegan Rd, Deerfield, IL 60015) at 35 lb/sq in. (240 kPa) N pressure allows packing of cooled, precoiled column.)

***E. Standardization***

Inject 3 μL of one of std solns with 10 μL syringe and det. elution time of ethylene glycol monomethyl ether. (Order is alcohol, $H_2O$, and glycol ether.) Adjust oven temp., if necessary, so latter elutes in 15–20 min. With satisfactory column, pen will return to within 1% of recorder 0 between alcohol and $H_2O$ peaks. Det. sample size for each std soln such that response for smallest peak is $\geq ^1/_4$ full scale on ×1 attenuation. Det. all attenuations necessary to keep all peaks on chart scale. (Too large samples will overload column and skew glycol ether peak.)

With some gas chromatographs, alcohol peak response will vary with time interval between emergence of glycol ether of previous injection and injection of sample. Thus, inject all samples (std curves and actual detns) at same time interval after emergence of glycol ether. This requires use of preliminary sample. If time sequence is broken, inject another preliminary sample to re-establish sequence.

Obtain chromatograms, in duplicate, and on same day, for each std soln, using sample sizes and attenuations detd above. Duplicate sample sizes to 0.1 μL and use same technic for injecting and withdrawing syringe needle. Det. peak ht of each component, correcting for attenuation.

Calc. ratios: peak ht $H_2O$/peak ht ethylene glycol monomethyl ether ($R_{PW}$), and wt $H_2O$/wt ethylene glycol monomethyl ether ($R_{WW}$). Average the 2 $R_{PW}$ values for each std soln and plot av. $R_{PW}$ values against corresponding $R_{WW}$ values. Draw best straight line thru 3 points. Make same calcns for alcohol stds, and plot corresponding $R_{PA}$ and $R_{WA}$ values. (Curves should be straight lines intersecting $x$ or $y$ axis near origin.)

***F. Determination***

Accurately weigh sample contg ca 100–400 mg $H_2O$ and/or alcohol into 125 mL g-s flask, add 24.50 ± 0.1 g ethylene glycol monomethyl ether, and mix thoroly. (Complete soln is unnecessary but glycol ether phase should contain all the $H_2O$ and alcohol.) Det. proper sample size as in **966.22E**, recording necessary attenuations. From good chromatogram, calc. $R_{PW}$ and $R_{PA}$ values. Read $R_{WW}$ and $R_{WA}$ values from std curves. From latter values prep. *final std soln* of 24.50 g ethylene glycol monomethyl ether plus $H_2O$ and/or alcohol which approximates (within 10%) curve of sample. Det., in sequence, (*a*) curve of proper size sample, (*b*) curve of adjusted *final std soln*, and repeat (*a*) and (*b*), in that order. Det. av. $R_{PW}$ and $R_{PA}$ values of unknown and final std solns, then $R_{WW}$ and $R_{WA}$ of std soln.

For $H_2O$: $R_{WW}$ (sample) = $R_{PW}$ (sample) × $R_{WW}$ (std)/$R_{PW}$ (std); wt $H_2O$ (sample) = wt ethylene glycol monomethyl ether (sample) × $R_{WW}$ (sample); and % $H_2O$ (sample) = (wt $H_2O$/wt sample) × 100.

For alcohol: $R_{WA}$ (sample) = $R_{PA}$ (sample) × $R_{WA}$ (std)/$R_{PA}$ (std); wt alcohol (sample) = wt ethylene glycol monomethyl ether (sample) × $R_{WA}$ (sample); and % alcohol (sample) = (wt alcohol/wt sample) × 100.

Ref.: JAOAC **49,** 718(1966).

## 970.61 Propylene Glycol in Cosmetics

### Gas Chromatographic Method

### First Action 1970
### Final Action 1971

(Applicable to all types of cosmetics)

### A. Apparatus

(a) *Distillation apparatus.*—All-glass, with ₮20/40 joints: 250 mL r-b flask, elec. heating mantle, 20 mL Barrett $H_2O$ trap with ₮ stopper, and driptip condenser.

(b) *Gas chromatograph.*—With H flame detector and capable of operating at ca 200°.

(c) *GC column.*—1.8 m (6′) × $^1/_4$″ od Cu or Al column packed with 80–100 mesh Chromosorb 101. Pack resin in column, using vibrator and column packer, **966.22D**, operated at 25–35 lb (170–240 kPa) pressure. Heat column overnight at 240° with He flow rate ca 100 mL/min. Condition column with propylene glycol as in **970.61D**. Inject enough aq. soln of propylene glycol-trimethylene glycol (1 + 1) to give $\geq ^1/_2$ full-scale response. If column is satisfactory, 2 symmetrical peaks will be obtained. Reject batches of Chromosorb 101 which give unsymmetrical peaks.

### B. Reagents

(a) *Propylene glycol.*—Eastman Kodak Co. No. 1321, or equiv.; assay by periodate oxidn: Place aliquot of aq. soln contg ≤45 mg propylene glycol in g-s flask, add 35 mL 0.02*M* $KIO_4$ soln, dil. to ca 100 mL with $H_2O$, and let stand 1 hr. Add ca 1.0 g $NaHCO_3$, 0.5 g KI, and 2.5 mL starch indicator (mix ca 1 g sol. starch with enough cold $H_2O$ to make thin paste, add 100 mL boiling $H_2O$, and boil ca 1 min while stirring). Titr. with 0.02*N* $KAsO_2$ soln to disappearance of blue. Stdze 25 mL 0.02*M* $KIO_4$ soln by same titrn, using $H_2O$ for sample, and calc. amt of $KIO_4$ reduced by sample. 1 mL 0.02*N* $KAsO_2$ = 0.76 mg propylene glycol.

(b) *Trimethylene glycol.*—Propylene glycol-free.

(c) *Isooctane (2,2,4-trimethylpentane).*—Bp 99–100°.

(d) *Propylene glycol and trimethylene glycol std solns.*—10 mg/mL. Prep. sep. std solns. Accurately weigh ca 1.0 g std, dissolve in $H_2O$, transfer to 100 mL vol. flask, and dil. to vol.

### C. Separation of Propylene Glycol by Co-distillation

Accurately weigh sample contg ca 2–40 mg propylene glycol into 250 mL r-b flask. Add 8–10 mL $H_2O$ and few boiling chips. Connect flask to distn app. and add, thru condenser, enough isooctane to fill $H_2O$ trap and provide 25–40 mL isooctane in distn flask. Adjust voltage on heating mantle so that isooctane distils at 5–10 mL/min. Continue distn 30 min after all $H_2O$ appears to be collected in trap. Drain as much $H_2O$ as possible (leave ca 0.25 mL in $H_2O$ trap) into small g-s container. (Stoppered 25 mL graduate is convenient.) Remove heat from distn flask and, when boiling stops, disconnect flask from app. and add 5 mL $H_2O$. Reconnect to app. and distil as before. Drain distillate into container contg first $H_2O$ distillate. Repeat with second 5 mL portion $H_2O$. Mix combined distillates.

### D. Preparation of Instrument

With Chromosorb 101 column in gas chromatograph, set column temp. at ca 180°, injection port and detector temps at ca 300°, and He flow rate near 70 mL/min. If necessary, adjust column temp. to elute propylene glycol in ca 6 min. Condition column by initial 0.5 μL injection of propylene glycol. Column must be conditioned in this manner once a day before use for detg propylene glycol. Use 5 μL aq. test soln contg ca 1 mg propylene glycol/mL and adjust H and air flow to flame detector until max. response is obtained. (*See* manufacturer's directions.) Note range and attenuation settings needed to keep peak on scale.

Establish rough calibration curve of peak ht response against wt propylene glycol by injecting known amts propylene glycol and observing response.

### E. Determination

Det. approx. propylene glycol content of aq. soln, obtained by codistn of sample with isooctane, by injecting known amt distillate into chromatograph. To sample soln add known aliquot of std trimethylene glycol soln (preferably sample soln should contain approx. equal wts propylene glycol and trimethylene glycol). Prep., from accurately measured aliquots of stds, soln contg approx. same wt propylene glycol and trimethylene glycol as prepd sample and dil. to approx. same vol. as sample soln.

Det., by trial injections, resp. vols of sample and std solns required to give nearly equal responses of ca $^3/_4$ full-scale for propylene glycol. Alternately inject these vols sample and known solns, making ≥2 injections of each soln. Det. sample and its corresponding std at same range and attenuation settings.

### F. Calculations

From chromatograms, calc. following peak ht ratios for sample, *R*, and std, *R*′, resp.:

$R$ = peak ht propylene glycol/peak ht trimethylene glycol

$R'$ = peak ht propylene glycol/peak ht trimethylene glycol

Using av. values of *R* and *R*′, calc. amt propylene glycol in sample.

$$\text{mg Propylene glycol} = (R/R') \times \text{mg propylene glycol (std)} \times [\text{mg trimethylene glycol (sample)}]/[\text{mg trimethylene glycol (std)}]$$

(If same aliquot of trimethylene glycol is used for prepd sample and std, last factor = 1.)

Ref.: JAOAC **53,** 82(1970).

CAS-57-55-6 (propylene glycol)

## 973.59 Eye Irritants in Cosmetic Constituents

### Bioassay

### First Action 1973

Use 6 albino rabbits of either sex, weighing 2.0–2.5 kg, randomly selected, for each substance. Facilities must be designed and maintained so as to exclude extraneous materials that might produce eye irritation. Examine both eyes of each animal before testing. Use only animals without defects or eye irritation.

Hold animal firmly but gently until quiet. Instill 0.1 mL test material onto cornea so that it flows into conjunctival sac of 1 eye of each animal by gently pulling lower lid away from eyeball to form cup into which test substance is dropped. Hold lids gently together 1 sec and release animal. Other eye, untreated, serves as control.

Examine eyes grossly or microscopically and record grade of ocular reaction of cornea, iris, and conjunctiva at 24, 48, and 72 hr, and 7 days. After 24 hr observation, further examine eyes after applying fluorescein-impregnated paper strips (Barnes-Hind, Ayerst, etc.). Eyes may be washed with sterile isotonic NaCl soln after 24 hr reading.

Independently grade each portion of eye (cornea, iris, and conjunctiva) without reference to total score, using definitions and color photographs in "Illustrated Guide for Grading Eye Irritation by Hazardous Substances," Superintendent of Documents, Government Printing Office, Washington, DC 20402.

Consider animal as exhibiting pos. reaction if test substance produces, at any of the readings, ulceration of cornea (other than fine stippling), or opacity of cornea (other than slight dulling of normal luster), or inflammation of iris (other than slight deepening of folds (or rugae) or slight circumcorneal injection of blood vessels), or if substance produces in conjunctivae (excluding cornea and iris) obvious swelling with partial eversion of lids or diffuse crimson-red with individual vessels not easily discernible. Basis for irritation is grade $\geq 1$ for cornea and iris and $\geq 2$ for redness and chemosis.

Sample is eye irritant if $\geq 4$ of 6 rabbits have irritation. Sample is not eye irritant if 0 or 1 of 6 rabbits has irritation. For combined parameters, eye is considered irritated if $\geq 1$ parameter has a grade considered to be eye irritant. If 2 or 3 animals exhibit pos. reaction, repeat test with 6 different animals. Second test is considered pos. if $\geq 3$ animals exhibit pos. reaction. If only 1 or 2 animals in second test exhibit pos. reaction, repeat test with 6 different animals. In third test, substance is considered irritant if any animal exhibits pos. reaction.

Ref.: JAOAC **56,** 905(1973).

## DEODORANTS AND ANTIPERSPIRANTS

### 938.11 Aluminum and Zinc in Deodorants

**Gravimetric Method**

**Final Action**

#### A. Reagents

(**a**) *8-Hydroxyquinoline soln.*—Dissolve 5.0 g 8-hydroxyquinoline in 12 mL HOAc, dil. to 100 mL with $H_2O$, and filter if not clear. Prep. fresh soln $\leq 2$ weeks.

(**b**) *Ammonium acetate soln.*—Approx. 2*N*. Dissolve 150–160 g $NH_4OAc$ in 1 L $H_2O$ and filter if not clear.

(**c**) *Hydrochloric acid.*—Approx. 2*N* (1 + 5).

(**d**) *Ammonium hydroxide.*—Approx. 2*N*. Vol. of $NH_4OH$ required to neutze 20 mL 2*N* HCl, (**c**), should be known to within $\pm 2$ mL.

#### B. Preparation of Sample

(**a**) *Liquids.*—Dil. 5 mL sample to 250 mL with $H_2O$ in vol. flask. If perfume oils sep., filter before taking aliquot for analysis.

(**b**) *Creams and pastes.*—Accurately weigh 2–3 g sample into 250 mL beaker. Add 5 mL HCl ($HNO_3$ if chlorides are to be detd) and ca 50 mL $H_2O$, and heat until oils liquefy and sep.; cool until oils solidify, and decant aq. layer thru fluted paper into 250 mL vol. flask. Return filter to original beaker and macerate thoroly. Repeat above extn twice, decant as before, and finally thoroly wash residue and paper with $H_2O$. (It is unnecessary to return filter paper to beaker after these extns.) Cool combined exts to room temp., dil. to vol. with $H_2O$, and mix.

(**c**) *Solids.*—Accurately weigh 2–3 g sample into 250 mL beaker, add 5 mL HCl ($HNO_3$ if chlorides are to be detd) and ca 50 mL $H_2O$, and heat to bp. Cool, and filter thru fluted paper into 250 mL vol. flask. If filtrate is cloudy, refilter thru fine quant. paper. Thoroly wash beaker and paper with $H_2O$. Cool flask and contents to room temp., dil. to vol. with $H_2O$, and mix.

#### C. Determination

(**a**) *Interfering metals absent.*—Take aliquot of sample soln contg 12–25 mg Al or 20–60 mg Zn. Add 1–2 drops phthln, and then add 2*N* $NH_4OH$ until neut. or until faint permanent turbidity results. Add 5 mL HOAc (1 + 9), dil. to ca 100 mL, and heat to 70–90°. Add 10 mL 8-hydroxyquinoline soln and then slowly add $NH_4OAc$ soln until 20 mL (*see Note*) in excess of vol. required to produce permanent ppt has been added. If permanent ppt forms on addn of 8-hydroxyquinoline, add only 20 mL $NH_4OAc$ soln. Heat below bp 2–5 min and set aside 30–60 min. (Moderate excess of 8-hydroxyquinoline is required for complete pptn. If enough reagent has been added, soln will be yellow at this point; if it is not, repeat detn, using larger vol. of 8-hydroxyquinoline soln.) Filter thru tared gooch, wash thoroly with $H_2O$, dry 1–2 hr at 130–140°, cool, and weigh. Dry again 30 min, cool, and weigh. Repeat to const wt ($\pm 0.3$ mg). (Alternatively, ppt may be dried overnight.)

$$\text{Wt ppt} \times 0.05871 = \text{Al}$$

$$\text{Wt ppt} \times 0.1848 = \text{Zn}$$

*Note:* Final pH of soln from which metals are pptd should be 4.9–5.1. Vol. of $NH_4OAc$ soln required to produce this pH should be detd experimentally each time new set of reagents is prepd. If $NH_4OAc$ is of usual purity, ca 20 mL soln will be required.

(**b**) *In presence of magnesium.*—Ppt as in (**a**) and set aside ca 30 min. Decant most of liq. thru quant. paper (part or all of ppt may be transferred to paper if necessary) and discard filtrate. Place beaker used for pptn under funnel and dissolve ppt on paper in hot 2*N* HCl (20 mL is usually enough if added in several small portions). Wash paper and funnel with 20–30 mL $H_2O$. Add 2 mL 8-hydroxyquinoline soln, 5 mL HOAc (1 + 9), and vol. of 2*N* $NH_4OH$ equiv. to 2*N* HCl used to dissolve ppt (do not use excess). Dil. to ca 100 mL, heat to 70–90°, and proceed as in (**a**), beginning ". . . slowly add $NH_4OAc$ soln . . ."

Refs.: Ind. Eng. Chem. Anal. Ed. **10,** 212(1938). JAOAC **28,** 734(1945).

CAS-7429-90-5 (aluminum)
CAS-7440-66-6 (zinc)

### 944.11 Zinc in Deodorants

**Gravimetric Method**

**Final Action**

#### A. Reagent

*8-Hydroxyquinaldine soln.*—Dissolve 5.0 g 8-hydroxyquinaldine in 12 mL HOAc, dil. to 100 mL with $H_2O$, and filter if soln is not clear. (Soln is stable ca 1 week. If only tech. grade base is available, purify by recrystn from alcohol (2 + 1), using 6 mL solv. for each g base, before prepg soln.)

#### B. Determination

Pipet aliquot of sample soln, **938.11B**, contg 20–50 mg Zn, into 400 mL beaker. Adjust soln to slight acidity, add 1 g $NH_4$ tartrate if Al is present, and then add 2 mL 8-hydroxyquinaldine soln for each 10 mg Zn present; dil. to 200 mL and heat to 60–80°. Neutze excess acid by adding $NH_4OH$ (1 + 4) until Zn complex salt that forms on addn of each drop just redissolves on stirring. Slowly add, with stirring, 45 mL $NH_4OAc$ soln, **938.11A(b)**, and let mixt. come to room temp.

Det. pH of soln; if pH is not 5.7–5.9, adjust with the $NH_4OH$ soln, and let mixt. stand 10–20 min to achieve equilibrium. Decant thru tared gooch and wash ppt in beaker twice with hot $H_2O$, decanting each wash into crucible. Finally transfer ppt to crucible and again wash with hot $H_2O$. (Total vol. washings should be >200 mL.) Dry crucible and ppt 2 hr at 130–140°, cool, and weigh. Reheat 30 min at 130–140°; cool, reweigh, and repeat heating, cooling, and weighing to const wt. Wt ppt × 0.1712 = wt Zn.

Refs.: Ind. Eng. Chem. Anal. Ed. **16,** 387(1944). JAOAC **33,** 371(1950).

CAS-7440-66-6 (zinc)

## 950.84 Aluminum in Deodorants

**Gravimetric Method**

**Final Action**

Multiply wt Zn found, **944.11B**, by 5.411 to obtain equiv. wt 8-hydroxyquinoline salt, multiply by appropriate factor for aliquot taken, and subtract from wt combined Al and Zn salts, **938.11C**. Difference × 0.05871 = wt Al.

## 976.24 Zirconium (Soluble) in Antiperspirant Aerosols

**Colorimetric Method**

**First Action**

### A. Reagents

(**a**) *Alizarin red S (sodium alizarin sulfonate) soln.*—Dissolve 1.5 g alizarin red S indicator (Allied Chemical Corp., or equiv.) in 300 mL hot $H_2O$. Cool and filter thru double layer of rapid, medium porosity paper (Whatman No. 12, 24 cm folded, or equiv.). Dil. filtrate to 1 L with $H_2O$, and refilter. Soln is stable ≥1 month.

(**b**) *Zirconyl chloride octahydrate.*—Fisher No. Z-80, or equiv. Assay as in **976.24B**.

### B. Assay of Standard

Accurately weigh 500–600 mg $ZrOCl_2.8H_2O$ into 400 mL beaker and dissolve in 50 mL $H_2O$. Add 4 g $NH_4NO_3$ and warm on steam bath to ca 50°. Slowly add, with stirring, 100 mL $NH_4OH$ and continue heating 20 min. Filter while hot thru 15 cm Whatman No. 42, or equiv., paper. Complete transfer of ppt with 2–3 portions 2% $NH_4NO_3$ in $NH_4OH$ (2 + 98). Carefully fold paper and place in ca 50 mL Pt crucible. Dry in oven at 105°. Partially cover crucible and gently heat with Meker burner until paper is well charred. Continue heating at max. burner temp. to const wt.

$$\% \text{ Zr} = \text{ZrO residue} \times 74.03/\text{wt } ZrOCl_2.8H_2O$$

### C. Preparation of Standard Curve

Dissolve $ZrOCl_2.8H_2O$ contg 200 mg Zr in 70 mL $H_2O$ in 200 mL vol. flask. Add 110 mL HCl, cool, and dil. to vol. with $H_2O$. Prep. this 1 mg/mL stock soln fresh weekly. Pipet 2, 5, 10, and 15 mL stock soln into 100 mL vol. flasks. Dil. each to vol. with HCl (55 + 45), and mix. Pipet 5 mL each dil. std and, as blank, 5 mL HCl (55 + 45) into sep. 100 mL vol. flasks for color development (0, 100, 250, 500, and 750 μg Zr/100 mL). Add to each flask 10.0 mL alizarin red S soln, (**a**), and 8 mL $H_2O$. Swirl and place in 75 ± 3° $H_2O$ bath. Monitor temp. of solns with thermometer in 100 mL vol. flask contg 23 mL $H_2O$ added at room temp. and placed simultaneously in bath. Swirl flasks occasionally while heating. After solns reach 70°, keep flasks in bath addnl 6.5 ± 1 min. Remove and let cool 20 min at room temp. Dil. each to vol. with $H_2O$ and mix. Measure $A$ in 2 cm cells against blank at 525 nm, scanning on recording spectrophtr from 700–460 nm. Plot $A$ against μg Zr/100 mL.

(If 750 μg Zr/100 mL cannot be read on $A$ scale, use higher range of spectrophtr or 14.0 mL aliquot (700 μg Zr/100 mL).)

### D. Preparation of Sample

Remove cap and any paper wrapping from aerosol can. Record wt of can to nearest 0.01 g. Replace cap and freeze contents by placing inverted can in beaker contg mixt. of solid $CO_2$ and acetone and cooling ≥1 hr. Transfer can to smaller beaker in exhaust hood and cautiously open bottom end with can opener, keeping end partially attached to can. Let volatile gases escape at room temp. Remove dip tube after initial thawing when gas evolution has subsided, but keep tube with can in beaker. Place beaker on steam bath, and heat gently to evolve higher boiling gases. Increase heat slowly and maintain until bubbling subsides. Place beaker contg can and dip tube in 70° forced-draft oven 45 min. Raise temp. to 115° and maintain 2.5 hr, stirring occasionally with stainless steel spatula. Remove from oven and let cool to room temp. Thoroly mix contents, including any portion clinging to sides, to form homogeneous conc. Weigh can plus contents, spatula, and dip tube. Stir contents ca 1 min and weigh again. Repeat stirring and weighing until wt is const to within 0.01 g. Record wt and immediately transfer contents to g-s weighing bottle (quant. transfer unnecessary). Stopper bottle and protect from further wt loss by opening only when necessary.

Remove cap from can and thoroly remove remaining contents from can, spatula, and dip tube with $H_2O$ and alcohol. Dry cap, can, spatula, and dip tube to const wt at 110°. Obtain wt of conc. by subtracting latter wt from wt previously recorded after drying contents. Obtain wt of intact can contents by subtracting wt of clean, dry can (without cap) and dip tube from gross wt initially recorded.

### E. Determination

Record gross wt of prepd sample in weighing bottle. Mix sample briefly and remove ca 1 g with spatula. Calc. wt removed as difference in gross wt.

Transfer sample as completely as possible from spatula to lower half of 600 mL beaker. Thoroly wipe spatula with small piece of filter paper and add paper to beaker. Add 5 mL alcohol and break up sample with glass rod. Slowly add 200 mL HCl while stirring vigorously. Heat to bp on steam bath. Boil 2–3 min and immediately transfer thru funnel to 1 L vol. flask. Complete transfer with 200 mL and 150 mL portions hot HCl. Warm flask on steam bath and shake vigorously 3–4 min. Rinse beaker with 450 mL $H_2O$, add to flask, and mix thoroly. Cool to room temp., dil. to vol. with HCl, and mix thoroly. Let undissolved material settle and coagulate. Filter portion thru double layer of 24 cm Whatman No. 12, or equiv., folded paper, discarding first 50 mL. Make appropriate dilns with HCl (55 + 45) to obtain Zr concn of 40–100 μg/mL. Pipet 5 mL into 100 mL vol. flask, and proceed as in **976.24C**, beginning "Add to each flask 10.0 mL alizarin red . . ."

$$\% \text{ Zr in intact can contents} = (C/W_s) \times (W_c/W_i) \times (F/10^4)$$

where $C$ = μg Zr/100 mL read from std curve; $W_s$, $W_c$, and $W_i$ = g sample, prepd conc., and intact can contents, resp.; and $F$ = appropriate diln factor.

Refs.: JAOAC **59,** 830, 1421(1976); **60,** 663(1977).

CAS-7440-67-7 (zirconium)

## 952.15 Boric Acid in Deodorants and Antiperspirants

**Ion Exchange Method**

**Final Action**

### A. Preparation of Ion Exchange Column

Provide glass tube 58 cm long × 2 cm diam. (23 × 0.75") with stopcock and outlet tube. Tamp 3 cm glass wool plug into bottom of tube, fill tube with $H_2O$, and add Amberlite IR-120(H) ion exchange resin slowly to form 20 cm column. Wash with HCl (1 + 9) and then with 50 mL portions $H_2O$ until effluent gives neg. Cl test.

Regenerate after use by transferring accumulated resin from number of detns to large glass tube and washing with HCl (1 + 9) until effluent gives neg. test for adsorbed cations, *e.g.*, Zn, Al. Then remove HCl from resin by washing with $H_2O$ until effluent gives neg. Cl test.

### B. Determination

Place sample contg 50–200 mg $H_3BO_3$ in 250 mL casserole, add 2 drops phthln, and make alk. with 10% NaOH soln. Evap. to dryness on steam bath under gentle air current, dry residue 1 hr at 140° in oven, and ash 1 hr at 550°. Cool to room temp., add ca 50 mL hot $H_2O$, acidify cautiously with HCl, and filter hot soln thru quant. paper into 250 mL beaker. Wash paper with little hot $H_2O$ and reserve filtrate (may be slightly cloudy).

Transfer paper to same casserole and make alk. by wetting with ca 10 mL $H_2O$ and few drops 10% NaOH soln. Evap. to dryness on steam bath, dry 1 hr at 140°, and ash 2 hr at 550°. Cool, add ca 50 mL hot $H_2O$, acidify with HCl, and filter into reserved filtrate. Wash casserole and paper thoroly with hot $H_2O$, and discard paper. (Total vol. soln should be ca 200 mL.)

Cool soln; add $NH_4OH$ until barely alk. to litmus paper or until flocculent ppt appears. Reacidify with HCl until slightly acid to litmus paper or until ppt just redissolves. Pass soln thru ion exchange column into 1 L flask at rate requiring 10–15 min for passage. Follow sample soln with several 50 mL portions $H_2O$ until effluent is only slightly acid to pH test paper. Add 5 drops Me red, **936.15D(a)**, make alk. with freshly prepd 10% NaOH soln, and then barely acid with HCl.

Connect flask to $H_2O$-cooled reflux condenser and boil 5 min. Wash down condenser with little $H_2O$ and cool soln to room temp. under running $H_2O$. Neutze to Me red with 0.1*N* NaOH, **936.16C**; add 4–5 g mannitol and ca 0.5 mL phthln. Titr. with 0.1*N* NaOH to pink color, add more mannitol, and if pink disappears, continue titrn until it reappears. Repeat addn of mannitol until there is no further change in color.

Det. blank as follows: To ca 350 mL $H_2O$ add vol. of freshly prepd 10% NaOH soln equal to that required to neutze sample after passing thru column. Barely acidify with HCl and proceed as above, beginning "Connect flask to $H_2O$-cooled reflux condenser . . ." Subtract blank titrn from sample titrn and calc. $H_3BO_3$ content of sample. 1 mL 0.1*N* NaOH = 0.00618 g $H_3BO_3$.

Refs.: Anal. Chem. **24,** 182(1952). JAOAC **36,** 791(1953).

CAS-10043-35-3 (boric acid)

## 951.04 Chlorides in Deodorants
### Gravimetric Method
### Final Action

Pipet aliquot of sample soln, **938.11B**, contg ca 100 mg Cl into 250 mL beaker. Dil. to 150 mL with $H_2O$, neutze to litmus with $NH_4OH$ (1 + 1), and acidify with 1 mL $HNO_3$ (1 + 1). If any undissolved ppt remains, add more $HNO_3$ (1 + 1) until clear soln is obtained. Add dropwise, stirring constantly, slight excess of 0.1*N* $AgNO_3$. (Excess should be ≤5 mL.) Pptn and succeeding operations must be carried out in subdued light. Heat mixt. to 90–95° and stir until ppt coagulates. Let ppt settle; add 1–2 drops 0.1*N* $AgNO_3$ to supernate to ensure presence of excess Ag. Let mixt. stand 1–2 hr in dark.

Decant thru tared gooch, wash ppt 2–3 times with 0.01*N* $HNO_3$ by decantation, and finally transfer ppt to gooch with 0.01*N* $HNO_3$. Continue washing ppt with 0.01*N* $HNO_3$ until washing gives neg. test for Ag when 1 drop 0.1*N* HCl is added. Complete washing by removing most of the $HNO_3$ with two 10 mL portions $H_2O$. Dry crucible 2 hr at 120–130° and weigh. Repeat drying to const wt (0.2 mg). Wt AgCl × 0.2474 = wt Cl.

Ref.: JAOAC **34,** 298, 299(1951).

## 951.05 Sulfates in Deodorants
### Gravimetric Method
### Final Action

Pipet aliquot of sample soln, **938.11B**, contg ca 100 mg sulfate into 600 mL beaker. Dil. to 350 mL with $H_2O$, neutze to litmus with $NH_4OH$ (1 + 1), and acidify with 2 mL HCl. If any undissolved ppt remains, add more HCl until soln is clear.

Heat 50 mL *1% $BaCl_2$ soln* almost to bp and add rapidly with stirring to sulfate soln which has also been heated to near bp. Let ppt settle, and add little $BaCl_2$ soln to ensure excess of Ba. Let mixt. stand 1–2 hr on steam bath. Decant thru tared gooch, wash ppt 4–5 times with small portions of warm $H_2O$ by decantation, and finally transfer ppt to gooch with warm $H_2O$. Continue washing ppt with warm $H_2O$ until washing gives neg. test for Cl. Dry crucible 2 hr at 110–120° and weigh. Repeat drying to const wt (0.2 mg). Wt $BaSO_4$ × 0.4116 = wt sulfate.

Ref.: JAOAC **34,** 298, 299(1951).

## 974.28 Hexachlorophene in Deodorants
### Spectrophotometric Method
### First Action 1974
### Final Action 1978

### A. Apparatus

(**a**) *Chromatographic equipment.*—Insert 2 cm plug of glass wool in bottom of tube 55 cm long × 2.5 cm od with constricted tip 2.5 cm long and 8 mm od. Provide with brass tamper of diam. slightly smaller than id of tube, and fittings, including pressure gage, for applying pressure to top of column.

(**b**) *Spectrophotometer.*—Capable of isolating band ≤5 nm in region 220–360 nm.

### B. Reagents

(**a**) *Silanized Celite.*—Weigh ca 700 g Celite 545 into 4 L beaker, add 3 L HCl (1 + 4), and stir thoroly. Heat on steam bath several hr, stirring occasionally. Filter slurry thru buchner under vac. and wash with $H_2O$ until washings are Fe- and Cl-free. Suck dry, transfer to beaker, and dry ca 15 hr at 135°. Transfer ca 150 g dried Celite to crystg dish, and let stand in air 30 min. In well ventilated hood, pour 25 mL GE SC-77 Dri-Film (General Electric Co., 1 River Rd, Schenectady, NY 12305) into bottom of large glass desiccator. Place dish contg Celite on porcelain support in desiccator, and let stand in closed desiccator 4 hr. Remove dish, and let stand in hood until residual HCl dissipates.

(**b**) *Immobile solvent.*—Mix equal vols $CCl_4$ and *n*-heptane.

(**c**) *Eluting solns.*—In sep. 250 mL vol. flasks, add 1 mL HCl to 25, 87.5, and 150 mL alcohol and dil. each to vol. with $H_2O$ (10, 35, and 60% alcohol, resp.).

(**d**) *Hexachlorophene std solns.—(1) Stock soln.*— 0.6 mg/mL. Accurately weigh ca 60 mg hexachlorophene USP and dil. to vol. in 100 mL vol. flask with acidified 60% alcohol, (**c**). *(2) Working std solns.*—Dil. 5, 10, and 20 mL aliquots stock soln to 100 mL with acidified 60% alcohol, (**c**) (0.03, 0.06, and 0.12 mg/mL).

### C. Preparation of Sample

(**a**) *For products containing sulfated surface-active agents.*—Accurately weigh, in weighing bottle, ca 1 g sample and transfer quant. to Ŧ 250 mL r-b flask with 75 mL 20% alcohol. Add 10 mL HCl and few boiling chips, attach $H_2O$-cooled condenser, and reflux 15 min. Cool to room temp. and transfer to 250 mL separator with ca 25 mL $H_2O$. Rinse flask with 30 mL $CHCl_3$ and transfer to separator. Shake well and drain $CHCl_3$ into another 250 mL separator. Rinse beaker with two addnl 30 mL portions $CHCl_3$, ext aq. soln with each, and combine $CHCl_3$ exts in 250 mL separator. Wash combined exts with 10 mL $H_2O$ acidified with HCl, and filter thru $CHCl_3$-wetted plug of cotton in powder funnel into 250 mL beaker. Wash cotton with 20 mL $CHCl_3$, and evap. $CHCl_3$ to ca 10 mL on steam bath under air current. Complete evapn to dryness at room temp. under air current.

(**b**) *For products not containing sulfated surface-active agents.*—Accurately weigh, in weighing bottle, ca 1 g sample and transfer quant. to 250 mL separator with 40 mL warm $H_2O$. Acidify with HCl, and ext with three 30 mL portions $CHCl_3$. Wash combined $CHCl_3$ exts with 10 mL $H_2O$ acidified with HCl, and continue as in (**a**), beginning, ". . . and filter thru $CHCl_3$-wetted plug . . ."

### D. Isolation of Hexachlorophene

Weigh two 12 g portions silane-treated Celite. To 1 portion in 250 mL beaker, add 7 mL $CCl_4$-*n*-heptane (1 + 1), mix well, and pack mixt. gently but firmly with tamper into chromatgc tube in ca 4 g portions.

To residue from **974.28C(a)** or (**b**), add 7 mL $CCl_4$-*n*-heptane (1 + 1), and stir well to dissolve or disperse residue. Add the second 12 g portion silane-treated Celite, stir thoroly, and pack mixt. into tube as before. Wipe beaker, stirring rod, and tamper with small piece of glass wool and lightly tamp it on top of completed column.

Elute column with 100 mL acidified 10% alcohol, then with 200 mL acidified 35% alcohol, and finally with 250 mL acidified 60% alcohol. Maintain flow at ca 2 mL/min with aid of air pressure and do not permit level of eluting liqs to fall below glass wool plug. Collect two 100 mL portions and then 50 mL portions in vol. flasks. Hexachlorophene should elute with acidified 60% alcohol.

### E. Determination

Add 1 drop HCl to each eluate in vol. flasks and obtain spectra over 220–360 nm in 1 cm cell against corresponding eluting soln. If necessary, dil. with corresponding eluting soln. Obtain spectra of working std solns similarly.

From curves, identify eluates contg hexachlorophene. Calc. hexachlorophene in each eluate by comparing $A$ at 297 nm of sample with that of std, using straight line background correction. Add amts in eluates to obtain amt in sample.

Ref.: JAOAC **57**, 563(1974).

CAS-70-30-4 (hexachlorophene)

## 952.16 Methenamine in Deodorants

**Titrimetric Method**

**Final Action**

### A. Reagent

*Borax-carbonate soln.*—Dissolve 5.0 g $Na_2CO_3$ and 4.0 g $Na_2B_4O_7.10H_2O$ in 100 mL $H_2O$.

### B. Determination

Pipet aliquot of sample soln, **938.11B**, contg 150–200 mg methenamine into 500 mL r-b flask and dil. to 30 mL with $H_2O$. Neutze to litmus with either NaOH soln or dil. $H_2SO_4$; then acidify with 1 mL $H_2SO_4$. Connect flask to $H_2O$-cooled condenser and reflux 30 min to hydrolyze methenamine. Dil. to 175 mL by adding $H_2O$ thru top of condenser, and disconnect condenser. Connect flask thru Kjeldahl trap to efficient straight-wall condenser and distil into 200 mL vol. flask contg 10 mL freshly prepd *10% $NaHSO_3$ soln*. Continue distn until residual vol. is ca 5 mL, taking care to avoid charring.

Wash down condenser with little $H_2O$ and cool distillate to room temp. Dil. distillate to vol. with $H_2O$, mix well, and let stand 30 min. Pipet 20 mL aliquot into wide-mouth 250 mL erlenmeyer, add 3–4 mL starch indicator (mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring const, and immediately discontinue heating. Add ca 1 mL Hg, shake, and let soln stand over the Hg), and destroy excess bisulfite with ca 1*N* I soln. Carefully adjust to starch-I end point with 0.5% $NaHSO_3$ soln and 0.05*N* I. Dil. to 50 mL with $H_2O$, add 10 mL borax-carbonate soln, and titr. with 0.05*N* I to permanent blue. 1 mL 0.05*N* I consumed in alk. titrn = 0.5841 mg methenamine.

Ref.: JAOAC **35**, 279(1952).

CAS-100-97-0 (methenamine)

## 952.17★ Phenolsulfonates in Deodorants

**Bromination Method**

**Final Action**
**Surplus 1970**

*See* **35.025–35.026**, 11th ed.

## 954.12 Phenolsulfonates in Deodorants

**Spectrophotometric Method**

**Final Action**

### A. Apparatus and Reagents

(**a**) *Spectrophotometer.—See* **974.28A(b)**.

(**b**) *Zinc phenolsulfonate std soln.*—10 mg/L in ca 0.1*N* NaOH. Dissolve 100 mg Zn phenolsulfonate, NF XI (equiv. to 62.67 mg phenolsulfonic acid), in 100 mL $H_2O$. Dil. 10 mL aliquot to 100 mL with $H_2O$. Pipet 10 mL aliquot into 100 mL vol. flask, add 4 mL freshly prepd 10% NaOH soln, and dil. to vol. with $H_2O$.

### B. Determination

(**a**) *In presence of sulfated surface-active agents.*—Accurately weigh sample contg 5–10 mg phenolsulfonic acid into 250 mL erlenmeyer. Add 10 mL $H_2O$ and 2 mL HCl, connect to $H_2O$-cooled condenser, and reflux 0.5 hr. Cool to room temp., transfer quant. to 100 mL separator with 20 mL $H_2O$, and proceed as in (**b**), beginning ". . . ext with three 30 mL portions $CHCl_3$."

(**b**) *In absence of sulfated surface-active agents*.— Accurately weigh, in weighing bottle, sample contg 5– 10 mg phenolsulfonic acid. Transfer quant. to 100 mL separator with aid of 30 mL $H_2O$. Acidify with HCl and ext with three 30 mL portions $CHCl_3$. Discard $CHCl_3$ exts. Filter aq. soln thru moistened quant. paper into 100 mL vol. flask and dil. to vol. with $H_2O$. Pipet 10 mL aliquot into 100 mL vol. flask, neutze to litmus paper with freshly prepd 10% NaOH soln, add 4 mL excess, and dil. to vol. with $H_2O$. Det. $A$ of sample soln and $A'$ of std soln at 253 nm in 1 cm cells, using 0.1$N$ NaOH as blank.

$$\% \text{ phenolsulfonic acid} = C \times A/[10\,A' \times (\text{g sample})]$$

where $C$ = concn phenolsulfonic acid (mg/L) in std soln.

Ref.: JAOAC **37**, 798(1954).

## 951.06 Urea in Deodorants

### Titrimetric Method

### Final Action

Pipet aliquot of sample soln, **938.11B**, contg 50–100 mg urea into ₮ 100 mL r-b flask. Acidify with HCl, adding 0.5 mL excess. Immerse flask in steam bath and evap. to dryness. Add 10 g cryst. $MgCl_2.6H_2O$ and 1 mL HCl, and connect flask to reflux condenser. Carefully heat mixt. with small flame until $MgCl_2$ dissolves in its $H_2O$ of crystn, and reflux slowly 2 hr so that rate of return of liq. from condenser is 9–14 drops/min.

Let soln cool, add $H_2O$ thru top of condenser, disconnect flask, and if necessary, heat to dissolve solids. Transfer soln to 1 L flat-bottom flask, dil. to ca 400 mL with $H_2O$, make alk. with 10% NaOH soln, and distil ca 275–300 mL into suitable portion of 0.1$N$ $H_2SO_4$ contg several drops of Me red, **936.15D(a)**. Titr. excess acid with ca 0.1$N$ NaOH, using more indicator if necessary. Stdze the 0.1$N$ NaOH against the std 0.1$N$ $H_2SO_4$, using Me red as indicator.

Correct for blank by refluxing 10 g cryst. $MgCl_2$ .$6H_2O$ and 1 mL HCl and proceeding as above. 1 mL 0.1$N$ $H_2SO_4$ = 3.003 mg urea.

Ref.: JAOAC **34**, 298, 299(1951).

CAS-57-13-6 (urea)

## DEPILATORIES

## 940.32 Sulfides in Depilatory Powders

### Titrimetric Method

### Final Action

Pipet 50 mL 0.1$N$ $As_2O_3$ soln, **939.12B**, into 250 mL g-s vol. flask. Weigh sample contg <0.12 g sulfide calcd as $H_2S$ and transfer to flask, washing down any material on sides of flask with $H_2O$. Add 20 mL HCl (1 + 1), stopper immediately, and shake vigorously until sample decomposes. (If sample contains $CaCO_3$, slowly add the 20 mL acid thru dropping funnel fitted with rubber stopper to fit flask. Shake gently, letting liberated $CO_2$ bubble up thru acid. When reaction subsides, drain remainder of acid into flask, remove funnel, stopper flask, and shake vigorously.)

Cool to room temp. and dil. to vol. with $H_2O$. Filter thru dry paper into dry flask. Pipet 100 mL filtrate into 300 mL erlenmeyer; add 5 mL starch soln, (mix ca 1 g sol. starch with enough cold $H_2O$ to make thin paste, add 100 mL boiling $H_2O$, and boil ca 1 min with stirring), and enough I soln to form blue soln. Make alk. with $NaHCO_3$, adding 1–2 g excess. Titr. to permanent blue with 0.1$N$ I, **939.13A**. Subtract mL 0.1$N$ I consumed in alk. titrn from mL 0.1$N$ $As_2O_3$ present in aliquot. 1 mL 0.1$N$ $As_2O_3$ = 0.005411 g CaS or 0.01271 g BaS.

Refs.: JAOAC **23**, 437(1940); **25**, 113(1942); **27**, 112(1944).

CAS-21109-95-5 (barium sulfide)
CAS-20548-54-3 (calcium sulfide)

## 950.85★ FACE POWDERS

### Final Action
### Surplus 1970

***A. Fats and Fatty Acids as Stearic Acid***

*See* **35.032**, 11th ed.

***B. Boric Acid***

*See* **35.033**, 11th ed.

***C. Zinc (Total)***

*See* **35.034–35.035**, 11th ed.

***D. Calcium (Acid-Soluble)***

*See* **35.036**, 11th ed.

***E. Magnesium (Acid-Soluble)***

Det. Mg in filtrate from acid-sol. Ca as in **973.54A**. $Mg_2P_2O_7 \times 0.3622$ = MgO.

***F. Barium Sulfate***

*See* **35.038– 35.039**, 11th ed.

***G. Titanium and Iron (Total)***

*See* **35.040–35.041**, 11th ed.

***H. Iron (Total)***

*See* **35.042–35.043**, 11th ed.

***I. Titanium (Total)***

% total ($TiO_2$ + $Fe_2O_3$) − % total $Fe_2O_3$ = % total $TiO_2$.

***J. Oxides of Iron, Titanium, and Aluminum (Total)***

*See* **35.045**, 11th ed.

***K. Aluminum (Total)***

% Total $Al_2O_3$ =
% Total ($Al_2O_3$ + $Fe_2O_3$ + $TiO_2$) − % total ($Fe_2O_3$ + $TiO_2$)

***L. Calcium (Acid-Insoluble)***

Det. Ca in filtrate from $NH_4OH$ ppt, **950.85J★**, as in **921.06B**, beginning ". . . heat to boiling . . ."

***M. Magnesium (Acid-Insoluble)***

Det. Mg in filtrate from acid-insol. Ca as in **920.200**. $Mg_2P_2O_7 \times 0.3622$ = MgO.

***N. Silica***

*See* **35.049**, 11th ed.

***O. Starch***

*See* **35.054,** 13th ed.

## HAIR PREPARATIONS

### 945.70★ 2,5-Diaminotoluene in Hair Dyes and Rinses

**Final Action 1965**
**Surplus 1970**

***A. Acetylation Method***

*See* **35.051**, 11th ed.

***B. Dichlorimide Method***

*See* **35.052–35.053**, 11th ed.

### 945.71★ Paraphenylenediamine in Hair Dyes and Rinses

**Final Action**
**Surplus 1970**

***A. Acetylation Method***

*See* **35.054–35.055**, 11th ed.

***B. Dichlorimide Method (Benzoquinone Method)***

*See* **35.056**, 11th ed.

### 952.18 Potassium Bromate and Sodium Perborate in Cold Wave Neutralizers

**Qualitative Tests**
**Final Action**

(**a**) *General tests.*—$KBrO_3$ and $NaBO_3$ are white cryst. salts sol. in $H_2O$. Aq. soln of $KBrO_3$ is slightly acid; of $NaBO_3$, slightly alk. In flame test, using Pt wire in slightly darkened room, $KBrO_3$ gives reddish violet flame when viewed thru Co glass; $NaBO_3$, typical yellow Na flame. Both compds give following test: Dissolve 0.1 g sample in 10 mL $H_2O$, acidify with HCl, and add 0.5 g KI. Liberation of I indicates presence of oxidizing agent.

(**b**) *Confirmatory test for bromate.*—To 1 mL 5% soln of sample in test tube, slowly add 2 mL $H_2SO_4$ with vigorous shaking. Note odor and color of liberated gas. (*Caution.*) Cool test tube, *carefully* add 2 mL $CS_2$, and shake. $CS_2$ layer becomes yellow or red if Br is present.

(**c**) *Confirmatory test for boron.*—Moisten 0.2 g sample in porcelain crucible with 1–2 drops $H_2SO_4$, add 2 mL MeOH, stir well, and ignite. Green flame indicates presence of B.

Ref.: JAOAC **35**, 285(1952).

CAS-7758-01-2 (potassium bromate)
CAS-7632-04-4 (sodium perborate)

### 923.10★ Pyrogallol in Hair Dyes

**Final Action**
**Surplus 1970**

***A. Qualitative Test***

*See* **35.058**, 11th ed.

***B. Colorimetric Method***

*See* **35.059–35.061**, 11th ed.

### 942.21★ Resorcinol in Hair Lotions

**Bromate Titration**
**Final Action**
**Surplus 1970**

*See* **35.062–35.063**, 11th ed.

### 945.72★ Salicylic Acid in Hair Lotions

**Bromate Titration**
**Final Action**
**Surplus 1970**

*See* **35.064**, 11th ed.

### 952.19 Thioglycolate Solutions in Cold Permanent Waves

**Final Action**

***A. Qualitative Test***

Dil. 2 mL sample to 10 mL with $H_2O$, acidify with 10% HOAc, add 5 mL excess, and shake well. Add 2 mL *10% $Cd(OAc)_2.2H_2O$ soln,* and shake. White gelatinous ppt forms if thioglycolic acid is present. Add excess of $NH_4OH$ (2 + 3) and shake. Ppt of Cd thioglycolate will dissolve.

***B. Quantitative Method***

(Applicable in absence of reducing substances other than thioglycolates)

Pipet sample aliquot contg 250–300 mg thioglycolic acid into wide-mouth 250 mL erlenmeyer. Dil. to 50 mL with $H_2O$, add 2–3 drops Me red (dissolve 0.1 g Me red in 50 mL alcohol, dil. to 100 mL with $H_2O$, and filter if necessary), and make slightly acid with HCl. Add 3–4 mL starch indicator (mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring const., and immediately discontinue heating; add ca 1 mL Hg, shake, and let soln stand over the Hg), and titr. with 0.1*N* I to purple end point. 1 mL 0.1*N* I = 0.009212 g thioglycolic acid.

Ref.: JAOAC **35**, 285(1952).

CAS-68-11-1 (thioglycolic acid)

### 970.62 Dithiodiglycolic Acid in Cold Permanent Waves

**Titrimetric Method**
**First Action 1970**
**Final Action 1971**

***A. Principle***

Soln contg mixt. of thioglycolic (TGA) and dithiodiglycolic (DTDGA) acids is titrd with std 0.1*N* I soln, which selectively titrs TGA. DTDGA is reduced to TGA in Jones reductor. Resulting total TGA is titrd; increased TGA represents DTDGA.

***B. Reagents***

(**a**) *Zinc metal.*—20–30 mesh. Mallinckrodt Chemicals Analytical Reagent grade has been found suitable.

(**b**) *Mercuric salt soln.*—2% aq. soln of $Hg(NO_3)_2$ or $HgCl_2$.

### C. Apparatus

(**a**) *Jones reductor*.—Glass tube, 50–65 cm long, 2 cm id, with stopcock, preferably Teflon, and delivery tip extending ca 8 cm below stopcock.

(**b**) *Magnetic stirrer*.—With glass- or Teflon-covered stirring bar.

### D. Preparation of Jones Reductor

Place 300 g Zn in 800 mL beaker; add 300 mL Hg soln and 2 mL $HNO_3$. Stir 10 min with glass rod. Decant supernate and repeat amalgamation with fresh portion Hg soln and 2 mL $HNO_3$. Wash amalgamated Zn 3 times, by decantation, with $H_2O$. (Zn should have silvery luster.) Maintain $H_2O$ layer over Zn thruout.

Fill tube with $H_2O$; then slowly add prepd Zn, draining excess $H_2O$. Pass 500 mL $H_2O$ thru column, maintaining $H_2O$ layer over Zn.

Det. suitability of reductor as follows: Pass 20 mL $H_2O$ thru column, followed by 200 mL $H_2SO_4$ (1 + 9), then 100 mL $H_2O$. Titr. combined washings as in **970.62E**(**c**). If titrn is >0.2 mL, wash column with addnl $H_2O$.

Det. reductor efficiency by treating ca 350 mg DTDGA, accurately weighed, in 5 mL $H_2O$ as in **970.62E**(**b**). Recovery must be ≥97%. Lower recovery indicates unsuitable reductor.

Prepd reductor may be stored 3 months before use, provided amalgamated Zn is always kept covered with $H_2O$. Prewash column with 200 mL $H_2SO_4$ (1 + 9) before use after overnight storage.

### E. Determination

(**a**) *Titration 1*.—Pipet sample aliquot contg 350–400 mg TGA and DTDGA into 500 mL Phillips flask contg mag. stirring bar; dil. to 100 mL with $H_2O$, add 2 drops Me red indicator (dissolve 0.1 g Me red in 50 mL alcohol, dil. to 100 mL with $H_2O$, and filter if necessary) and just acidify with $H_2SO_4$ (1 + 1). Add starch indicator soln (mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring const., and immediately discontinue heating; add ca 1 mL Hg, shake, and let soln stand over the Hg) and titr. soln with 0.1*N* I std soln, **939.13A**.

$$1 \text{ mL } 0.1N \text{ I} = 9.212 \text{ mg TGA}$$

(**b**) *Reduction*.—Arrange reductor column to deliver into 1 L suction flask contg mag. stirring bar. Connect flask to vac. outlet which can be regulated to desired flow rate.

Dil. sample aliquot equal to that used in *Titration 1* with 200 mL $H_2SO_4$ (1 + 9).

Add 50 mL $H_2SO_4$ (1 + 19) to column, apply gentle suction, and elute at ca 10 mL/min. When liq. reaches top of amalgam, immediately add dild sample soln, increase vac., and elute at ca 17–20 mL/min. When liq. reaches top of amalgam, rinse sample container with 100 mL $H_2SO_4$ (1 + 19) in several portions and add to column. Follow with three 100 mL portions $H_2O$ at ca 50 mL/min. Release vac., rinsing column tip into flask with $H_2O$.

(**c**) *Titration 2*.—Place suction flask contg reduced sample soln on mag. stirrer; add 5 drops Me red indicator and set stirrer at medium speed. Add $NH_4OH$ to yellow indicator color (ca 70 mL); then add $H_2SO_4$ (1 + 1) dropwise just to indicator red color. Stopper flask and place in ice bath. Cool sample soln with min. swirling to ≤25°. Add 5 mL starch indicator, place on mag. stirrer, and titr. with 0.1*N* I to purple end point.

Perform sep. blank detns for *Titrations 1* and *2* and make appropriate corrections.

$$\text{mg DTDGA in sample aliquot} = 9.111\ (N - M)$$

where $M$ = mL 0.1*N* I from *Titration 1* corrected for blank and $N$ = mL 0.1*N* I from *Titration 2* corrected for blank.

Ref.: JAOAC **53,** 78(1970).

## gamma-BHC in Technical BHC, Pesticide Formulations, and Lindane Shampoos and Lotions

*See* **984.05**.

# SUNTAN PREPARATIONS

## 970.63 Amyl *p*-Dimethylaminobenzoate in Suntan Preparations

**Spectrophotometric Method**

**First Action 1970**
**Final Action 1971**

### A. Apparatus

(**a**) *Spectrophotometer*.—Cary Model 11 (replaced by Models 14 and 17, Varian Instrument Group) recording spectrophtr, or equiv., with 1 cm quartz cells.

(**b**) *Chromatographic equipment*.—(*1*) Glass chromatgc tube, 55 cm × 22 mm id with glass wool plug in constricted tip. (*2*) Brass tamper to fit chromatgc tube. (*3*) Source of variable air pressure to regulate column elution rate at ca 2 mL/min.

### B. Reagents

(**a**) *Solvent*.—(*1*) *Immobile solvent*.—*n*-Heptane-$CCl_4$ (1 + 1). (*2*) *Mobile solvents*.—Acidified 50% and 60% alcohol, contg 2 mL HCl/500 mL.

(**b**) *Silanized Celite*.—*See* **974.28B**(**a**).

(**c**) *Amyl p-dimethylaminobenzoate*.—0.01 mg/mL. Dil. 10 mg std to 1 L with $NH_4OH$-60% alcohol (1 + 99).

### C. Preparation of Samples

In weighing bottle, weigh sample contg 10–20 mg amyl *p*-dimethylaminobenzoate. Transfer quant. to 250 mL separator with 50 mL $H_2O$, slightly acidify with HCl, using test paper, and ext with four 35 mL portions $CHCl_3$. Combine exts, wash with 10 mL $H_2O$, and filter thru $CHCl_3$-washed cotton plug in powder funnel, into 250 mL beaker. Wash cotton with 10 mL $CHCl_3$. Evap. to ca 1 mL on steam bath under air jet. Remove beaker from steam bath and continue evapn under air jet until all $CHCl_3$ has evapd. Reserve prepd residue for chromatgy.

### D. Chromatography

(**a**) *Column preparation*.—Prep. column in 2 layers as in **974.28C**, par. 1 and 2. (Proper prepn of columns may be checked by eluting known amt of std from column, noting recovery.)

(**b**) *Elution*.—Elute column first with 100 mL acidified 50% alcohol at 2 mL/min, then with 350 mL acidified 60% alcohol at same flow rate. Collect eluates in consecutively numbered 50 mL vol. flasks. Add few drops $NH_4OH$ to each flask, mix well, test for alky, and dil. to vol.

### E. Determination

Obtain spectra at 220–360 nm for each eluate. For blanks, use corresponding alcohol solns consisting of either $NH_4OH$-50% alcohol (1 + 99) or $NH_4OH$-60% alcohol (1 + 99). Dil. as necessary with $NH_4OH$-alcohol solns of proper concn. For quant. detn, obtain spectra of this compd in basic soln, not acid soln. Calc. amt material in each eluate by comparing *A* at 314 nm of sample with std amyl *p*-dimethylaminobenzoate soln in $NH_4OH$-60% alcohol (1 + 99), (**c**), using straight line background correction.

Ref.: JAOAC **53,** 84(1970).

CAS-14779-78-3 (amyl *p*-dimethylaminobenzoate)

## 942.22 Vanishing Cream

**Final Action**

### *A. Test for Type of Emulsion*

Dust small amts of finely ground oil-sol. and $H_2O$-sol. dyes on sep. portions of sample. If color of oil-sol. dye spreads rapidly, $H_2O$-in-oil emulsion is indicated; if color of $H_2O$-sol. dye spreads, oil-in-$H_2O$ emulsion is indicated.

### *B. Water*

Transfer 5–20 g sample to erlenmeyer; add 50 mL toluene, few glass beads, and ca 2 g *lump rosin*. Connect flask to Dean and Stark distg tube receiver, and distil until no more $H_2O$ collects in receiver. Cool, read vol. $H_2O$ under the toluene at room temp., and from this vol., calc. % $H_2O$.

### *C. Ash*

Place 2–10 g sample in flat-bottom Pt dish, and remove $H_2O$ and volatile material by placing dish on steam bath or in 100° oven. Ignite sample at low temp. and finally at 600° to const wt.

### *D. Chloroform-Soluble Material*

Place 2–10 g sample in separator, add 25–50 mL $H_2O$, acidify slightly with $H_2SO_4$ (1 + 9), and ext with successive portions $CHCl_3$, collecting all exts in second separator. (Usually 4–5 portions $CHCl_3$, each ca 35 mL, are enough to remove all $CHCl_3$-sol. material.) Wash combined $CHCl_3$ exts with 10 mL $H_2O$, filter thru cotton plug placed in separator stem, and collect filtrate in weighed dish. Shake aq. washing with small vol. of $CHCl_3$, and filter this $CHCl_3$ into dish. Evap. $CHCl_3$ on steam bath and dry residue for 15 min intervals at 100° to const wt.

**Glycerol**

### *E. Reagents*

(**a**) *Potassium periodate soln.*—0.02 *M*. Dissolve 4.6 g $KIO_4$ in ca 500 mL hot $H_2O$. Dil. to ca 900 mL with $H_2O$, cool to room temp., and dil. to 1 L.

(**b**) *Sodium hydroxide std soln.*—0.02*N*. *See* **936.16**.

(**c**) *Bromocresol purple indicator.*—Dissolve 0.1 g bromocresol purple in 100 mL alcohol.

(**d**) *Propylene glycol.*—Bp 85–86°/10 mm.

(**e**) *Arsenious oxide soln.*—0.02*N*. Dil. 100 mL 0.1*N* $As_2O_3$, **939.12B**, to 500 mL with $H_2O$.

### *F. Isolation and Oxidation of Glycerol*

(**a**) *Isolation of glycerol.*—Place 2–10 g sample in separator, add 25–50 mL $H_2O$, acidify slightly with $H_2SO_4$ (10 g/100 mL), and ext with successive portions $CHCl_3$. (Usually 4–5 portions, each ca 35 mL, remove all $CHCl_3$-sol. material.) Wash combined $CHCl_3$ exts with 10 mL $H_2O$. Filter aq. soln and wash $H_2O$ thru cotton plug to remove droplets of $CHCl_3$, and collect filtrate in 250 mL vol. flask. Add 3 drops bromocresol purple indicator to filtrate and neutze with $CO_2$-free alkali (0.1*N* NaOH is satisfactory), making final adjustment with 0.02*N* NaOH. Dil. almost to vol. with $H_2O$, and if necessary, add more alkali to keep soln light but definite purple; then complete diln to vol. and mix.

(**b**) *Periodate oxidation.*—Transfer aliquot neut. soln, preferably contg 30–40 mg glycerol, to 100 mL vol. flask, and add 50 mL $KIO_4$ soln. Dil. to vol. with $H_2O$ and let stand ca 1 hr. Test for excess periodate, which must be present in oxidn mixt., by adding $NaHCO_3$ and KI to test portion. If excess is present, I is liberated.

### *G. Determination*

(**a**) *By titration of formic acid.*—(Applicable in absence of substances yielding acid on periodate oxidn.) Transfer 50 mL aliquot of oxidized mixt. to titrn flask, add 10 drops propylene glycol (ca 0.5 mL), mix well, wash down sides of flask with $H_2O$, and let stand 10 min. Add 3 drops bromocresol purple indicator and titr. with NaOH soln to light purple end point. 1 mL 0.02*N* NaOH = 1.842 mg glycerol.

(**b**) *From periodate consumed.*—Transfer 20 mL aliquot of oxidized mixt., **942.22F(b)**, to titrn flask and dil. with ca 50 mL $H_2O$. Add ca 1.0 g $NaHCO_3$, 0.5 g KI, and 5 mL starch indicator (mix ca 1 g sol. starch with enough cold $H_2O$ to make thin paste, add 100 mL boiling $H_2O$, and boil ca 1 min with stirring). Titr. immediately with $As_2O_3$ soln to disappearance of blue. Stdze 10 mL $KIO_4$ similarly. Difference between the 2 titrns represents amt of periodate reduced in 20 mL aliquot taken. To obtain amt of periodate reduced in original aliquot obtained from 250 mL flask, multiply above difference by 5.

$$1 \text{ mL } 0.02N\ As_2O_3 = 0.4605 \text{ mg glycerol}$$

Refs.: JAOAC **25,** 903(1942); **26,** 249(1943); **27,** 462(1944); **30,** 507, 651(1947); **31,** 580(1948); **33,** 362, 367(1950).

CAS-56-81-5 (glycerol)

SPECIAL REFERENCE

"Newburger's Manual of Cosmetic Analysis," 2nd ed., AOAC, Arlington, VA 22201 (1977).

# 16. Extraneous Materials: Isolation

**Jack L. Boese and Ruth Bandler, *Associate Chapter Editors***
*Food and Drug Administration*

## GENERAL

### 945.75 Extraneous Materials (Foreign Matter) in Products
#### Isolation Techniques

#### *A. Definition of Terms*

*Extraneous materials.*—Any foreign matter in product associated with objectionable conditions or practices in production, storage, or distribution; included are filth (*see* **970.66A(a)–(d)**), decomposed material (decayed tissues due to parasitic or nonparasitic causes), and miscellaneous matter such as sand and soil, glass, rust, or other foreign substances. Excluded are bacterial counts.

#### *B. Apparatus*

(Avoid use of polyethylene beakers, funnels, containers, etc., as insect fragments and rodent hairs adhere to app. made from this material.)

(**a**) *Aerator, water.*—For attachment to faucet to provide smooth-flowing, aerated $H_2O$ stream. Remove lower screen. (Available from Faucet-Queens, Inc., 550 Palwaukee Dr, Wheeling, IL 60090, No. 00200.)

(**b**) *Autoclave.*—*(1) Slow exhaust type.*—Set "slow exh" to lower pressure from 15 to 0 in 15–20 min. *(2) Non-slow exhaust.*—Let cool to 0 psi before opening or venting.

(**c**) *Blenders.*—*(1) High-speed.*—Use 1 L, 4-lobe jar fitted with 4-blade assembly, 2 blades tilted upward ca 30° with diam. 60 mm and 2 blades tilted downward ca 25° with diam. 55 mm. Operate at speed specified in method, using variable transformer. Use tachometer to measure speed of blending jar blades or blade shaft, after removing blades. *(2) High-speed overhead.*—Alternative to high-speed blender: Mixer with 6 canted, sharp-edge stainless steel blades rotating on shaft of suspended motor and speed control. Blades rotate at bottom of stainless steel cup having 4 indentations, forming lobes. Sorvall Omni-Mixer (DuPont Instrument Co., Sorvall Operations, Peck's Ln, Newtown, CT 06470), or equiv., meets these requirements.

(**d**) *Bolting cloth.*—Silk cloth woven to std size opening and thickness which is used in flour mills. Number of silk specifies number of mesh/linear in. "X," "XX," or "XXX" after number refers to thickness of thread from which cloth is woven; this also affects size of opening in cloth. Therefore, follow designation exactly as to both number and "X" of bolting cloth. (Available from Tetko, Inc., 420 Saw Mill Rd, Elmsford, NY 10523.)

Prep. disks by boiling large squares of silk before cutting them into circles. Circles cut from unboiled silk shrink and become misshapen. Make rulings ca 5–7 mm apart with India ink or other permanent marking material, using fine pen, on boiled and pressed cloth marked off in circles ca 85 mm diam.

When needed, dye ruled cloth by placing in hot (80–85°) soln of 50 mg FD&C Blue No. 1 in 1 L $H_2O$ contg 2.5 mL HOAc, and holding at this temp. ca 15 min with frequent stirring. Rinse well and store in dark.

(**e**) *Butter stirrer.*—*See* Fig. **945.75A**.

(**f**) *Centrifuge.*—International type EXD centrf. (International Equipment Co.) with 8-place No. 240 head, No. 320 shield, No. 325 trunion ring, and No. 571 cushion, or other centrf. giving equiv. max. relative centrifugal force. The following formula may be used to det. equiv. centrf.: $N_1^2r_1 = N_2^2r_2$, where $N_1$ = 2200 rpm and $r_1$ = 19.6 cm (distance from center of centrf. head to bottom of horizontal centrf. tube).

(**g**) *Cyclone.*—Laboratory cyclone or pulper consists of cylindrical perforated metal screen in which revolves paddle which forces soft material from food product out thru openings in screen. Tough materials such as seeds, skins, and stems are moved along and out opening in end of cylinder. Use as power source $^1/_4$ horsepower, 110 v, 1725 rpm elec. motor. Screen is 22 gage material, 400 holes/sq in., each 0.027″ diam. Screen is 2.5″ id and length of effective screen is 3″. Paddle has 2 fins, each $^{25}/_{32}$″ wide, set alternately and extending $1^3/_{16}$″ from center of shaft. Pulper is fed thru hopper which leads into basin 3.5″ long and 2.5″ id. Portion of paddle with fins inserted at 30° angle forces material from basin into screening compartment. Cyclone is so constructed that waste opening may be closed, as needed. Sieved material is caught in shield and delivered thru spout to container. Machine may be readily disassembled for washing. (Blueprints available from Div. of Microbiology (HFF-237), Food and Drug Administration, 200 C St, SW, Washington, DC 20204.)

(**h**) *Extraction vessels.*—*(1) Kilborn funnel.*—1 L, 3.5″ od by 9.5″ high, 8 mm opening at tip. Rubber tubing $^3/_8$″ id and pinch clamp provides convenient cut-off.

*(2) Percolator.*—2 L, Corning Glass Works No. 7040, or equiv., conforming to following general size and shape: 115 mm id × 400 mm long, ca 90 mm id at 200 mm down from top, with 8–9 mm bore tip, with cut-off as in *(1)*. Use stirring rod 370 × 10 mm diam, when specified, to prevent compacting of sample in drain opening.

*(3) Percolator with oversize bore tip.*—Use std percolator as in *(2)* but replace std bore tip (8–9 mm id) with 17–18 mm id bore tip and appropriate size rubber tubing and pinch clamp. Use stirring rod described in *(2)*.

*(4) Trap flask. — Wildman.*—Consists of 1 or 2 L erlenmeyer into which is inserted close-fitting rubber stopper or wafer stopper (Entomological Supply Co., Inc., 2411 S Harbor City Blvd, Melbourne, FL 32901) supported on stiff metal rod 5 mm ($^3/_{16}$″) diam. and ca 10 cm longer than ht of flask. (Rod of greater diam. is not desirable because of its greater displacement of liq.) Rod is threaded (#10–32) at lower end and furnished with nuts and washers to hold it in place on stopper. Countersink lower nut and washer in the rubber to prevent striking flask. *See* **970.66B(b)** and Fig. **945.75B**.

(**i**) *Filter paper.*—Use smooth, high wet-strength, rapid-acting filter paper ruled with oil-, alcohol-, and water-proof lines 5 mm apart. S&S No. 8 is satisfactory.

(**j**) *Filter paper defatting cup.*—Center S&S 588 folded filter paper, or equiv., over bottom of smaller beaker specified in method. Partially shape paper over bottom of beaker and gently insert beaker and paper into larger specified beaker. Remove smaller beaker and transfer weighed sample into formed paper cup.

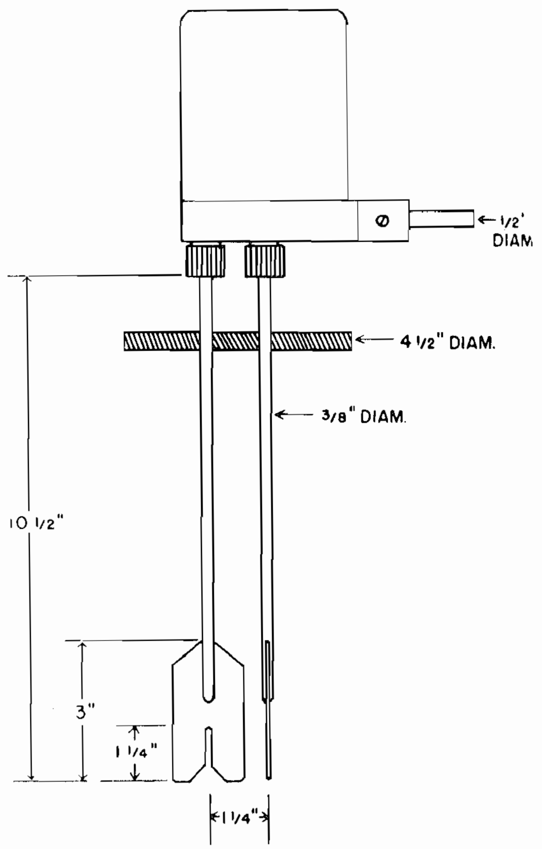



FIG. 945.75A—Mechanical butter stirrer

(**k**) *Funnels for filtration with suction.*—Use funnels with filter papers or bolting cloth cupped up on sides to eliminate loss of solids. Use rapid filter paper for filtration thru Hirsch funnel.

Use of wire screen or bolting cloth between perforated funnel plate and filter paper accelerates filtration and gives more uniform distribution of solids.

(**l**) *Illuminators for widefield stereoscopic microscopes.—Filth examination.*—Illuminator for this purpose should have:

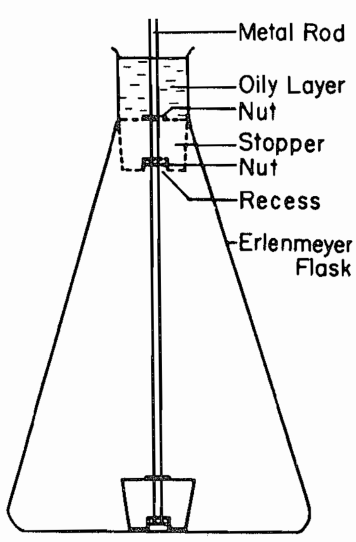



FIG. 945.75B—Wildman trap flask

compactness and flexibility; transformer or resistor to vary light intensity; focusing adjustment to give uniformly lighted field of view; blue-white color from cool low-voltage source.

(**m**) *Howard mold-counting apparatus.*—(*1*) *Howard mold-counting slide.*—Glass slide of one-piece construction with flat plane circle ca 19 mm diam. or rectangle 20 × 15 mm surrounded by moat and flanked on each side by shoulders 0.1 mm higher than plane surface. Cover glass is supported on shoulders and leaves depth of 0.1 mm between underside of cover glass and plane surface. Central plane, shoulders, and cover glass have optically worked surfaces. To facilitate calibration of microscope, newer slides are engraved with circle 1.382 mm diam. or with 2 fine parallel lines 1.382 mm apart.

(*2*) *Reticle* (*accessory disk*) *for Huygenian eyepiece.*—Glass disk that fits into microscope eyepiece, ruled into squares each side of which is equal to $^1/_6$ of diam. of field. Since limiting diaphragm is eyepiece field stop, rulings equal $^1/_6$ of this diaphragm opening. Field viewed on slide with mold-counting microscope has diam. of 1.382 mm at magnification of 90–125×. Reticles (accessory disks) available for widefield oculars may have field of view >1.382 mm in diam. Center portion of field of view is used for mold counting and is delineated by inscribed circle having 1.382 mm specimen field diam. Inscribed circle is ruled into squares each having side equal to $^1/_6$ diam. of circle.

(**n**) *Magnetic stirring bar and stirrer-hot plate.*—Teflon-covered bars ca 47 mm long × 9 mm od; use with hot plate having independent, continuously variable heat and speed controls. *See* also **970.66B(c)**.

(**o**) *Microscopes.*—(*1*) *Compound microscope.*—For mold counting and other filth and decomposition work, microscope should have following min. specifications: binocular body with inclined oculars; 4 parfocal achromatic objectives of ca 4, 10, 20, and 40×; revolving 4-place nosepiece; Abbe condenser with N.A. of 1.25; 10× Huygenian or widefield eyepieces; fine adjustment; mech. stage.

(*2*) *Widefield stereoscopic microscope recommended for filth examination.*—Microscope should have following min. specifications: binocular body with inclined oculars; sliding or revolving nosepiece to accommodate 3 objectives; 3 parfocal objectives 1×, 3×, and 6 or 7.5×; paired 10× and paired 15× widefield oculars; mounted on base and capable of illumination by transmitted or reflected light. 30× is ordinarily used for routine examination of filter papers. Verification at higher magnification may be required.

(**p**) *Pipet for tissue transfer.*—Use 1 mL measuring pipet with bore 3.0 ± 0.5 mm and tip cut off at 1.0 mL mark. In pipetting, draw material slightly above 0.5 mL mark and let it drop slowly to mark.

(**q**) *Rot fragment counting plate and cover preparation.*—Glass plate; 55 × 100 mm, 1.5–4.0 mm thick with cover 50 × 85 mm, ca 1.5 mm thick. Carefully paint on coat of resist over the entire surface, avoiding pinholes. Asphaltum varnish makes excellent resist; paraffin wax may also be used. Carefully scribe crosswise parallel lines, 4.5 mm apart with 15 mm space at each end, thru resist. If asphaltum varnish is used, lines may be scribed with new steel-wheel glass cutter.

Place coated scribed slides face down over HF in polyethylene container. Det. proper acid fume exposure by trial and error. Following etching, remove resist by placing slide in $H_2O$ contg detergent. If resist is not easily scrubbed off, use toluene for cleanup.

Alternatively, use clear plastic plate; 55 × 100 mm, 4–6 mm thick with glass cover 50 × 85 mm, ca 2 mm thick. With sharp needle, carefully scribe crosswise parallel lines, 4.5 mm apart with 15 mm spaces at each end. Several slides can be made at one time by using strip of plastic 100 mm wide and any multiple of 55 mm long, allowing extra length to compensate for each cut of 2–3 mm thickness.

Fasten $^1/_2$ of square cover slip, ca 22 mm on side and ca 0.25 mm thick, at each end of counting plate to raise cover plate above ruled plate. *See* Fig. **945.75C**. Glass slides are available on special order from Ace Glass, Inc.

(**r**) *Sieves*.—*See* "Definitions of Terms and Explanatory Notes. Sieves of No. 100 or finer should be "plain (not twill) weave" of stainless steel. Plain weave is woven with one wire alternately over and under next.

(**s**) *Thin layer chromatographic apparatus*.—

(*1*) *Desaga/Brinkmann standard model applicator, or equiv.*

(*2*) *Desaga/Brinkmann standard mounting board, or equiv.*

(*3*) *Desaga/Brinkmann drying rack, or equiv*.—Accommodates ten 8 × 8″ plates.

(*4*) *Desaga/Brinkmann model 51 stainless steel desiccating cabinet, or equiv.*

(*5*) *Window glass*.—8 × 8″, double strength window glass plates of uniform width and thickness; smooth off corners and edges with file or other tool.

(*6*) *Chromatographic tank and accessories*.—Metal instead of glass troughs.

(*7*) *Dipping tank and accessories*.—Stainless steel, $8^1/_2$ × $8^1/_2$ × $^1/_4$–$^3/_{16}$″ inside width with metal supports and close-fitting U-shaped cover ca 9 × $^1/_2$″. Capacity ca 300 mL.

(*8*) *Spotting pipets*.—1 μL.

(*9*) *Spray bottle*.—8 oz. (Thomas Scientific No. 2753-J10 or Lurex Scientific, Inc., No. 131-0514, 250 mL).

(*10*) *Chromatography spray flask*.—250 mL (Microchemical Specialties Co., 1825 Eastshore Hwy, Berkeley, CA 94710, No. S-4530-D).

(*11*) *Tank liner*.—Cut 2 pieces, $12^1/_4$ × $8^3/_4$″, from desk blotter, white or colored, and bend into L-shape to fit tank.

(*12*) *Strong ultraviolet light source*.—Such as germicidal lamps (General Electric Co., Nela Park, Cleveland, OH 44112), either (*1*) two 30 watt, 36″ tubes, No. G30T8, mounted in std 30 watt reflector fixture ca 20 cm above papers; or (*2*) two 15 watt, 18″ tubes, No. G15T8, mounted in std 15 watt desk lamp fixture placed ca 10 cm above papers. Shield to protect eyes and skin at all times.

### C. Reagents

(**a**) *Acid-alcohol soln*.—HCl and 60% alcohol (1 + 9) or HCl and 40% isopropanol (1 + 9).

(**b**) *Alcohol*.—95% com. ethanol (not denatured) unless otherwise specified. Make all dilns by vol.

(**c**) *60% Alcohol-calcium chloride soln*.—To each 3 L 60% alcohol (amt for 1 analysis), add 200 g anhyd. $CaCl_2$. Stir until salt dissolves. Cloudiness from traces of $CaCO_3$ will clear up during analysis when soln is acidified.

(**d**) *Allantoin std soln*.—Prep. aq. soln contg 2 mg/mL. Pipet 1 mL aliquot into 10 mL vol. flask and dil. to vol. with acetone. Stable ca 3 months.

(**e**) *Antifoam soln*.—1 g Dow Corning Antifoam A compd dild with 20 mL EtOAc. Use supernate and keep tightly closed.

(**f**) *Carob bean soln*.—Blend 0.75% carob bean gum in $H_2O$. Boil 2 min and cool to 20–25°. Add 2 mL HCHO/100 mL and stir gently. Let settle and use clear supernate.

(**g**) *Cellulose powder*.—TLC grade, MN 300 (Brinkmann Instruments, Inc., Cat. No. 66 00 100-8).

(**h**) *Crystal violet soln*.—Dissolve 10 g dye (Colour Index 42555) in 100 mL alcohol and filter.

(**i**) *Detergent soln*.—Prep. aq. Na lauryl sulfate soln as required.

(**j**) *Emulsifiers*.—Nonionic, $H_2O$-sol. surfactants. (*1*) *Nonylphenoxypoly(ethyleneoxy)ethanol*.—Igepal CO-730 (GAF Chemicals Corp., 1361 Alps Rd, Wayne, NJ 07470). (*2*) *Dialkylphenoxypoly(ethyleneoxy)ethanol*.—Igepal DM-710 (GAF Corp.). (*3*) *Nonylphenoxypoly(ethyleneoxy)ethanol*.—Igepal CO-630 (GAF Corp.).

(**k**) *Flotation liquid*.—Mineral oil, (**p**), and heptane, (**l**), (85 + 15).

(**l**) *Heptane*.—Com. *n*-heptane contg <8% toluene.

(**m**) *Indoxyl sulfate* (*urinary indican*) *std soln*.—Approx. 0.1 mg/mL. Available from Sigma Chemical Co. Stored in light-resistant container in refrigerator, soln is stable ca 1 month.

(**n**) *Isopropanol saturated with heptane*.—To 600 mL isopropanol add 45 mL heptane and 430 mL $H_2O$, mix, and let stand overnight. Siphon from below interface.

(**o**) *Kerosene, deodorized*.—Sargent-Welch, Cat. No. C12978, or equiv.

(**p**) *Mineral oil*.—Paraffin oil, white, light, 125/135 Saybolt Universal Viscosity (38°), sp gr 0.840–0.860 (24°). Request supplier to select lot meeting specifications and provide certificate of analysis.

(**q**) *Pancreatin soln*.—Use USP or sol. pancreatin kept refrigerated at 10°. Use fresh soln. Mix at rate of 5 g/100 mL $H_2O$ at ≤40°. Use special soln for cheese, 10 g/100 mL. Stir with malted milk unit or blender 10 min, or let stand 30 min with frequent shaking. Centrf. at 1500 rpm and filter supernate thru S&S No. 8 paper, or equiv. Alternatively, filter thru cotton pads 10–13 cm thick and then thru rapid No. 8 paper in Hirsch funnel with suction.

(**r**) *p-Dimethylaminobenzaldehyde* (*pDMAB*) *spray*.—Mix 30 mL MeOH and 10 mL HCl and let cool. Dissolve with stirring 0.5 g pDMAB in 25 mL cooled mixt. Stable ca 1 month. (*Caution:* Spray reagent is toxic and corrosive. *See* safety notes on spraying chromatograms.)

(**s**) *Sodium acetate spray*.—Satd aq. soln.

(**t**) *Sodium oleate*.—Tech. grade.

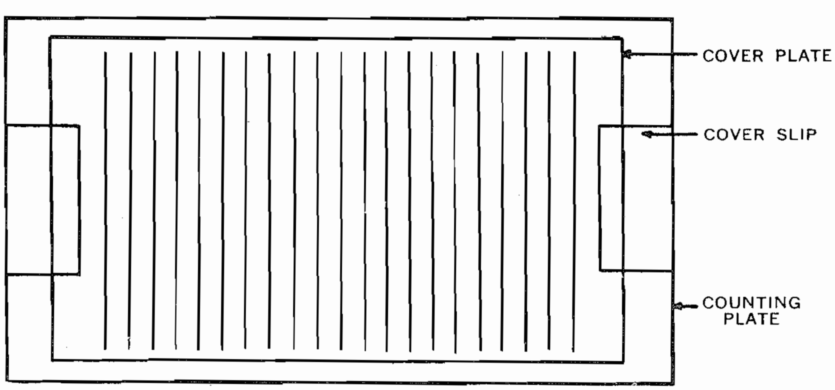



**FIG. 945.75C—Rot fragment counting slide**

(**u**) *Sodium phosphate soln.*—Tech. grade $Na_3PO_4$. Prep. 5% soln.

(**v**) *Stabilizer solns.*—0.5% Na carboxymethylcellulose preferred (Hercules Inc., Cellulose and Protein Products Dept, 1313 Market St, Wilmington, DE 19899). Place 500 mL boiling $H_2O$ in high-speed blender. With blender running, add 2.5 g Cellulose Gum and 10 mL ca 37% HCHO soln w/w, and blend ca 1 min. Alternatives: 3–5% pectin or 1% algin. Add required amt of stabilizer directly to $H_2O$ while agitating in high-speed blender. Treat soln with vac. or heat to remove air bubbles. Add 2 mL HCHO soln/100 mL soln as preservative. (If blender is not available, mix dry stabilizer with alcohol to facilitate incorporation with $H_2O$.) Adjust to pH 7.0–7.5. Filter soln thru 8 μm membrane filter (Millipore No. SCWP-047-00, or equiv.) using suitable vac. filtration app. (Millipore No. XX15-047-00, or equiv.).

(**w**) *Tween 80–60% alcohol soln.*—To 40 mL polysorbate 80 (ICI Americas, Inc.), add 210 mL 60% alcohol, mix, and filter. (Proportionate vols may be prepd.)

(**x**) *Tween 80–40% isopropanol soln.*—To 40 mL polysorbate 80 (ICI Americas, Inc.), add 210 mL 40% isopropanol, mix, and filter. (Proportionate vols may be prepared.)

(**y**) *Tetrasodium EDTA–alcohol soln.*—Dissolve 5 g $Na_4EDTA$ in 100 mL $H_2O$, add 150 mL alcohol, mix, and filter. (Proportionate vols may be prepd.)

(**z**) *Tetrasodium EDTA–40% isopropanol soln.*—Dissolve 5 g $Na_4EDTA$ in 150 mL $H_2O$, add 100 mL isopropanol, mix, and filter. (Proportionate vols may be prepd.)

(**aa**) *Urea std soln.*—20 mg/mL $H_2O$. Stable ca 3 months.

(**bb**) *Wetting agents.*—(*1*) *Tergitol Anionic 7.*—Na heptadecyl sulfate (Sigma Chemical Co.). (*2*) *Triton X-114.*—Alkylaryl polyether alcohol (Rohm & Haas Co.).

## 970.66 Light and Heavy Filth

### General

#### A. Definition of Terms

(**a**) *Filth.*—Any objectionable matter contributed by animal contamination of product such as rodent, insect, or bird matter; or any other objectionable matter contributed by insanitary conditions.

(**b**) *Heavy filth.*—Heavier filth material sepd from product by sedimentation based on different densities of filth, food particles, and immersion liqs such as $CHCl_3$, $CCl_4$, etc. Examples of such filth are insect and rodent excreta pellets and pellet fragments, sand, and soil.

(**c**) *Light filth.*—Lighter filth particles that are oleophilic and are sepd from product by floating them in an oil-aq. liq. mixt. Examples are insect fragments, whole insects, rodent hairs, and feather barbules.

(**d**) *Sieved filth.*—Filth particles of specific size ranges sepd quant. from product by use of selected sieve mesh sizes.

#### B. Special Technics

(**a**) *Wet sieving technique.*—Use clean sieve of correct diam. (8″ min.), mesh type (plain *not* twill weave) and mesh number (100, 140, 230, etc). Hold sieve under aerator, **945.75B(a)**, spray of specified temp. $H_2O$ at approx. 30° angle. Pour well-mixed sample, portionwise (not so much that clogging or excessive foaming results), onto sieve so that moderate pressure spray of $H_2O$ contacts material on sieve. Increase $H_2O$ pressure to achieve max. spray action on sieve, but not so violent that sample froths over lip of sieve. Keep sample material washed to lower inside edge of sieve (while held at 30° angle) and direct $H_2O$ spray onto sample material until majority of detergent foaming subsides and thru $H_2O$ is essentially clear. Repeat portionwise addn of sample and wash sample container thoroly on final addn. Continue washing material on sieve until *all* detergent foaming subsides and thru $H_2O$ is clear. Quant. transfer sieve retainings as specified in method. Clean sieve inside walls using rubber policeman and direct $H_2O$ spray on screen, held at angle, to collect all sample residues at lower edge of sieve. Repeat sidewall and screen washing, as necessary, to ensure quant. transfer of sieve retainings.

(**b**) *Operation of Wildman trap flask.*—Unless otherwise directed in specific method, cool mixt. in flask to room temp. Bring vol. of liq. to ca 900 mL in 2 L flask and to ca 600 mL in 1 L flask. Add vol. of flotation liq. as stated in method by pouring down stirring rod. Stir mag., **970.66B(c)**. Add enough liq. to bring flotation liq. well into neck of flask. (*Note:* Deaerate all flotation liqs before use.)

Unless otherwise stated, let mixt. stand 30 min, intermittently stirring bottom layer every 3–6 min during first 20 min of standing. Spin stopper (wafer) to remove sediment and trap off by raising stopper (wafer) as far as possible into neck of flask, being sure that oil layer and ≥1 cm of liq. below interface are above stopper (wafer). Hold stopper (wafer) in place and pour off liq. into beaker. Rinse out material on rod and in neck of flask with liq. extn medium in which floating was performed and add to beaker.

Do not wash out neck of flask with 95% alcohol or other liq. which may interfere with surface relationships of the 2 phases; this will cause loss in recovery in subsequent trappings.

Filter trapped material and rinsings with suction thru rapid paper in Hirsch funnel. Add flotation liq. as specified to trap flask and stir vigorously. Add enough liq. extn medium to bring flotation liq. into neck of flask. Trap off again, rinse, and filter as above.

(**c**) *Operation of magnetic stirrer.*—To disperse flotation liq. thru sample, dil. liq. extn medium to vol. specified in method and bring to proper temp. Add mag. stirring bar, **945.75B(n)**, and proper vol. of flotation liq. Slowly bring unit to max. speed that does not produce visible or audible splashing (central portion of stirring bar is usually just visible at bottom of vortex) and stir for time stated in method. Time stirring interval after achieving proper speed and vortex.

(**d**) *Filtration technic.*—(Treatment of trapped-off material.) If material trapped off in beaker contains appreciable starchy debris, add enough HCl to make soln 1–2% of HCl (1 + 99 − 49), bring to boil, and filter while hot. If fats or colloidal material retard filtration, hasten by playing stream of hot $H_2O$ over paper during filtration.

(**e**) *Clearing of plant materials.*—With sedimentation or flotation procedures, some food material may be trapped off with filth particles. By proper clearing, filth may be made to stand out in contrast with white background of filter paper by one of following technics:

(*1*) For heavy filth, moisten paper with $H_2O$ or 50% alcohol. (This method does not clear material completely, but it leaves rodent pellets and other filth soft and pliable.)

(*2*) For light filth examination, wet paper with glycerol-alcohol (1 + 1) immediately after filtering. Place enough liq. on paper to fill fibers but not enough to cause flowing of extd materials. This clearing agent does not harden filth material on paper, as do many oils which might be used as clearing agents.

(*3*) Clove oil can be used for clearing plant materials. This oil has high refractive index and clears more completely than does alcohol-glycerol soln.

(**f**) *Illumination for the widefield stereoscopic microscope.*—(*1*) *By direct light.*—Focus and adjust light to strike paper from above at ca 70° angle from horizontal. Light may come from right or left.

(**g**) *Microscopic examination of filter papers.*—Make examination at 30× (unless otherwise specified), using widefield stereoscopic microscope, on properly cleared paper on opaque white background. Continually tease and probe particles while observing thru microscope. Turn over all large pieces of material, such as bran, which might obscure filth elements. Examine all doubtful pieces of material at 60–75×. At least twice magnification used in original examination is necessary to show new details not observable at lower power. If doubt still remains, mount piece, clear thoroly, and examine under compd microscope. *Thoro knowledge of appearance of authentic materials is assumed.*

(**h**) *Counting insect and other animal filth.—Diagnostic characteristics of insect fragments:* Count as of insect origin any fragment showing one or more of the following characters: (*1*) characteristic shape of whole or portion of specific appendage or body part; (*2*) articulation point (various types of joints); (*3*) one or more body hairs or setae; (*4*) one or more setal scars; (*5*) surface pattern (sculpturing) characteristic of a specific insect; (*6*) one or more sutures present (various types separating body plates or sclerites). *Diagnostic characteristics of animal hairs: See* Vazquez, A. W., Structure and Identification of Common Food-Contaminating Hairs, JAOAC **44,** 754(1961).

(**i**) *Format for reporting filth.—Container:* Describe size, type, and closure(s) of immediate container and note condition if not intact. *Product:* Common name, if identity is known, or simple description. *Code(s)*: Manufacturer's or distributor's name and identification marks. *Method(s)*: Cite AOAC paragraph number(s) and note any modifications made. *Amount examined:* Number subsamples analyzed and amount per sub. If amount is variable, report for each sub under Findings. *Findings*—Report findings on analyst's worksheet by subsample number. Use *only* categories that apply and report any filth element that is found under no more than one category. Within categories, group filth elements by identity, when known, and then by size or other appropriate descriptive feature. If amt filth present makes exact count impractical, report either approximate or minimal figure rather than term "too numerous to count." Summarize sample results by category totals and averages. Note whether or not sample was fumigated before shipment or on receipt at laboratory if there are whole insects, mites, or other arthropods.

(*1*) No. whole insects or equivs (i.e., sep. heads or body portions with head attached). Distinguish whole insects and equivalents in subtotals. Give identity, stage of life cycle, and size (mm). State whether whole insects are *alive* or *dead*.

(*2*) No. insect cast skins. Give identity (if known), size (mm), and state whether nymphal, larval, or pupal. Distinguish whole cast skins (with head portion) and cast skin fragments.

(*3*) No. insect eggs. Give identity, if known.

(*4*) No. insect fragments, other than sep. setae. Give identity (if known), dimensions or size range (mm), and name of part. State whether identified fragments are from adult or immature insects.

(*5*) No. setae (if fly, state).

(*6*) Insect excreta. Report wt (mg) and/or count of excreta pellets with dimensions or size range (mm). Give identity, if known.

(*7*) Insect penetration of container. Report number, size (mm), and direction. Note container integrity and completeness of closures and seams.

(*8*) No. mites. Give identity, if known. State whether *alive* or *dead*. Report mite fragments here as subcategory.

(*9*) No. arthropods other than insects and mites. State whether *alive* or *dead*. Give identity (e.g., spiders, pseudoscorpions). Report fragments here as subcategory.

(*10*) No. rat or mouse fecal pellets (state which or give length and diameter in mm). Give wt (mg) if from condimental seeds, spices, cocoa beans, coffee, or grains.

(*11*) No. rat or mouse fecal pellet fragments. Give basis for identification. Give dimensions or size range (mm). Give wt (mg) if from condimental seeds, spices, cocoa beans, coffee, or grains.

(*12*) Other mammalian feces. Report size (mm) and wt (mg). Give identity (e.g., cat, cow), if known, and basis for identification.

(*13*) No. rat or mouse hairs or hair fragments. Report length (mm) of each hair and hair fragment or, if numerous, group into categories of limited size range.

(*14*) No. other hairs and hair fragments. Report length (mm), grouping as in (*13*). If unidentified, state whether striated or nonstriated.

(*15*) No. feathers, feather fragments, and barbules. Give dimensions or size range (mm) of feathers and fragments.

(*16*) Urine on container or food (state which). Report odor of urine, if detected. Give number and dimensions or size range (in.) of stains and note if penetration is to product. Report component(s) detected by AOAC test.

(*17*) Bird excreta on container or food beneath (state which). Report amount as wt (mg) or no. and dimensions of droppings, as appropriate.

(*18*) Other extraneous materials (describe and report each type by appropriate quantitative figure).

## BEVERAGES AND BEVERAGE MATERIALS

### 965.38 Filth in Cocoa, Chocolate, and Press Cake

**Flotation Method**

**First Action 1965**
**Final Action 1988**

#### *A. Apparatus and Reagent*

(**a**) *Hirsch porcelain funnel with plug.*—Size 0, fitted with fixed perforated filtering plate. Diam. at top, 94 mm; diam. of plate, 56 mm. Fit stem end of funnel with rubber tubing ca 10 cm (4") long which can be plugged with plastic or cork stopper.

(**b**) *Sodium hypochlorite soln.*—Approx. 0.25%. Dil. 5 mL com. NaOCl soln, 5.25% by wt, with 95 mL $H_2O$. Prep. fresh daily.

#### *B. Determination*

(**a**) *Cocoa.*—Mix 50 g cocoa into 500 mL hot (55–70°) 2% detergent soln, **945.75C(i)**, or 500 mL hot 2% Igepal CO-630, **945.75C(j)**(*3*). Pour portionwise onto No. 230 sieve, **945.75B(r)**, and wash with forcible stream of 55–70° tap $H_2O$, using aerator, **945.75B(a)**. Remove fat by tilting sieve ca 20° and play $H_2O$ thru liq. which collects at side. When fat and fine material have washed thru and foam is gone, transfer residue to 2 L trap flask, **945.75B(h)**(*4*), with $H_2O$. Add ca 500 mL $H_2O$ and boil 10 min. Cool to room temp. and add $H_2O$ to total vol. of 1 L. Pour 50 mL heptane, **945.75C(l)**, down stirring rod. Lower mag. stirring bar, **945.75B(n)**, into flask on stirring rod stopper. Raise rod above liq. and secure with clamp. Stir mag., **970.66B(c)**, 5 min. After stirring, fill flask with $H_2O$. Let stand 30 min, gently stirring bottom layer every 4–5 min with stirring bar for first 20 min. Trap off heptane. Add 35 mL more heptane, stir by hand gently 1 min, let stand 15 min, and again trap off. Filter combined trappings, using

Hirsch funnel. Remove paper and examine microscopically. If debris on filter paper is excessive, proceed as in (**d**) after examining paper for hairs only.

(**b**) *Chocolate.*—Use 100 g finely shaven chocolate and proceed as in (**a**).

(**c**) *Press cake.*—(*1*) *Method I.*—Heat sample (usually very hard lumps) 2–3 hr at 60–70° and break into ≤0.5" (1 cm) pieces. Mix 50 g into 500 mL hot 2% detergent soln, **945.75C(i)**, or 500 mL hot 2% Igepal CO-630, **945.75C(j)**(*3*), in 800 mL beaker. Stir with butter stirrer, **945.75B(e)**, or mag. stirrer, at low speed 2–3 hr until completely dispersed, or let soak overnight. Stir thoroly, pour portionwise onto No. 230 sieve, **945.75B(r)**, and proceed as in (**a**).

Refs.: JAOAC **48,** 543(1965); **50,** 496(1967); **57,** 957(1974).

(*2*) *Method II.*—Break sample into ≤1 cm (0.5") pieces, using hammer or similar implement. Weigh 50 g into 1 L beaker and add 100 mL peanut oil. Add mag. stirring bar, and heat with gentle stirring to 150°. Transfer to cool mag. stirring unit and stir 10 min at speed where no splashing occurs. Add 500 mL aq. 5% Triton X-114 soln, **945.75C(bb)**(*2*), and stir 5 min. Proceed as in (**a**), beginning "Pour portionwise onto No. 230 sieve, . . ." If oil drops are visible after completion of sieving, wash material on sieve with 25–50 mL 2% aq. Triton X-114 soln by spraying from wash bottle. Repeat as necessary. Continue sieving until surfactant is removed and proceed as in (**a**).

Ref.: JAOAC **54,** 567(1971).

(**d**) *Bleaching technic.*—Return paper to Hirsch funnel in suction flask. Wash thoroly with $H_2O$. (If paper contains alcohol and glycerol from examination for hairs, wash first with alcohol and then with $H_2O$.) Apply vac. until paper appears dry, turn off vac., and plug rubber tubing with stopper. Cover paper with ca 3 mm (5–7 mL) NaOCl soln and let stand until bleaching of cocoa tissue is complete, but <30 min. Maintain level of soln entire period and do not let soln flow over rim of paper. Turn on vac., which will remove stopper. Wash paper with $H_2O$. Examine microscopically for insect fragments and other extraneous materials other than hairs.

Refs.: JAOAC **58,** 1302(1975); **61,** 400(1978).

## 988.16 Filth in Ground Coffee and Coffee Substitutes

### Sedimentation and Flotation Method

### Final Action 1988

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

(**a**) *Heavy filth, sand, and soil.*—Weigh 100 g sample in 600 mL beaker, add 350 mL $CHCl_3$, and boil 15 min, stirring occasionally. Wash down sides of beaker with $CHCl_3$. Let mixt. cool and settle 15 min with occasional stirring of top layer. Carefully decant $CHCl_3$ and floating tissue onto smooth ca 15 cm filter paper in buchner without disturbing heavy residue on bottom of beaker. Repeat decanting with small amts of $CHCl_3$ until practically no plant tissue remains with residue on bottom of beaker. (Sp gr of $CHCl_3$ may be increased by addn of $CCl_4$, if necessary to float plant tissue. Do not add $CCl_4$ beyond 1 part $CCl_4$ to 1 part $CHCl_3$.) Transfer residue from beaker to ashless filter paper and examine for filth. If residue is appreciable, ignite filter and det. wt sand, soil, etc.

(**b**) *Light filth* (*ground coffee*)—Make filter paper cup from 24 cm paper and 150 and 250 mL beakers, **945.75B(j)**. Weigh 25 g sample into this cup. Add 100 mL $CHCl_3$ by pouring most of liq. outside paper cup into the 250 mL beaker. Press cup down into $CHCl_3$ and place on steam bath. Boil gently 5 min, avoiding excessive loss of $CHCl_3$. Lift paper, clamp with clothespin, and let drain to slow drip. Discard solv. and repeat extn with two 100 mL portions $CHCl_3$. Position paper on Hirsch funnel and aspirate to apparent dryness. Complete by drying (*1*) overnight in hood, (*2*) 1 hr in 80° convection oven, or (*3*) 30 min in 80° vac. oven at >5" (13 cm, 16.9 kPa) vac.

Wash sample into 2 L trap flask with total of 400 mL $H_2O$. Boil gently 15 min, remove from hot plate, and set aside. Dil. to 600 mL with $H_2O$, add 400 mL undild isopropanol while stirring mag., heat to bp, and boil 2–3 min. Add 40 mL mineral oil, **945.75C(p)**, and heat to vigorous boil. Transfer to cool stirrer and stir mag., **970.66B(c)**, 5 min. Slowly fill flask with 40% isopropanol by letting liq. flow down rod while top of stopper is held just above contents. Stir with rotary swirl to resuspend solids, and trap off after 2 min. Filter onto ruled paper. Add 30 mL mineral oil, stir by hand 30 sec, and trap off after 5 min. Filter onto another ruled paper and examine papers microscopically.

If filter paper debris is excessive, examine for rodent hairs and then bleach as in **965.38B(d)**.

Ref.: JAOAC **55,** 57(1972).

(**c**) *Light filth* (*other substitutes except chicory*).—Air dry decanted material on paper overnight or for 1 hr in oven at ca 80°, transfer dried material to 2 L trap flask, and add 400 mL hot $H_2O$. Boil 15 min and, if necessary, add small amts cold $H_2O$ intermittently to prevent foaming. Cool mixt. to <20°. Trap off twice, using 35 and 25 mL portions heptane, **945.75C(l)**, resp. In first trapping, after stirring heptane, let stand 5 min before filling flask. Filter and examine microscopically.

(**d**) *Light filth* (*ground chicory*).—Add 50 g sample to 1 L beaker contg soln of 5 g Na lauryl sulfate and 10 g $NaHCO_3$ in 500 mL $H_2O$. Stir and place *in* steam bath. Heat 20 min, stirring twice at 5 min intervals, and wash down sides with few mL $H_2O$ after each stirring. Transfer to No. 230 sieve and wash until foam is gone. Rinse sieve retainings with ca 100 mL 40% isopropanol. Transfer (use of teaspoon suggested) to 2 L trap flask with 40% isopropanol. Dil. to 800–900 mL with 40% isopropanol. Boil 4 min with gentle stirring. Add 50 mL mineral oil and heat until boiling starts again. Move to cool mag. stirrer and stir 5 min. Fill trap flask with 40% isopropanol added down rod with stopper held just above liq. Stir immediately to resuspend settlings. Stir 2 more times at 3 min intervals. Raise rod; wash with few mL 40% isopropanol and clamp with stopper ca 75 mm below interface. Let stand 4 min. Trap off, rinse neck, and filter onto ruled paper. Add 25–30 mL mineral oil, stir by hand 45 sec at moderate speed, and add 15–20 mL $H_2O$. Let stand 20 min, trap off, rinse flask neck with undild isopropanol, and filter onto sep. ruled papers. Examine papers at 20–30×.

Ref.: JAOAC **54,** 571(1971).

## 967.23 Aphids in Hops

### Flotation Method

### First Action 1967
### Final Action 1988

### *AOAC-ASBC Method*

#### *A. Reagents and Apparatus*

(**a**) *Flotation soln.*—Satd $Na_2B_4O_7$ soln, 100 g borax/L $H_2O$.

(**b**) *Iodine stain.*—Dissolve 0.5 g I and 1.5 g KI in 25 mL $H_2O$.

(c) *Blender*.—"Intensifier" Twin Shell Blender, Patterson-Kelley Co., Div. of Harsco, 100 Burson St, PO Box 458, East Stroudsburg, PA 18301-0458, or equiv.

### B. Preparation of Sample

Place sample in blender, using 4 qt (3.8 L) size shell for small samples or 8 qt (7.6 L) size shell for large samples. Activate blender and "intensifier" for 1 min intervals until blending and breakage of sprigs are complete. Draw off 10 g samples from bottom plate.

### C. Determination

Mix 10 g representative sample in 100 mL satd borax soln in 2 L Wildman trap flask. Bring to *slow* boil. Keep mixt. from boiling onto sides of flask by keeping boiling to min. and by washing down sides with $H_2O$. Boil 1.5 hr, and cool to room temp. Fill flask to 1600 mL with $H_2O$ and 35 mL heptane, **945.75C(l)**, and stir vigorously 10 sec. Fill flask with $H_2O$, let stand 30 min, and trap off. Perform second trapping, using 25 mL heptane, stirring 10 sec, and letting flask stand 15 min. Wash neck of flask with isopropanol. Pour trappings onto ruled paper(s), add 10–12 drops I stain, and examine microscopically. If excess plant tissue is present in trappings, pour trappings thru 5" No. 10 sieve held over paper. Wash plant tissue on sieve with alcohol onto filter paper to remove any adhering insects.

Count as aphid any whole aphid or part contg head. Count individually aphid cast skins and other insects.

Ref.: JAOAC **50,** 499, 520(1967).

## 970.67 Filth in Tea
### Direct Sieving Method
### First Action

See **960.51**.

## 970.68 Heavy Filth in Tea
### Sedimentation Method
### First Action

See **975.48**.

## 981.18 Light Filth in Tea
### Flotation Method
### First Action 1981
### Final Action 1988

### A. Reagents

*Sequestering agent*.—Mix 50 mL Tween 80–40% isopropanol, **945.75C(x)**, 50 mL $Na_4EDTA$-40% isopropanol, **945.75C(z)**, and 200 mL 40% isopropanol.

### B. Determination

Weigh 10 g sample into 800 mL beaker, add ca 300 mL boiling $H_2O$, heat to boiling, and simmer 6 min with mag. stirring using Teflon-coated bar. Wash material from beaker to No. 230 sieve with forcible stream of aerated $H_2O$, but do not let mixt. splash out of sieve. When rinse $H_2O$ is clear, drain residue and quant. transfer with 40% isopropanol to 2 L Wildman trap flask. Rinse sides of flask with 40% isopropanol, and bring total vol. in flask to 900 mL with 40% isopropanol. Pour 50 mL flotation oil, **945.75C(k)**, down stirring rod, with top of disk or rubber stirring stopper held just below surface of liq. Clamp rod above liq. Mag. stir 6 min. Let stand 2–3 min. Pour 300 mL sequestering agent down stirring rod, with top of disk or rubber stirring stopper held just below surface of liq. Mix 1 min by gently swirling with stirring rod beneath surface of liq. Pour 40% isopropanol slowly down stirring rod to fill flask. Rinse rod with 40% isopropanol and clamp so that stopper is held above layer of settled material. Let stand 5 min. Rotate stopper to free settled material. Let stand 25 min and trap off, rinsing neck with isopropanol until all residue is removed. Rinse neck with 40% isopropanol and add rinse to trappings. Add 30 mL flotation oil by pouring down stirring rod into trap flask. Hand-stir 1 min to bring flotation oil down. Add 40% isopropanol to refill flask. Let stand 30 min, and trap off into same beaker of first trappings. Rinse neck with isopropanol until all residue is removed. Transfer combined trappings onto one or more ruled filter paper(s). Examine paper(s) microscopically at 30× for insect fragments and rodent hairs.

Ref.: JAOAC **64,** 287(1981).

# DAIRY PRODUCTS

## 952.21 Sediment in Milk
### Sediment Test Method
### Final Action 1965

### A. Apparatus and Materials

(a) *Tester*.—Simply constructed, easily cleaned, and adjustable between samplings to permit sanitary removal of used disk and replacement with clean disk. Before using, check tester for reproducibility as in **952.21B**. Milk or sediment must not bypass disk. Select type according to method of sampling:

(*1*) *For mixed sample method*.—Pressure, gravity, or vac. type: (a) For 1 gal. sample use any suitable device that will filter sample thru disk with exposed area $1^1/_8$" diam. (b) For 1 pt sample, equip single-unit, off-bottom tester with No. 125 BNC 0.40" safety head (available from Sediment Testing Supply Co., 7366 N Greenview Ave, Chicago, IL 60626) having filtering area 0.40" diam., or use any suitable device having filtering area 0.40" diam.

(*2*) *For off-bottom method*.—Single-unit type for intake of 1 pt on upstroke of plunger and discharge thru disk on down stroke, or 2-unit type, contg 1 unit for removal of 1 pt milk from bottom of can and another for filtering sample. Use sampling device long enough to permit reaching bottom of milk can, with filtering area $1^1/_8$" diam.

(b) *Cotton sediment disks*.—Std lintine cotton disks or pads, $1^1/_4$" diam., for use over flat wire screen in tester to expose filtration area $1^1/_8$" diam. Disk must not contain phenolic resins or other chem that may contaminate milk.

Test sediment disks as follows: Filter 12 mg std sediment mixt. (60 mL aliquot (**d**)) thru pad, using clean flask to catch filtrate. Transfer filtrate to beaker, rinse flask 3 times with $H_2O$, and add rinsings to beaker. Filter fitrate thru 7 or 9 cm S&S White Ribbon paper (or equiv.) that has been washed with ca 200 mL $H_2O$, dried to constant wt at 100°, and cooled in covered dish in desiccator before weighing. Thoroly rinse beaker and paper with $H_2O$ and dry to const wt as above. Test ≥3 disks; av. wt sediment passing thru each disk should be ≤2.8 mg. In addn, std disk prepd from fine mixt. should not appear to have sediment buried beneath surface.

(c) *Sediment filtering apparatus*.—(*1*) *For $1^1/_8$" diameter stds*.—App. must hold $1^1/_4$" sediment disk and have effective filtering area $1^1/_8$" diam. This $1^1/_8$" area must be unobstructed except for wire screen or wire screen and perforated plate support for filter disk. App. should be supported in filter flask so vac. can be used for rapid filtration or flask air outlet can be closed to stop filtration. App. should have ca 80° funnel with capacity of ≥80 mL but ≤450 mL. Test app. by filtering $H_2O$ suspension of C thru std disk. Disk should have clean, sharply defined border. When sediment suspension is filtered, sediment should be evenly distributed over disk with no pattern formation. Figs **952.21A** and **B** show suitable app.

(2) *For 0.40″ diameter stds.*—Vac. type that holds $1^1/_4$″ sediment disk and uses only 0.40″ diam. filtering area. Test app. as in (*1*).

(**d**) *Preparation of coarse std sediment disks.*—Prep. uniform mixt. of oven-dried (100°) materials which meet following screening specifications. Grind all materials by hand with mortar and pestle.

| | |
|---|---|
| Cow manure, thru No. 40 | 53% |
| Cow manure, thru No. 20, retained on No. 40 | 2% |
| Garden soil, thru No. 40 | 27% |
| Charcoal, thru No. 40 | 14% |
| Charcoal, thru No. 20, retained on No. 40 | 4% |

Place 2.00 g above mixt. in 100 mL vol. flask, thoroly wet with 4–6 mL *0.1% Aerosol OT soln* (prep. 1–2% soln in acetone and dil. with $H_2O$) or other suitable wetting agent, add 46 mL 0.75% carob bean gum soln prepd as in **945.75C(f)**, and bring level of liq. just into neck of flask by adding *50% (by wt) sucrose soln*. Let stand ≥30 min, add few drops alcohol, and dil. to vol. with the sucrose soln. Mix thoroly, pour into 250 mL beaker or other suitable container, and stir with mech. or mag. stirrer at speed (ca 200–300 rpm) such that mixt. is thoroly agitated but very little air is whipped into suspension. Observe with bright reflected light to see that suspension is uniformly stirred.

Transfer, while stirring, 10 mL portion (200 mg std sediment) with large-tip, graduated pipet to 1 L vol. flask, and dil. to vol. with the 50% sucrose soln. When thoroly mixed, each mL contains 0.2 mg sediment. Mix, pour into 1.5 L beaker, and stir as above. If particles accumulate on side of beaker, wash down with portions of sediment suspension or push under with tip of pipet. While stirring, pipet definite vols of sediment mixt. and add to $^3/_4$ pt filtered sweet skim milk. Mix thoroly and pass mixt. thru std sediment disk in filtering app., (**c**)(*1*). Gently pour milk down side of filtering app. and filter with very little or no suction. Wash container promptly with $^1/_4$ pt filtered skim milk. Let last portion of milk flow thru pad with no suction applied. If sediment does not appear to be evenly distributed over pad, add 15 or 20 mL skim milk and let it filter thru without suction. Repeat addn until sediment appears evenly distributed. Suck air thru disk ca 1 min to remove excess skim milk.

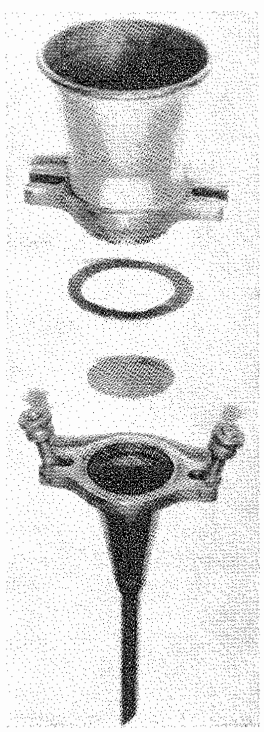


**FIG. 952.21A—Sediment filtering apparatus, unassembled**

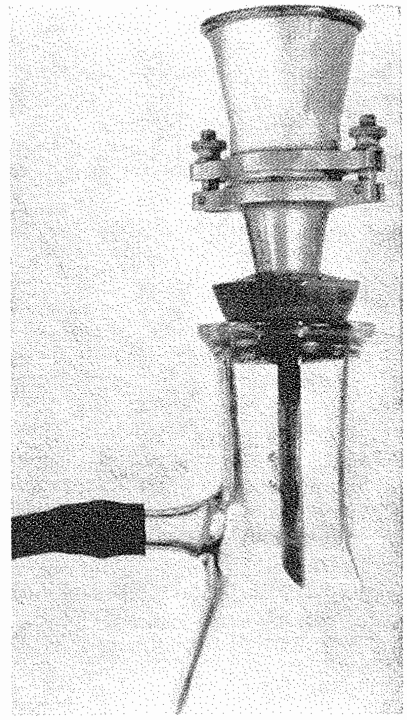


**FIG. 952.21B—Sediment filtering apparatus, assembled**

For permanent record, mount and spray disks with 40% HCHO soln or with alc. soln contg 2.5 g each of menthol and thymol in 100 mL. Alternatively, if most of milk is removed by thoro aspiration, no preservative is needed. Dried pads may be coated with colorless plastic cement dild with 1–3 vols acetone so that mixt. is thin enough to pour easily. If acetone dissolves pigment from paper and stains pads, place pads on flat glass plate for treating with dild cement. Move pads while drying to prevent sticking to glass. When pads are almost dry, place light wt (*e.g.*, petri dish) directly on them to prevent curling. Pads may be mounted with plastic cement. (Std disks made from manure contg large amt of chlorophyll cannot be coated with plastic cement, as solv. exts chlorophyll and stains pad green. Use this method of preserving pads only if there is no leaching of pigment from sediment on addn of dild plastic cement.)

Following above method, prep. series of disks contg sediment remaining from 0.0, 0.2, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 12.0, and 14.0 mg std mixt. Mark disks to show mg of sediment used to prep. each pad. Do not use as std any pad on which sediment is not evenly distributed.

For comparison with tests on samples, entire series of disks may be used, but usually it is more convenient to select few disks denoting variations in grade that are applicable to particular investigations being made. If grading charts are prepd and reports made, indicate on chart and report whether mixed or off-bottom sample was used. If stds are to be handled or used for appreciable length of time, place them under glass, transparent plastic sheets, or other suitable materials. In using stds, use either of following methods: (*1*) Grade sediment disk to nearest std disk regardless of whether actual amt of sediment is above or below std; or (*2*) grade sediment disk of sample as "more than __ mg" or "less than __ mg." Choose stds to fit method of grading. When grading disks, disregard gross pieces of material (whole flies, hairs, large chunks of dirt or manure, etc.) but if such matter is present, list each sep. on report.

(**e**) *Preparation of fine std sediment disks.*—(*1*) *$1^1/_8$" diameter stds.*—Grind oven-dried (100°) cow manure, garden soil, and wood charcoal (not powd) in impact mill, Wiley mill, or other suitable type, using fine screen in mill. Pass cow manure thru Wiley mill or similar type 2 or 3 times. Sift materials sep. in max. batches of 50 g as follows: Dry 25–50 g at 100° for 3–4 hr. While still warm, place in 8" No. 140 sieve nested over No. 230. Add cover and receiver. Shake nested sieves by hand 5 min at ca 120 strokes/min. Sep. sieves and brush off material adhering to underside of No. 230 and discard before emptying sieve. Dry material retained on No. 230 ca 2 hr (max. batch 20 g) and resift 5 min as above. Sep. sieves and brush off material adhering to underside of No. 230 before emptying. Use "on 230" fractions from second siftings and mix uniformly in following proportions: cow manure 66%, garden soil 28%, and charcoal 6%.

Combine above 2 "on 140" fractions of each of the 3 materials and resift as above, except use No. 120 sieve nested over No. 140. Resift new "on 140" fraction, retaining "on 140" fraction from second sifting. (Dry before each sifting and brush material from underside of No. 140 sieve before emptying.) Mix manure, soil, and charcoal in above proportions.

Place 1.80 g mixt. from "on 230" fractions and 0.20 g mixt. from "on 140" fractions in 100 mL vol. flask. Proceed as in (**d**), beginning ". . . thoroly wet with 4–6 mL *0.1% Aerosol OT soln* . . ." except use $H_2O$ instead of 50% sucrose soln for dilg 10 mL aliquot to 1 L.

Where (**d**) states "While stirring, pipet definite vols . . ." proceed as follows: Det. approx. funnel capacity of filtering app., (**c**)(*1*), by pouring $H_2O$ into assembled app. with filter flask air outlet closed. Include $H_2O$ that filters thru as part of funnel capacity. While stirring, pipet aliquots of sediment suspension into beakers. Add $H_2O$ to make total vol. 20–50 mL less than funnel capacity, using total vol. of ≥60 mL but ≤400 mL.

With filter flask air outlet closed to prevent filtration, mix dild aliquot and pour into app., (**c**)(*1*), fitted with wet std disk, (**b**). (Use alcohol or wetting agent if necessary to wet disk.) Add 20–50 mL $H_2O$ to beaker and rinse by swirling. Pour into funnel, keeping lip of beaker touching surface of $H_2O$ if possible. (Rinse $H_2O$ should nearly fill funnel if capacity is ≤450 mL.) Open flask air outlet. After $H_2O$ has filtered thru pad, apply vac. and aspirate disk for ca 1 min. Remove pad and let dry in covered dish. If sediment is not evenly distributed, discard pad. After some practice, ca 75% of pads prepd should be acceptable. No preservative is required. Pads may be coated with dild plastic cement and used as in (**d**).

(*2*) *0.40" diameter stds.*—While stirring, pipet 100 mL above dild fine suspension into suitable container and dil. to 800 mL with $H_2O$. Each mL contains 0.025 mg sediment and when filtered thru 0.40" diam. area is equiv. to 0.2 mg filtered thru $1^1/_8$" diam. area. Prep. series of stds by filtering suitable aliquots thru disks in app., (**c**)(*2*). Dil. each aliquot to 50–60 mL and filter with min. suction. Rinse beaker with small vol. $H_2O$ and add to funnel. Carefully rinse side of funnel with small vol. $H_2O$. Use min. suction necessary to remove excess $H_2O$ from disk. Designate 0.40" diam. stds as "__ mg equiv." and use in grading 1 pt mixed sample test disks in same manner as $1^1/_8$" diam. stds are used.

(**f**) *Photographic stds.*—Photographic stds (obtainable from Photography Div., Office of Governmental and Public Affairs, US Dept of Agriculture, Washington, DC 20250) may be used as guide in grading sediment pads, but it is preferable to use actual disks prepd as in (**d**) or (**e**). Stds that more nearly resemble disk being graded should be used in each case. Do not use photographs that have become faded, stained, soiled, or otherwise damaged.

### B. Checking Sediment Testers

To check sediment testing devices, proceed as follows: Measure actual vol. of milk delivered to assure that 1 pt is withdrawn and passes thru disk. Transfer 10 mL 2% sediment suspension in sucrose soln, **952.21A(d)**, using large-tip, graduated pipet, to 10 gal. clean filtered $H_2O$ in clean milk can. After thoroly agitating mixt., remove 1 pt with clean pt measure and filter thru $1^1/_8$" diam. area of sediment disk, **952.21A(b)**, mounted on suitable funnel of correct size, *e.g.*, **952.21A(c)**(*1*). After thoro agitation of contents of milk can, again remove pt sample with the sediment testing device and pass thru sediment disk in exactly same manner as for testing milk. Repeat this operation with the tester several times to det. whether all disks so obtained give same sediment as disk obtained by filtering thru funnel, **952.21A(c)**(*1*).

### C. Collection of Sample

(**a**) *Mixed sample method.*—For retail containers, 5 to 10 gal. cans, and storage tanks, use 1 pt or 1 gal. samples. Before mixing milk, transfer with small strainer any floating extraneous matter, such as flies, hairs, large chunks of debris, etc., to mounted disk, **952.21D(a)**, or mount on sep. disk, properly identified. Thoroly mix milk in container before removing test portion. Avoid contamination of sample with foreign matter on stirrers or by any other means. For retail containers take 1 pt from mixed container or composite sufficient number to make 1 gal. Proceed as in **952.21D(a)**.

(**b**) *Off-bottom method.*—For 5 to 10 gal. cans take pt sample with either type of off-bottom tester from unstirred can of milk. Before withdrawing sample, remove with small strainer any floating extraneous matter as in (**a**). Take sample ≤$^1/_4$" off bottom of unstirred can of milk by inserting sampler and, during upstroke of plunger, drawing head of instrument once across diam. of can bottom or around circumference if can has high center. Expel milk with gun in can and then with short stroke remove excess fluid from pad. Proceed as in **952.21D(b)**.

### D. Determination

(**a**) *Mixed samples.*—Pass sample thru properly adjusted disk, **952.21A(b)**, held in correct position in tester. Warm 1 pt sample to 90–100°F and filter thru restricted area 0.40" diam., **952.21A(a)**(*1*). If single-unit off-bottom tester with special head is used, warm sample larger than 1 pt to 90–100°F and withdraw 1 pt with tester while stirring, or draw 1 pt into tester and warm milk by holding tester under running hot $H_2O$ before discharging milk thru disk.

Warm 1 gal. sample to 80–90°F or filter cold thru $1^1/_8$" diam. area of disk, **952.21A(b)**. If milk is filtered at temp. <90°F, rinse disk by filtering ca $^1/_2$ pt sediment-free warm (90–100°F) $H_2O$ thru disk before removing from tester. If milk is to be salvaged, do not dil. with $H_2O$. (Milk varies in its rate of flow thru disks; pasteurized milk may be more difficult to filter than raw milk. Other factors influencing rate of flow are temp., fat content, degree of clumping of fat globules, stage of lactation, presence of mastitic milk, and amt of sediment in sample.)

Remove disk from tester and mount on special sized paper or store in individual transparent waxed envelope. (If disk is placed on paper or in envelope while still moist, drying milk acts as adhesive.) Grade by comparison with std disks, **952.21A(d)** or **(e)**, and indicate on report whether pad was graded wet or dry. (Character of sediment may be detd by microscopic examination.)

To prevent decomposition on storage, disk may be sprayed with HCHO soln or alc. menthol-thymol soln as in **952.21A(d)**. Do not use glue to affix disk to paper; if disk becomes detached, moisten with few drops $H_2O$ and remount. Protect from contamination.

**(b)** *Off-bottom samples.*—Remove disk from tester, **952.21C(b)**, and proceed as in **(a)**, third par., beginning ". . . mount on special sized paper . . ."

Refs.: JAOAC **35**, 340(1952); **36**, 310(1953); **37**, 117(1954); **48**, 559(1965). Am. J. Public Health **37**, 728(1947).

## 948.27 Sediment in Dairy Products

### Sediment Test Method

### Final Action 1988

**(a)** *Rapid method for sweet cream and cream in which curd is easy to disperse and in absence of mold.*—Place 1 pt (500 mL) sample in beaker or pan of convenient size, ca 2 L, and add ca 500 mL hot $H_2O$ (70–90°). More or less $H_2O$ may be added so that mixt. when ready for filtration is at 45–60°. Remove whole flies or other large filth particles which float to surface and which would be broken up by stirrer. Place these on sediment pad when completed. Place pan under malted milk stirrer, and add, while stirring, 25 mL 40% Na hexametaphosphate soln, if necessary, add more Na hexametaphosphate soln to make mixt. alk. to litmus. Stir 30–60 sec or until curd is broken up. Filter with vac. thru std sediment disk, **952.21A(b)**. If pad clogs, filter remaining portion thru fresh disk. Rinse pan and funnel with hot $H_2O$ onto sediment disk.

**(b)** *Other dairy products.*—Proceed as in **960.49C** or **D**, and filter thru std sediment disk, **952.21A(b)**. Violent mech. agitation, such as is provided by malted milk stirrer, may be used to facilitate dispersion of product.

Compare with std sediment disks, **952.21A(d)**, **(e)**(*1*), or **(f)**.

Ref.: JAOAC **31**, 93(1948).

## 960.49 Filth in Dairy Products

### Filtration Methods

### First Action 1960

Use following methods independently or in various combinations. Weigh 225 g, except in **960.49F**, into suitable container and use S&S ruled No. 8 paper for filtration. Cut hard cheese into small pieces.

#### *A. Evaporated Milk, Condensed Milk, Sweet Cream, Spray-Dried Whole or Skim Milk*

Reconstitute dried or concd products. Dil. reconstituted product with equal vol. hot $H_2O$, hot 3% $Na_2C_2O_4$ soln, or hot 2% $Na_2CO_3$ soln, and filter with suction. During filtration, continually wash paper with stream of near boiling $H_2O$ to prevent accumulation of layer of particles which clogs paper. Examine paper microscopically.

#### *B. Butter*

Place container in $H_2O$ bath or oven at ca 80°. When fat seps, filter directly thru paper with suction, retaining most of curd and $H_2O$ in container. After fat passes thru, filter remaining material. To facilitate filtration of curd, wash paper with near boiling $H_2O$ during filtration. (For butter not filterable by this process, use **960.49C**.) Examine paper microscopically.

#### *C. Soft and Semi-Soft Cheese and Sour Cream; Some Dried Whole and Skim Milks; and Butter That Cannot Be Filtered by 960.49B*

Cut 225 g cheese into 6 mm cubes and add to 800–1000 mL boiling $H_3PO_4$ (1 + 40) in 1.5–2 L beaker, stirring continuously with slow speed mech. stirrer, **945.75B(e)**, or on mag. stirrer-hot plate, **945.75B(n)**, with stirring bar ca 75 × 12 mm, until sample is dispersed (usually >20 min). Filter, without letting mixt. accumulate on paper, and continually wash filter with stream of near boiling $H_2O$ to prevent clogging. When filtration is impeded, add $H_2O$, dil. (1–5%) alkali, $H_3PO_4$ (1 + 40), or hot alcohol, until paper clears; then resume addn of sample suspension and $H_2O$. Examine paper microscopically.

#### *D. Hard Cheeses, Hard Skim, Part Skim Milk Cheeses (Romano, Ricotta, Feta, Pecorino, Sardo, Goats' Milk Cheeses, Sbrinz, Goya, Whey Cheeses, etc.)*

(Not applicable to cheese contg herbs, spices, or molds thruout)

Prep. cheese for sampling by trimming and discarding thin layers to remove all "old" cut surfaces and to keep paraffin coating and mold out of sample. Cut and break up 225 g trimmed cheese into 4 L beaker. Add ca 700 mL ca 55° filtered $H_2O$. Set beaker under mech. stirrer, **945.75B(e)**, and stir 15 min, maintaining mixt. at 55°. Add 100 mL 20% aq. $Na_4EDTA$ soln, stir, and adjust mixt. to pH 8 with $NH_4OH$ or dil. HCl (1 + 2). Rinse sides of beaker free of adhering cheese particles with ca 60° $H_2O$. Maintain pH 8 by addn of $NH_4OH$ and keep adding ca 60° $H_2O$ to dil. cheese mixt. to ca 3 L. If foaming occurs, place wet vegetable parchment paper, 27 lb wt, formerly Patapar paper (available from James River Corp., KVP Group, 100 Island Ave, Parchment, MI 49004), or Parafilm M (Cat. No. 13-374, Fisher Scientific Co.), split to accommodate stirrer blades, over top of beaker to break foam. Continue stirring until cheese becomes finely dispersed.

Cool dispersion to 40° and adjust to pH 8 with $NH_4OH$ or HCl (1 + 2). Add 300 mL pancreatin soln, **945.75C(q)**, (except use 600 mL pancreatin soln for ricotta). Let mixt. digest at ≤40° with continued stirring ca 1.5 hr. Maintain pH 8 by addn of $NH_4OH$.

After digestion, place beaker on hot plate and heat to 65–68°, continuing mech. stirring. Adjust to pH 6.0 ± 0.2 with HCl (1 + 2). Carefully adjust stirrer blades close to bottom of beaker to pick up any cheese particles which settle. Continue stirring 15 min or until cheese appears completely solubilized. Rinse inside of beaker, stirrer blades, etc., with ca 65° $H_2O$ and filter thru ruled paper, using ca 65° $H_2O$ and then alcohol to rinse beaker. If filtration becomes slow (e.g., cream cheese), let paper clear, wash with alcohol, and use addnl paper. Mixt. will filter more easily if No. 60 screen (ca 5 cm diam.) is placed under paper and small amt of mixt. is allowed to suck dry before filtering is continued. Examine paper microscopically.

Ref.: JAOAC **50**, 501(1967).

### E. Cheese Containing Mold, Plant Tissues, and Spices

Disperse cheese by **960.49C** or first par. of **960.49D**. Pour thru No. 140 sieve, **945.75B(r)**, washing thoroly with forcible stream of $H_2O$. Transfer material retained on sieve to beaker. Add 200 mL $H_3PO_4$ (1 + 49), boil until lumpy residue dissolves, and pour again thru No. 140 sieve, washing thoroly with forcible stream of hot $H_2O$. Transfer material on sieve with ca 200 mL 60% alcohol to trap flask and cool. Trap off, using heptane, **945.75C(l)**, and $H_2O$, filter, and examine microscopically.

### F. Casein

Weigh 50 g sample into 1 L beaker. Slowly stir in 170 mL 20% $Na_4EDTA$ soln until well mixed with sample. With constant stirring, bring vol. to 1 L with hot tap $H_2O$ (55–70°). Wet sieve on No. 230 sieve, **945.75B(r)**, with forcible spray of hot tap $H_2O$ until foam subsides. Wash sieve retainings into beaker and filter thru ruled filter paper. Examine papers microscopically.

Ref.: JAOAC **53,** 552(1970).

## NUTS AND NUT PRODUCTS

## 968.33 Filth in Shelled Nuts

**Final Action 1988**

(Not applicable to pecans)

### A. Heavy Filth by Sedimentation —First Action 1968

(*Caution:* See safety notes on distillation, flammable solvents, and petroleum ether.)

Weigh 100 g sample into 600 mL beaker. Add ca 350 mL pet ether and boil gently 30 min, adding pet ether to maintain original vol. Decant solv., taking care not to lose any coarse nut tissue, and discard. Add ca 300 mL $CHCl_3$ to beaker and let settle 10–15 min. Pour off floating nutmeats and ca $^2/_3$ of the $CHCl_3$, and discard. Repeat sepn with smaller vols of mixt. of $CHCl_3$ and $CCl_4$ (1 + 1) until residue in beaker is relatively free of nutmeat particles. Transfer residue in beaker to ashless paper and examine for heavy filth. If appreciable amt of sand and soil is present, ignite paper in weighed crucible at ca 500° and weigh.

Ref.: JAOAC **51,** 527(1968).

### B. Light Filth by Flotation —First Action 1970

(*Caution:* See safety notes on flammable solvents, toxic solvents, ethanol, and chloroform.)

*Nutmeats, all sizes, except pecans.*—Weigh 100 g sample into 1.5 L beaker. Add 600 mL $CHCl_3$; boil 15 min. Prep. ≥24 cm paper for 100 mm plate diam. buchner by moistening with $H_2O$ and forming around base of 1 L beaker. Place 7 cm disk of bolting cloth, **945.75B(d)**, (mesh size not critical) in buchner, insert paper, apply vac., and press moistened paper until good seal is obtained. Rinse paper with isopropanol. Quant. transfer nutmeats and $CHCl_3$ onto previously prepd paper. Maintain suction on nutmeats in buchner 5 min after visible dripping ceases. Release vac., add isopropanol until nutmeats are covered, let stand few min, and reapply vac. until dripping ceases. Repeat isopropanol wash step and aspirate 5 min after visible dripping ceases. Quant. transfer nutmeats on paper to 2 L trap flask, **945.75B(h)**(*4*). Scrape all fines from paper with spatula and finally rinse paper clean with 60% alcohol-$CaCl_2$ soln, **945.75C(c)**. Bring vol. to 1 L with 60% alcohol-$CaCl_2$ soln and add 50 mL HCl. Add mag. stirring bar, **945.75B(n)**, to flask, place flask on mag. stirring hot plate, and heat to *full boil* with gentle stirring. Immediately transfer flask to cool stirring unit and add 40 mL mineral oil, **945.75C(p)**, by pouring down stirring rod. Stir mag., **970.66B(c)**, 2 min.

Fill with 60% alcohol-$CaCl_2$ soln and gently stir 5–10 sec with stirring rod. Let stand 2 min and trap off. Add 25 mL mineral oil, hand stir gently 30 sec, and let stand 10 min. Repeat trapping. Wash flask neck thoroly with isopropanol, and transfer washings to beaker with trappings. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **53,** 553(1970).

## 968.34 Filth in Pecans

**Final Action 1988**

### A. Light Filth —First Action 1968

(*Caution:* See safety notes on toxic solvents and chloroform.)

Form cup, using 32 cm paper with 1–1.5 L beaker, **945.75B(j)**. Weigh 100 g sample into filter paper cup and place in 1.5 L beaker. Add 400 mL $CHCl_3$ and boil 5 min. After cooling few min, lift paper and drain. Repeat 5 min boil and drain with two addnl 400 mL portions $CHCl_3$. Proceed as in **968.33B**, beginning "Place 7 cm disk of bolting cloth, . . ."

Ref.: JAOAC **51,** 527(1968).

### B. Curculio Larvae in Pecan Pieces —First Action 1970

Weigh 115 g (ca $^1/_4$ lb) sample into 1.5 L beaker and add mag. stirring bar, **945.75B(n)**. Add 300 mL undild isopropanol and stir on mag. stirrer 5–10 sec. Add $H_2O$ (200 mL for midget pieces and 300 mL for small, small medium, medium, and mixed pieces) and stir 5–10 sec on stirrer. After few sec, gently agitate settled nutmeats with stirring rod to release any entrapped curculio. Remove all floating material and examine for curculio larvae. Reclaim flotation soln by pouring thru No. 12 sieve and use for one addnl sample.

Ref.: JAOAC **53,** 550(1970).

## 978.19 Filth in Coconut (Shredded)

**First Action 1978**
**Final Action 1988**

(**a**) *Heavy filth.*—Proceed as in **941.16A**, using 100 g sample in 800 mL beaker.

(**b**) *Light filth.*—Weigh 100 g sample into 1.5 or 2.0 L beaker. Add 1 L detergent soln, **968.35E**. Heat in steam bath 10 min, stirring immediately and after ca 5 min.

Pour entire sample onto 8″ No. 230 sieve, rinse beaker with hot $H_2O$, and add rinse to sieve. Wash sieve with forcible stream of hot $H_2O$ until all foam is gone; then rinse well with 40% isopropanol and let drain. Place wide stem funnel in 2 L trap flask, and transfer bulk of sample to flask with spoon. Rinse remaining material to edge of sieve with aerator spray, and transfer quant. to trap flask with 40% isopropanol. Dil. to 1 L with 40% isopropanol, add mag. stirring bar, **945.75B(n)**, and place on mag. stirrer-hot plate, **945.75B(n)**. Add 40 mL mineral oil, **945.75C(p)**, and stir vigorously 1 min. Turn stirrer to slow rate, add 50 mL HCl, and heat to vigorous boil.

(*Caution:* Soln may froth violently upon reaching bp with high heat input.)

Place sample flask on cool stirrer and stir mag., **970.66B(c)**, 3 min. Let stand 2 min; then slowly fill flask with 40% isopropanol added down stirring rod to bring oil interface 1 cm above fully raised stopper or wafer. Lower stopper to midpoint of flask, clamp, and let stand undisturbed 2 min. Trap off into beaker, rinsing neck of trap flask with 40% isopropanol. Add 25 mL flotation liq. **945.75C(k)**, and stir vigorously by hand 1 min. Adjust oil level as above, and let stand undisturbed 10 min. Trap off into second beaker, rinsing neck of flask well with isopropanol.

Filter each trapped off layer onto sep. identified papers, rinsing beakers with isopropanol, and examine papers microscopically at 30×.

Ref.: JAOAC **61,** 898(1978).

## 968.35 Filth and Extraneous Material in Peanut Butter

### Sedimentation/Flotation Methods

### Final Action 1988

***A. Preparation of Sample⋆***
***—Surplus 1970***

*See* **40.031**, 11th ed.

***B. Water-Insoluble Inorganic Residue ("WIIR") and Excreta⋆***
***—Surplus 1970***

*See* **40.032**, 11th ed.

***C. Rocks and Decomposed Peanuts⋆***
***—Surplus 1970***

*See* **40.035**, 11th ed.

***D. Glass⋆***
***—Procedure 1960***
***—Surplus 1970***

*See* **40.036**, 11th ed.

### *Light Filth*

### *First Action 1968*

***E. Reagent***

*Detergent soln*—Dissolve sep. 20 g USP Na lauryl sulfate and 10 g tech. $Na_2B_4O_7.10H_2O$ in $H_2O$, combine, and dil. to 1 L.

***F. Determination***

Weigh 100 g sample into 1.5 L beaker and heat on steam bath until softened. Add 1 L filtered hot detergent soln, and stir well. Heat 10 min *in* steam bath. Stir well, pour portionwise onto No. 230 sieve, **945.75B(r)**, and wash with forcible stream of 55–70° tap $H_2O$, using aerator, **945.75B(a)**. When foam is gone, transfer material on sieve to 2 L trap flask, **945.75B(h)**(*4*), with 55% alcohol (or 40% isopropanol) and bring vol. to 1 L. Add 50 mL HCl. Lower mag. stirring bar into flask on stirring rod stopper. Heat to bp and boil 10 min while slowly stirring on mag. stirring hot plate **945.75B(n)**.

Transfer flask to unheated stirring unit and immediately add 40 mL mineral oil, **945.75C(p)**, by pouring down stirring rod. Stir mag. 2 min. Fill with deaerated 55% alcohol (or 40% isopropanol) and gently stir 5–10 sec with stoppered rod. Let stand 5 min. Trap off. Add 25 mL mineral oil, stir by hand gently 30 sec, and let stand 5 min. Repeat trapping. Wash flask neck thoroly with isopropanol. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **51,** 531(1968).

# GRAINS AND THEIR PRODUCTS

## 950.86 Light Filth (External) in Grains and Seeds

### Flotation Method

### Procedure

Transfer 225 g sample to 2 L trap flask, **945.75B(h)**(*4*). Add 600 mL 40% alcohol and boil gently, with frequent stirring, 5 min. Cool, trap off, using heptane, **945.75C(l)**, and 40% alcohol, filter, and examine microscopically.

## 982.31 Insect Infestation (Internal) of Wheat

### Cracking Flotation Method

### First Action 1982
### Final Action 1988

***A. Preparation of Sample***

Mix grain by passing 6 times thru Jones sampler, recombining sepns before each pass. Sep. slightly >50 g and weigh 50 g. Transfer weighed sample, small amt at a time, to 5 or 8 in. No. 12 sieve, and with stiff bristle brush, work insects thru sieve as completely as possible.

Grind screened sample in cutting-type mill set at 0.061 in. (see **985.36B**). Dry damp or tempered grain in forced-draft oven 1 h at 70–80° or 2 h in oven without draft.

***B. Isolation***

Transfer cracked grain, including any residue in mill, to 2 L glass beaker contg mag. stirring bar. **945.75B(n)**, and mixt. of 600 mL $H_2O$ + 50 mL HCl. Stir *gently* while boiling 15 min on hot plate.

Transfer sample to No. 100 sieve, **945.75B(r)**, with gentle stream of hot tap $H_2O$. Wash material on sieve with *very gentle* stream of hot (55–70°) tap $H_2O$ until washings show no acidity when tested with blue litmus paper.

Add mag. stirring bar, **945.75B(n)**, to 2 L trap flask, **945.75B(h)**(*4*). Place wide-stem funnel in flask opening and quant. transfer residue on sieve to flask with 40% isopropanol. Add 40% isopropanol to total vol. of 800 mL.

Clamp stirring rod so stopper or wafer is above liq. in flask. (Trap flask may stand overnight at this point.) Stir *gently* while boiling 7 min ± 10 s on mag. stirring hot plate. Remove flask from hot plate and wash down sides with min. of 40% isopropanol and immediately add 100 mL 1 + 1 mixt. Tween-80 and 40% isopropanol soln, **945.75C(x)**, and $Na_4EDTA$ and 40% isopropanol soln, **945.75C(z)**, slowly down rod. Hand-stir *gently* 1 min and let stand 3 min.

Add 50 mL mineral oil, **945.75C(p)**, down stirring rod. Stir mag. **970.66B(c)**, 5 min on cool mag. stirrer, and let stand 3 min.

Fill flask with 40% isopropanol, added slowly down stirring rod to avoid mixing or agitation of flask contents, and let stand 20 min *undisturbed*. Trap off, rinsing neck of flask with 40% isopropanol, and add rinse to trappings in beaker.

Add 35 mL mineral oil to flask and hand-stir 1 min. Clamp stirring rod so stopper or wafer is at midpoint of flask. Let stand 5 min, spin stirring rod to free settlings from stopper or wafer, and adjust oil level with 40% isopropanol to ca 1 cm above fully raised stopper. Let stand *undisturbed* 15 min. Trap off, and combine trappings in beaker. Rinse neck of flask well with isopropanol, adding rinsings to beaker. Filter trappings on ruled filter paper, rinsing beaker well with isopropanol. Examine papers at 15×, counting only whole or equiv. insects and cast skins.

Ref.: JAOAC **64,** 1408(1981).

## 985.36 Insect Infestation (Internal) of Oats

### Cracking Flotation Method

### First Action 1985

#### *A. Reagent*

*Tween 80–$Na_4$EDTA [(ethylenedinitrilo)-tetraacetic acid tetrasodium salt] premix soln.*—Measure 420 mL 40% isopropanol in 500 mL graduate. Add 80 mL Tween 80 (polysorbate 80) to 100 mL g-s graduate. Invert 100 mL graduate over 2 L glass beaker and drain briefly. Rinse 100 mL graduate with several portions of the 420 mL 40% isopropanol, pouring each rinse into beaker. Add rest of 40% isopropanol to beaker, add mag. stirring bar, **945.75B(n)**, and start mag. stirring. Add 10 g $Na_4$EDTA to beaker while stirring rapidly. Add 500 mL 40% isopropanol and stir until uniform. Mixed reagent stored in g-s flask is stable 1 week.

#### *B. Preparation of Sample*

Mix grain by passing 6 times thru Jones sampler, recombining sepns before each pass. Sep. slightly >25 g and weigh 25 g. Transfer weighed sample, small amt at a time, to 5 or 8 in. No. 12 sieve, and with stiff bristle brush, work insects thru sieve as completely as possible.

Grind screened sample in cutting-type mill set at 0.061 in. (Elec. coffee grinder can be used.) Particle size of cracked oats, ca 0.061 in., can be checked by passing the 25 g cracked oats thru No. 14 sieve, **945.75B(r)**. Amt of cracked oats retained on sieve should range from 75 to 80% by wt and amt passing thru sieve should range from 20 to 25% by wt. When using Labconco mill, rotate blade adjusting wheel counterclockwise until cutting blades are touching. Then rotate adjusting wheel clockwise $^3/_4$ revolution. This setting should give cracked oats within particle size range mentioned above. Dry damp or tempered grain in forced-draft oven 1 h at 70–80° or 2 h in oven without draft.

#### *C. Isolation*

Transfer cracked grain, including any residue in mill, to 2 L glass beaker contg mag. stirring bar, **945.75B(n)**, and add 600 mL isopropanol. Cover beaker and stir *gently* while boiling 5 min on hot plate.

Transfer sample to No. 100 sieve, **945.75B(r)**, with *gentle* stream of hot tap $H_2O$. Wash material on sieve with *very gentle* stream of hot (55–70°) tap $H_2O$ to remove isopropanol. Quant. transfer material on sieve to original 2 L beaker with $H_2O$. Add mag. stirring bar, **945.75B(n)**, and mixt. of 600 mL $H_2O$ + 50 mL HCl. Cover beaker and mag. stir contents *gently* while boiling 15 min on hot plate. Transfer material to No. 100 sieve, **945.75B(r)**, with *gentle* stream of hot tap $H_2O$. Wash material on sieve with *very gentle* stream of hot (55–70°) tap $H_2O$ until washings show no acidity when tested with blue litmus paper.

Add mag. stirring bar, **945.75B(n)**, to 2 L trap flask, **945.75B(h)(4)**. Place wide-stem funnel in flask opening and quant. transfer material on sieve to flask with 40% isopropanol. Add 40% isopropanol to total vol. of 800 mL.

Clamp stirring rod so stopper or wafer is above liq. in flask. (Trap flask may stand overnight at this point.) Stir *gently* while boiling 7 min ±10 s on mag. stirring hot plate. Remove flask from hot plate and wash down sides with min. of 40% isopropanol and immediately add 100 mL Tween 80–$Na_4$EDTA soln slowly down rod. Hand-stir *gently* 1 min and let stand 3 min.

Add 50 mL mineral oil, **945.75C(p)**, down stirring rod. Stir mag., **970.66B(c)**, 5 min on cool mag. stirrer, and let stand 3 min.

Fill flask with 40% isopropanol, added slowly down stirring rod to avoid mixing or agitation of flask contents, and let stand 20 min *undisturbed*. Trap off into beaker, rinsing neck of flask with 40% isopropanol, and add rinse to trapping in beaker.

Transfer trapping to ruled filter paper, rinsing beaker well with isopropanol. Add 35 mL mineral oil, **945.75C(p)**, to flask and hand-stir 1 min. Clamp stirring rod so stopper or wafer is at midpoint of flask. Let stand 5 min, spin stirring rod to free settlings from stopper or wafer, and adjust oil level with 40% isopropanol to ca 1 cm above fully raised stopper. Let stand *undisturbed* 15 min. Trap off into beaker, rinsing neck of flask well with isopropanol and adding rinsings to trapping in beaker. Transfer trapping to ruled filter paper, rinsing beaker well with isopropanol. Examine papers at 15 ×, counting only whole or equiv. insects and cast skins.

Ref.: JAOAC **68**, 699(1985).

## 955.42 Insect Infestation (Internal) of Grains and Seeds (Except Wheat and Oats)

### Cracking Flotation Method

### First Action

Mix grain by passing 6 times thru Jones sampler, recombining sepns before each pass. Sep. slightly >100 g and weigh 100 g. Transfer weighed sample, small amt at time, to 5″ or 8″ No. 12 sieve, and with stiff bristle brush, work insects thru sieve as completely as possible.

Grind screened sample in cutting-type mill set at 0.061″. (Dry damp or tempered grain in forced-draft oven 1 hr at 70–80° or 2 hr in oven without draft.) Transfer cracked grain, including any residue in mill, to 2 L trap flask, **945.75B(h)(*4*)**, trap as in **970.66B(b)**, using 60% isopropanol satd with heptane, **945.75C(n)**, and heptane, **945.75C(l)**, as solvs, and filter on 10XX bolting cloth, **945.75B(d)**. If considerable starchy material is in ext, hydrolyze with HCl as in **970.66B(d)**. Examine as in **970.66B(g)** except use 15× as lower limit of magnification. Count only whole and equivalent insects and cast skins.

## 955.43 Foreign Matter in Brewer's Grits

#### *A. Rodent Excreta—Procedure*

*See* **941.16A**.

#### *B. Light Filth—First Action*

*See* **941.16B**.

## 971.31 Light Filth in Cracked Wheat and Flours

### Flotation Methods

### First Action 1971

### Final Action 1973

(Applicable to rye, pumpernickel, and buckwheat flours)

(*Caution*: See safety notes on distillation, toxic solvents, and chloroform.)

Weigh 25 g sample into 94 × 33 mm Soxhlet thimble and cover with pad of glass wool. Add ca 300 mL $CHCl_3$ and 3–4 glass beads to 500 mL Soxhlet extn flask. Ext in Soxhlet extractor at medium rate ca 90 min (counting time from first overflow and siphoning ca every 5 min). Place extn thimble in 250 mL beaker and dry with air on steam bath until no $CHCl_3$ odor remains.

Quant. transfer contents of thimble and any material adhering to glass wool, with spatula and acid-alcohol rinses, **945.75C(a)**, to 1 L trap flask for trap flask method, **(a)**, or to 1–1.5 L beaker for percolator method, **(b)**.

Place trap flask or beaker on mag. stirrer, add stirring bar, and slowly add acid-alcohol soln, stirring constantly to form smooth slurry. Dil. to ca 600 mL with acid-alcohol soln and add ca 40 mL mineral oil, **945.75C(p)**. Mag. stir mixt. 10 min, **970.66B(c)**. Continue as in **(a)** or **(b)**.

**(a)** *Trap flask method*.—At end of stirring period, fill flask with acid-alcohol soln and let stand 30 min; stir gently every 3–4 min for first 25 min. Trap off, rinsing neck of flask with acid-alcohol soln. Add ca 30 mL mineral oil and stir mag. ca 5 min. Let stand 30 min, stirring and trapping as above. Combine trappings in beaker and transfer quant. to Kilborn funnel, **945.75B(h)**(*1*) or percolator (*2*), contg ca 125 mL $H_2O$. Retain beaker. Add tap $H_2O$ to ca 1 cm from top of funnel. Let oil layer sep. 5–10 min and drain until interface is ca 5 cm above constriction, discarding lower aq. layer. Repeat $H_2O$ washes until lower phase is clear. Drain interface-oil layer into retained beaker, washing sides of funnel with ca 50–100 mL $H_2O$. If product residue is present in retained beaker contents, add ca 10 mL HCl. Boil ca 5 min on hot plate and filter thru ruled paper. After mineral oil layer has passed thru paper, rinse all glassware used (except trap flask) with alcohol followed by $H_2O$, then 5% detergent soln, **945.75C(i)**, and finally with $H_2O$. Filter rinses thru original filter paper. Examine microscopically.

**(b)** *Corning percolator method*.—Place stirring rod in drain opening inside percolator, **945.75B(h)**(*2*). Add ca 250 mL $H_2O$ to percolator. Quant. transfer beaker contents to percolator. Add acid-alcohol soln to ca 6 cm from top. Let stand ca 30 min; gently stir contents with stirring rod every 3–4 min during first 25 min in such manner as to prevent product dropping into drain opening. After 30 min, raise stirring rod and drain, discarding lower layer but leaving ca 250 mL in percolator. Rinse stirring rod with acid-alcohol soln and add rinsings to percolator contents. Retain stirring rod for further rinsings.

Add acid-alcohol soln to percolator to ca 6 cm from top and let stand ca 30 min, stirring as before. Drain and discard lower layer as before to 250 mL. Add $H_2O$ to ca 6 cm from top and proceed as in **(a)**, beginning "Let oil layer sep. 5–10 min . . ."

Refs.: JAOAC **53**, 723(1970); **54**, 903(1971).

## 981.19 Light Filth in Corn Meal (White and Yellow)

### Brine Flotation Method

### First Action 1981

Weigh 50 g sample into 600 mL beaker, add stirring bar [**945.75B(n)**], and 300–400 mL alcohol. Boil 2–3 min with const stirring on mag. stirrer plate [**945.75B(n)**]. Transfer to 230 mesh sieve [**945.75B(r)**], wash with ca 100 mL alcohol, then with hot tap $H_2O$, $\leq 80°$, and then with brine [prepd by dissolving ca 360 g rock salt, $H_2O$-softener salt, ice cream salt, or equiv. per L tap $H_2O$ and filtering]. Transfer to 2 L trap flask [**945.75B(h)**(*4*)] and dil. to 800 mL with brine. Add 30 mL olive oil and mag. stir [**970.66B(c)**] 10 min. Remove from stirrer, insert trap rod, wash insides of flask with brine, and wait 3–5 min. Fill flask very slowly, ca 50 mL/min, with tap $H_2O$ down trap rod. Percolator method given below is most efficient way to do this:

Set trap flask on base of ring stand and suspend percolator [**945.75(h)**(*2*)] in ring directly above so that tip is ca 20 in. above base. Attach 6 in. piece of 1/4 in. id rubber tubing to tip and close with pinch cock capable of adjusting flow. Insert rod of trap flask into tubing and, after filling percolator with $H_2O$, open pinch cock only slightly and let $H_2O$ flow down trap rod into flask. By inserting 6 in. piece of 1/8 in. rod or wire (spent refill of ballpoint pen works well) into tubing along with trap rod, tendency for stream of $H_2O$ to spin off trap rod is eliminated.

After filling flask, stir bottom brine layer with rotary motion to release any trapped oil. Do not stir entire contents of flask, because it is desirable at this point to maintain integrity of the 2 layers, brine/$H_2O$. Let flask remain still 30 min before trapping. Trap oil layer and rinse neck of flask with $H_2O$. Add 35 mL olive oil and stir in. Repeat trapping but, at this time, hand-stir entire flask contents with plunger with rotary motion to release any oil adhering to flask sides. Wait 30 min, trap into beaker contg first trapping, and rinse neck of flask, rod, and plunger with alcohol, then $CHCl_3$ to remove all adhering oil. Filter on ruled paper, rinsing beaker as above, and examine microscopically.

Ref.: JAOAC **64**, 191(1981).

## 986.28 Mammalian Feces in Corn Meal

### Alkaline Phosphatase Detection Method

### First Action 1986

#### *A. Principle*

Intestinal tract of most mammals contains alk. phosphatase enzyme. Enzyme at test pH and temp. splits phosphate radicals from substrate/pH indicator phthln diphosphate to produce light pink to red-purple color from free phthln.

#### *B. Apparatus*

**(a)** *Hot water bath*.—Maintained at 42 ± 1°.

**(b)** *Hot plate stirrer and 41 mm ovoid stirring bar*.—Fisher 1451158A or equiv.

**(c)** *Petri dishes*.—Plastic disposable, 150 × 20 mm or 150 × 15 mm (Falcon 1058, available from BBL Microbiology Systems, or plastic/glass equiv.).

**(d)** *Weighing boats*.—8.1 × 8.1 × 1.9 cm, 100 mL capacity (Fisher Scientific Co., Cat. No. 02210B, or approx. size equiv.).

#### *C. Reagents*

**(a)** *Magnesium chloride soln*.—Dissolve 0.203 g $MgCl_2.6H_2O$ and dil. to 500 mL with $H_2O$. Indefinite shelf life.

**(b)** *Stock test reagent*.—To prep. soln, dissolve 19.0 g borax ($NaB_4O_7.10H_2O$) and 6.28 g anhyd. $Na_2CO_3$ in 1 L $H_2O$ with stirring. Add 0.94 g phthln diphosphate and stir while adding 2 mL $MgCl_2$ soln. Prepn is stable ca 4 months at room temp. Soln should be colorless and ca pH 9.5. Discard if not colorless. Degraded phthln diphosphate produces pink color in reagent. Store phthln diphosphate in desiccator below 0°. (Phthln diphosphate, Sigma Chemical Co., No. P 9875.)

**(c)** *Liquid test agar*.—Prep. fresh before using, 150 mL per 10 g sample to be analyzed. Measure equal vol. of stock test reagent, **(b)** (half of total test agar vol. needed), and $H_2O$ into sep. appropriate size beakers. Beaker for $H_2O$ must be large enough to accommodate 2 times vol. of $H_2O$. Reserve stock test reagent. Place beaker of $H_2O$ on hot plate stirrer, add stirring bar (ovoid 41 mm), and, with rapid stirring, add sufficient agar to $H_2O$ to yield 2% agar soln (1.5 g agar/75 mL $H_2O$). Continue stirring, and heat to boil (watch for foam-over). Cover beaker with cover glass to prevent heat loss. When agar begins to foam, add reserved stock test reagent, pouring reagent down side of beaker to prevent agar from coming out of soln. Stir rapidly with heat ca 1 min.

### D. Determination

Weigh 10 g corn meal into weighing boat from each well mixed subsample. Prep. appropriate amt of liq. test agar, (**c**). Cool boiled test agar by placing beaker of test agar into larger beaker of cold $H_2O$. Continually *stir* test agar and *maintain* temp. check until soln is 55°. Pour test agar into petri dish, ca 150 mL per dish. Immediately distribute monolayer of corn meal onto surface of test agar. This is accomplished by gently tapping weighing boat held so that corn meal flows over one side, not from corner, while tilting and moving boat above agar surface as corn meal flows. Let corn meal become wet with test agar and sink before adding another layer. Continue in this manner until entire 10 g sample has been added. Distribution time should be ca 1 min per 10 g corn meal sample. Best sepn of corn meal and excreta occurs while test agar is hot. Multiple samples can be added to resp. dishes, one at a time or a little of each sample to its resp. dish sequentially, until all of each sample has been distributed.

Let test agar gel (requires ≥20 min). Agar is gelled when no agar flows when dish is slightly tipped. (*Note:* Take care not to disturb dispersed material in liq. test agar. If particles are moved, color concn around particles will be diffused and pos. spots will be missed.) When gelling is complete, check for pink spots, viewing plate against white background. Mark spots on lid of dish, using grease pencil. Mark lid and bottom of dish, using $H_2O$-proof marker, so that lid can always be placed in same position.

Incubate petri dish at 42° in $H_2O$ bath 10 min. Submerge plate in $H_2O$ bath just enough to cover agar level in dish. When incubating several dishes at one time, place plates in $H_2O$ in pairs, staggering times so that reading delays are avoided, and small, rapidly diffusing pink spots are not missed. Remove plate from $H_2O$ bath after 10 min. Wipe inside lid to remove fog and hold lid so that bottom edge of lid is 2–3 mm above top edge of petri dish base while reading plate. Replace lid and repeat 10-min incubation 2 times, marking addnl pink spots on the petri dish lid after each period. Tally and record number of spots as fecal particles/10 g sample. Spots which appear and then are not seen on subsequent checks and spots which are seen on bottom of petri dish with corn meal are to be counted in tally.

### E. Positive Control for Feces and Test Agar Medium

Scatter some ground known rodent feces on petri dish of liq. test agar in place of corn meal sample and continue with method. One control plate is needed for each batch of test agar prepd.

### F. Response

Amt, intensity, and range of color (light pink to red-purple) observed will vary depending on size of fecal particles, species source, and diet of animal. Particles as small as 250 μm can be identified.

Ref.: JAOAC **69**, 496(1986).

## 941.16 Filth in Grain Products

**First Action**

(Applicable to corn grits, rye and wheat meal, whole wheat flour, farina, semolina)

### A. Rodent Excreta

(*Caution:* See safety notes on toxic solvents, carbon tetrachloride and chloroform.)

Weigh 50 g sample in 250 mL hooked-lip beaker. Add $CHCl_3$ to within ca 1 cm of top, mix thoroly, and let settle ≥30 min, stirring surface layer occasionally. Carefully decant $CHCl_3$ and float tissue onto buchner, without disturbing heavy residue in bottom of beaker. Before decanting, take care that floating layer has not become so compact as to render this operation difficult. Add amt of $CCl_4$ equal to amt of $CHCl_3$ and tissue left in beaker, let settle again, and decant as before. Repeat this process with mixt. of equal parts $CHCl_3$ and $CCl_4$ until very little tissue remains in beaker. Do not decant any rodent excreta fragments that may be present. Wash residue in beaker onto 7 cm ruled paper with stream of $CHCl_3$ or $CCl_4$ and examine microscopically. Retain decanted floating tissues for light filth analysis.

Ref.: Cereal Chem. **18,** 655(1941).

### B. Light Filth

(Not applicable to whole and degerminated corn meal)

Draw air thru material in buchner, **941.16A**, until liq. evaps. Air dry overnight, or dry in oven at ca 80°. (*Caution:* In oven drying, phosgene is liberated and adequate ventilation must be provided.) Transfer residue to 1 L trap flask, **945.75B(h)**(*4*). Add 100 mL 60% isopropanol satd with heptane, **945.75C(n)**, and mix thoroly. Wash down sides of flask with isopropanol-heptane soln until ca 400 mL is added, and soak 30 min. Trap off twice with 20–30 mL heptane, **945.75C(l)**, for each trapping and 60% isopropanol satd with heptane as liq. extn medium. In first trapping, let stand 5 min after stirring in heptane before filling flask. Filter, and examine both trappings microscopically.

## 970.69 Light Filth in Wheat Germ

**Flotation Method**

**First Action 1970**
**Final Action 1973**

(Applicable to raw or processed wheat germ)

(*Caution:* See safety notes on toxic solvents and chloroform.)

Form paper cup, using 32 cm paper and 250 and 400 mL beakers, **945.75B(j)**. Tare 400 mL beaker and paper, weigh 50 g sample into filter paper in beaker, and add ca 150 mL $CHCl_3$. Boil on steam bath 5 min, occasionally rinsing down sides of filter paper with $CHCl_3$ to maintain original level. Remove from heat. Carefully lift paper contg sample from beaker so as to prevent any loss of sample. Let most of $CHCl_3$ drain into beaker; then discard drainings. Repeat operation 2 addnl times beginning ". . . add ca 150 mL $CHCl_3$." After last $CHCl_3$ defatting, place paper contg sample in buchner. Apply vac. until draining slows to drip. Rinse sides of paper and sample with undild isopropanol and apply vac. until draining has ceased. Turn off vac. Add ca 50–60 mL undild isopropanol to sample. Let stand 2 min; then apply vac. until dripping ceases and sample appears dry.

Transfer sample from paper to 1 L beaker with hot tap $H_2O$ (55–70°). Fold filter paper in half and rub together; then wash with hot tap $H_2O$ into beaker. Repeat several times until paper appears clean. Discard paper and bring vol. of hot $H_2O$ to 600 mL. Add 30 mL HCl and 1 mL antifoam, **945.75C(e)**. Boil on stirrer-hot plate, **945.75B(n)**, 10 min with const stirring; then remove from heat. Pour contents of beaker onto No. 230 sieve, **945.75B(r)**, and wash with forcible hot $H_2O$ spray (55–70°), **945.75B(a)**, until all starchy material has passed thru and only bran remains (color of sample will change from light tan to dark brown). Transfer material from sieve to 2 L trap flask with 55% alcohol or 40% isopropanol, dil. to ca 1 L, and add 50 mL HCl. Heat to 60–70° on hot plate (do not boil), remove flask from heat, and add 50 mL mineral oil, **945.75C(p)**. Stir mag., **970.66B(c)**, 3 min. Fill flask with same dil. alcohol used previously, stir gently by hand 1 min, let flask stand 10 min, and trap off, rinsing neck of flask with same dil. alcohol used previously. Perform second extn, using 25 mL mineral oil. Stir gently by hand 1 min, let stand 15 min, and trap off. Rinse neck of flask with undild isopropanol or alcohol. Filter trappings thru ruled paper and examine microscopically.

Ref.: JAOAC **53,** 560(1970).

## 972.32 Light Filth (Pre- and Post-Milling) in Flour (White)

### Flotation Method

**First Action 1972**
**Final Action 1988**

Digest 50 g flour in 2–2.5 L beaker with ca 600 mL HCl (3 + 97) by autoclaving 5 min at 121°. Immediately transfer digest to 1 L beaker, using HCl (3 + 97) at room temp. to asist in transfer. Add 50 mL mineral oil, **945.75C(p)**, and stir mag. **970.66B(c)**, 5 min. Quant. transfer to Kilborn funnel, **945.75B(h)**(*1*), or percolator, (*2*), retaining beaker. Let stand 30 min, stirring gently with long glass rod several times during first 10 min. Drain lower layer to ca 3 cm of interface, wash sides with cold tap $H_2O$, and let layers sep. ca 2–3 min. Repeat drain and $H_2O$ wash until lower phase is clear. After final wash, drain oil layer into retained beaker, rinsing sides of funnel with $H_2O$ and alcohol. Add HCl to ca 3% (v/v) and boil 3–4 min on hot plate. Filter hot soln thru ruled paper, and thoroly rinse beaker and funnel with $H_2O$, alcohol, and 5% detergent soln, **945.75C(i)**. Filter each rinse sep. thru same paper. Examine microscopically.

Ref.: JAOAC **55,** 514(1972).

## 940.34 Insect Eggs in Flour

### Sieving Method

**First Action**

Transfer 50 g flour to No. 100 sieve (if >ca 0.1 g residue is obtained, No. 60 or No. 80 sieve should be used to prevent slow filtration after digestion) and sift gently until no more flour passes thru. Transfer residue on sieve to 250 mL beaker and wet with 2–3 mL alcohol. Add 30 mL $H_2SO_4$ (1 + 19), cover beaker, and heat on steam bath 10 min. Filter thru paper on suction funnel, using min. suction necessary to filter. Keep beaker partially inverted over funnel and rinse with $H_2O$. Turn off suction. Add 15–20 mL ca 0.1*N* I to paper in funnel. Allow 10–15 sec for I to stain contents. Apply gentle suction. After I passes thru filter, wash paper with 25–30 mL 1% $H_2SO_4$, followed by several small $H_2O$ washes. Transfer paper to petri dish and examine at once under 20× magnification.

Ref.: Food Ind. **12,** 36(1940).

## 943.06 Insect Excreta in Flour

**Final Action 1988**

**(a)** *Optional for 1–4 samples*.—Weigh 0.20 g flour on weighed flat glass disk 7–7.5 cm diam. Add clove oil and spread mixt. into thin uniform layer. (Enough oil should be present to clear flour and present smooth surface of oil, but not so much that mixt. flows off disk.) Place wire grid over disk and examine microscopically with dark background and intense reflected light. Depending upon size of plate, larger amts of flour and ruled glass plate can be used and oil-flour mount covered with glass, *e.g.*, use 0.5 g flour on tomato rot count plate, **945.75B(q)**. Weigh flour in counterbalanced scoop or directly on plate. Thoroly sat. flour on counting plate, cover with glass, and count insect excreta. To move or turn suspected particles, gently apply pressure or move cover slightly while observing thru microscope.

**(b)** *Optional in multiple-sample schedule*.—Tare 2–8 small numbered vials on each balance pan and weigh by shifting weights from one side to other. (If desired, larger portion may be weighed in beaker and some of flour floated off in $CHCl_3$-ether or $CHCl_3$-toluene mixt., sp gr 1.40, before transferring to filter paper.) Rinse contents of each vial onto smooth-surface, ruled paper in Hirsch funnel with $CHCl_3$ or $CCl_4$. Transfer paper to petri dish, flood with clove oil, and examine with dark background and intense reflected light.

Ref.: JAOAC **26,** 257(1943).

## 982.32 Light Filth in Rice Flours (Powders), Extruded Rice Products, and Rice Paper

### Flotation Method

**First Action 1982**
**Final Action 1988**

### A. Sample Preparation

**(a)** *Rice flours (powders)*.—Preheat hot plate to max. heat. Add mag. stirring bar to 2 L beaker, and tare. Add 100 g sample. With forceful stream, add ca 100 mL hot tap $H_2O$. Add 75 mL HCl and fill to 800 mL mark with hot tap $H_2O$. Place hot mixt. on hot plate and, with mag. stirring, bring mixt. to vigorous boil. Boil 5 min. In small increments, transfer hot mixt. to No. 230 sieve. Reserve 2 L beaker. Wash residue with forceful stream of hot tap $H_2O$ until foaming has subsided and $H_2O$ is clear. Transfer residue to reserved 2 L beaker with 40% isopropanol. Add mag. stirring bar. Fill with 40% isopropanol to 800 mL mark. With mag. stirring, bring to boil on hot plate. Add 95 mL mineral oil, **945.75C(p)**, and boil and stir 3 min.

**(b)** *Extruded rice products and rice paper*.—Preheat hot plate to max. heat. Add mag. stirring bar to a 2 L beaker, and tare. Break up 225 g extruded product or paper while weighing into tared beaker. Add ca 1450 mL hot tap $H_2O$ mixed with 150 mL HCl. Hand-stir with glass rod while heating to vigorous boil. When product is fluid enough, use mag. stirring. Boil 10 min. In small increments, transfer hot mixt. to No. 230 sieve. Reserve 2 L beaker. Wash residue with forceful stream of hot tap $H_2O$ until foaming has subsided and $H_2O$ is clear. Transfer residue to reserved 2 L beaker with 40% isopropanol. Add mag. stirring bar. Fill with 40% isopropanol to 800 mL mark. With mag. stirring, bring to boil on hot plate. Add 95 mL mineral oil, **945.75C(p)**, and boil and stir 3 min.

### B. Filth Analysis

**(a)** *Rice flours (powders)*.—Clamp off rubber hose on percolator, **945.75B(h)**(*2*). Add 300 mL 40% isopropanol. Transfer hot sample mixt. from above to percolator. Rinse 2 L beaker with 40% isopropanol and pour rinse into percolator. With same beaker, add enough 40% isopropanol (ca 1 L) to fill percolator within 3 cm of top. Let stand 5 min and drain contents to 5 cm from bottom of oil layer. Repeat fill and drain steps at 2 min intervals with hot tap $H_2O$ until aq. phase is clear. Drain as above. Drain oil layer into 1 L beaker. Rinse percolator sides with several alternate washes of $H_2O$, 40% isopropanol, and isopropanol, collecting rinsings in same 1 L beaker. A 1% sodium lauryl sulfate soln may also be used if needed for final rinse. Filter onto ruled filter paper and examine microscopically at ca 30×.

(**b**) *Extruded rice products and rice paper*.—Proceed as in (**a**), except for second cycling. Refill percolator with 20% isopropanol. Let stand addnl 5 min. Drain oil layer into 1 L beaker and rinse percolator as above.

Ref.: JAOAC **65**, 1086(1982).

## 965.39 Light Filth in Flour (Corn)

**Final Action 1989**

### *A. Pancreatin Digestion Method*

*Light filth*.—Weigh 50 g flour into 600 mL beaker; stir into smooth slurry with 50 mL pancreatin soln, **945.75C(q)**, dild with 100 mL $H_2O$. Dil. with $H_2O$ to total vol. of ca 400 mL, and adjust to pH 8 with $Na_3PO_4$ soln, **945.75C(u)**. Readjust pH after ca 15 min and again in ca 45 min. Add, with stirring, 3 drops HCHO soln and digest 16–18 hr at room temp. or ≤40°. Transfer to 2 L trap flask and ext as in **970.66B(b)**, using 30 and 20 mL deodorized kerosene, **945.75C(o)**, and $H_2O$ as solvs. Combine trappings and rinsings in beaker, transfer to 2 L trap flask, and trap off as above. If considerable starchy material is in ext, hydrolyze with HCl as in **970.66B(d)**. Examine papers microscopically.

### *B. Acid Hydrolysis Method*

*Light filth*.—Disperse 50 g flour in 1 L beaker with ca 400 mL HCl (5 + 95) and 20 mL mineral oil, **945.75C(p)**. Place on hot plate, bring to rolling boil with stirring, and boil 10 min. Remove from heat and transfer quant. to extn vessel, **945.75B(h)**(*1*) or (*2*). Rinse beaker and rod with ≤50 mL hot $H_2O$, transfer rinsings to extn vessel, and retain beaker and rod. Fill extn vessel with cold $H_2O$ to ca 3 cm from top. Let settle 30 min, and drain carefully without forming vortex, until upper layer is ca 5 cm from bottom. Add 25 mL kerosene, **945.75C(o)**, to extn vessel and drain oil layer into retained beaker. If excessive starchy material has sepd with oil layer, hydrolyze with 100–200 mL HCl (5 + 95) before continuing. Wash sides of extn vessel with 5% detergent soln, **945.75C(i)**, in wash bottle, and collect washings in retained beaker. Filter entire contents of beaker thru ruled paper, **945.75B(i)**, in Hirsch funnel. Rinse beaker with 5% detergent soln, and filter. Examine microscopically at 30×.

### *C. Rodent Excreta*

Proceed as in **941.16A**.

Ref.: JAOAC **48**, 554(1965).

## 972.33 Light Filth in Flour (Soy)

**Flotation Method**

**First Action 1972**
**Final Action 1988**

Weigh 50 g sample into 500 mL beaker and add ca 50 mL isopropanol, with stirring. Add mag. stirring bar and, with stirring, slowly add 300 mL satd NaCl soln at room temp. Stir to thin slurry and transfer in small increments to No. 230 sieve, **945.75B(r)**. Wash residue with forceful stream of cold $H_2O$, using aerator, **945.75B(a)**, until effluent is clear and foaming has subsided. Let residue drain in sieve. Wash residue with isopropanol and let drain. Transfer residue to 1.5 L beaker with 40% isopropanol and dil. to 800 mL with 40% isopropanol. Heat to bp with mag. stirring, and add 50 mL mineral oil, **945.75C(p)**. Stir mag., **970.66B(c)**, 3 min while continuing to boil.

Transfer quant. to 2 L percolator, **945.75B(h)**(*2*). Retain 1.5 L beaker as vessel to fill percolator with $H_2O$ during refill cycles. Fill percolator with 40% isopropanol to within 3 cm of top. Let stand 5 min and drain contents to within 3 cm of bottom of oil layer. Repeat drain and refill steps at 3 min intervals with hot tap $H_2O$ (55–70°) until aq. phase is clear. Drain most of aq. phase and discard. Drain oil layer into 400 mL beaker. Wash percolator with alternate washes of $H_2O$ and isopropanol, and collect washings in 400 mL beaker. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **55**, 60(1972).

## 972.34 Light Filth in Wheat Gluten

**Flotation Method**

**First Action 1972**
**Final Action 1988**

Add 900 mL 40% isopropanol, 100 mL HCl, and mag. stirring bar to 2 L trap flask, **945.75B(h)**(*4*). Using mag. stirrer, without heat, stir at max. speed where no splashing occurs, and slowly add 50 g sample thru long-stem funnel with wide diam. bore to avoid getting sample on stoppered rod or sides of trap flask. Stir 3 min, and then boil 15 min with gentle stirring on mag. stirrer-hot plate. Immediately transfer flask to cool stirring unit and add 40 mL light mineral oil, **945.75C(p)**. Stir mag., **970.66B(c)**, 3 min. Slowly fill flask with 40% isopropanol by letting liq. flow down stoppered rod while top of stopper is maintained just above flask contents. After filling flask, gently stir settled material 5–10 sec with stoppered rod. Let stand undisturbed 5 min and immediately trap off. Add 25 mL light mineral oil, stir gently by hand 30 sec, and let stand 10 min. Repeat trapping. Wash flask neck thoroly with isopropanol and transfer washings to beaker contg trappings. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **55**, 64(1972).

## 972.35 Light Filth in Starch

**Sieving Method**

**First Action 1972**
**Final Action 1988**

Weigh 225 g sample into 2 L beaker, add 1.2 L cold $H_2O$, and stir well to disperse lumps. Pour onto No. 230 sieve, **945.75B(r)**, and wash with forcible stream of cold tap $H_2O$, using aerator, **945.75B(a)**. (If excessive residue remains on sieve, wash into beaker with $H_2O$, add HCl to make to (1 + 9), bring to bp, and repeat sieving.) Quant. transfer sieve retainings to beaker, filter onto ruled paper, and examine microscopically.

Ref.: JAOAC **55**, 62(1972).

# BAKED GOODS

## 972.36 Light Filth in High Bran Content Breads

**Flotation Method**

**First Action**

*Post-milling contamination*.—Add 225 g sample to 2 L beaker contg ca 1 L $H_2O$ and 50 mL HCl. Stir well. Add 1 mL antifoam soln, **945.75C(e)**. Autoclave ca 20 min as in

**945.75B(b)**(*1*) or ca 15 min as in (*2*). Transfer digest in small portions onto No. 140 sieve, **945.75B(r)**, with hot tap $H_2O$ (55–70°) until amt of residue remains const. Place sieve in pan, cover residue to depth of ca 2 cm with alcohol or isopropanol, let stand 5 min, and drain. Repeat this step 3 times with $CHCl_3$, then twice more with alcohol or isopropanol, and drain completely. Promptly transfer sieve retainings quant. to 1 L beaker with acid-alcohol, **945.75C(a)**, dilg contents to ca 600 mL with acid-alcohol. Add 50 mL mineral oil, **945.75C(p)**, and stir mag., **970.66B(c)**, 5 min.

Completely transfer beaker contents to percolator, **945.75B(h)**(*2*), or Kilborn funnel, (*1*), retaining beaker. Let stand 30 min, stirring gently ca every 5 min with long glass stirring rod for first 20 min; drain contents to ca 250 mL. Add acid-alcohol to ca 3 cm of top and let stand 30 min, stirring as before. Again drain to ca 250 mL. Fill funnel with cold tap $H_2O$, let settle ca 1.5 min, and drain to ca 250 mL. Continue drain and refill cycles until lower aq. phase is clear and free of suspended material.

After last wash, drain oil-$H_2O$ interface into retained beaker. Promptly wash sides of percolator with 50–100 mL portions hot tap $H_2O$, isopropanol or alcohol, and 5% detergent soln, **945.75C(i)**, if necessary. Filter thru ruled paper, washing sides of beaker as above, using rubber policeman, if necessary. Examine microscopically.

Ref.: JAOAC **55,** 516(1972).

## 972.37 Light Filth in Baked Goods with Fruit and Nut Tissues

**First Action 1972**

### *A. Pancreatin Digestion Method*

*Post-milling contamination.*—Weigh 225 g sample into 2 L beaker, add enough hot $H_2O$ to soften and sat. material, and proceed as in (**a**). If lumps persist or if $H_2O$ is not immediately absorbed uniformly thru entire mass, proceed as in (**b**).

(**a**) Adjust mixt. to pH 7–8 with ca 5% $Na_3PO_4$ soln. Stir and break up material as much as possible. Cool to 40° and add 100 mL pancreatin soln, **945.75C(q)**. Stir thoroly and readjust to pH 7–8. Let stand 30 min, stir, and readjust pH.

(**b**) Est. vol. of mixt. and add HCl to ca 1 + 49 concn. Boil until solids become finely divided and so digested that mixt. will not froth over when covered during boiling. Neutze to ca pH 6 with NaOH soln; then add $Na_3PO_4$ soln to pH 8 and continue as in (**a**).

For white flour products, add 0.2 mL or 4 drops HCHO and digest overnight. For products made from whole wheat and rye flours and from similar materials of high bran content, digest only 2–3 hr.

Pour digested material thru 5″ or 8″ No. 140 sieve, **945.75B(r)**. While pouring, play forcible stream of hot $H_2O$ from tap on this material. Wash well with large stream of hot $H_2O$. After complete washing (no starchy material visible unattached to bran), wash twice alternately with alcohol and $CHCl_3$ in that order, and then rinse thoroly with alcohol and finally with $H_2O$.

Transfer material to filter paper if little residue remains or to 1 or 2 L trap flask, **945.75B(h)**(*4*), if large amt remains. Transfer bulk of material with spoon. Rinse residue from screen with 60% alcohol from wash bottle. Wash screen with forcible stream of hot $H_2O$, collecting final residue at one edge of screen and transferring to trap flask with stream of 60% alcohol as above. Add 400 or 900 mL 60% alcohol, depending on size of trap flask.

Boil 20 min. Cool to <20° and add 20 or 40 mL heptane, **945.75C(l)**; fill flask with 60% alcohol, and trap off twice. Use care in stirring and adding alcohol to prevent emulsions or inclusion of air. If residue in flask tends to rise, stir material down 2 or 3 times. Filter trapped-off material and examine microscopically.

### *B. Acid Hydrolysis Method*

(Rapid method; also applicable to flours)

*Post-milling contamination.*—Add 225 g sample to 2 L beaker contg ca 1 L $H_2O$ and 30 mL HCl. Wet product completely and, for flour, stir until slurry is practically lump-free. Add antifoam soln, **945.75C(e)**, cover with watch glass, and heat 15–20 min in autoclave at 121°. Let pressure fall to 0 before opening vent valve. Transfer digest in small portions to 5″ or 8″ No. 140 sieve, **945.75B(r)**, washing thoroly between addns with needle spray from aerator. After all sample has been transferred, continue washing until there is no further reduction in amt of residue. Proceed as in **972.37A**, par. 5, beginning "After complete washing . . ."

Ref.: JAOAC **45,** 660(1962).

## 950.94★ Light Filth (Pre- and Post-Milling) in Baked Goods with Fruit and Nut Tissues

**Pancreatin Digestion Flotation Method**

**First Action**
**Surplus 1975**

*See* **44.056**, 12th ed.

## 970.70 Light Filth in White Breads and High-Fat Products

**Flotation Method**

**First Action 1970**
**Final Action 1988**

Add 1 L hot (55–70°) tap $H_2O$ to 2 L beaker. Add 20 mL emulsifier, **945.75C(j)**(*2*), and 5 mL (**j**)(*1*), and mix well. Add 225 g sample, breaking any crust to <1 sq in. Stir well. Proceed with either autoclave, (**a**), or steam bath, (**b**), digestion.

(**a**) *Autoclave.*—Add 30 mL HCl with stirring. Add 1 mL antifoam soln, **945.75C(e)**. Autoclave as in **945.75B (b)**(*1*) or (**b**)(*2*) for 30 min at 121°.

(**b**) *Steam bath.*—Add 90 mL HCl with stirring. Heat in steam bath 10 min. Add 1 mL antifoam soln. Boil 15 min on mag. stirrer-hot plate, **945.75B(n)**, keeping beaker covered with watch glass.

Wet sieve on No. 230 plain weave sieve, **945.75B(r)**, with hot $H_2O$ (55–70°). Sieve until effluent is clear and foam is gone. Transfer sieve retainings to original beaker. (*Caution:* Do not allow sample in beaker or sieve to cool.) Add 30 mL HCl and dil. to 1 L with $H_2O$. Stir on stirrer-hot plate, and bring to bp. Boil 6 min, add 50 mL mineral oil, **945.75C(p)**, and continue heating until boiling resumes. Transfer beaker to cool mag. stirrer and stir mag., **970.66B(c)**, 3 min.

Promptly transfer beaker contents to percolator, **945.75C(h)**(*2*), contg ca 250 mL $H_2O$. Rinse beaker into percolator and bring vol. to 1700 mL mark with $H_2O$. After 1 min, stir percolator contents with glass rod. Place rod in beaker and set aside to receive final oil drain. Let stand 2 min. Drain oil to 250 mL mark and discard drainings. Refill percolator with $H_2O$. Continue drain and refill cycles until lower aq. phase is almost clear. Drain oil to 250 mL mark. Drain oil into original beaker. Wash percolator sides with min. of 50 mL $H_2O$ and alcohol or isopropanol. If sides do not appear clean, follow with $H_2O$ and 5% detergent wash, **945.75C(i)**. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **53,** 562(1970).

## 975.46 Light Filth in Breading of Frozen Food Products

**Flotation Method**

**First Action 1975**
**Final Action 1988**

Using clip tongs, place each portion individually in $H_2O$ bath maintained at 17–49°C (63–120°F). Let remain until breading becomes soft (5–110 sec for portions held in storage at −18°C (0°F) and can easily be removed with round tip, 10 cm (4") blade spatula or table knife. *Limit dip time in >100°F $H_2O$ to 15 sec max.*

(*Note*: Several preliminary trials may be necessary to det. dip time required for debreading sample units. *For these trials only,* prep. satd soln of $CuSO_4.5H_2O$ (450 g (1 lb)/2 L tap $H_2O$). Correct dip time is min. time of immersion in $CuSO_4$ soln required before breading can be easily scraped off, provided that debreaded portions are still solidly frozen, and only slight trace of blue color is visible on surface of debreaded portions. As guide, use lower temps with raw and higher temps with precooked products.)

After immersion, remove portion and blot lightly with double thickness paper towel. Complete this step in ≤7 sec. Scrape and remove breading and batter with spatula, removing material from narrow sides and ends in initial movements followed by removal from wide flat surfaces. If breading is difficult to remove, redip partially debreaded portion in $H_2O$ at room temp. (17–30° C; 63–86° F) ca 2 sec. Blot with towel and remove residual batter and breading material.

Weigh 50 g sepd breading in 1 L beaker. Add ca 300 mL hot tap $H_2O$ (55–70°), 5 mL emulsifier CO-730, **945.75C(j)**(*1*), 20 mL emulsifier DM-710, **945.75C(j)**(*2*), and 60 mL HCl. Fill beaker to 600 mL with hot tap $H_2O$, stir well, and heat beaker in steam bath 20 min, stirring at 5 min intervals. Add 1 mL antifoam soln, **945.75C(e)**. Boil 15 min on preheated hot plate, keeping beaker covered with watch glass. Wet-sieve on No. 230 plain weave sieve, **945.75B(r)**, with hot $H_2O$, until effluent is clear and foam has dispersed. Transfer sieve retainings to original beaker with HCl-60% alcohol soln (1 + 9), and bring vol. to ca 600 mL. Add 50 mL mineral oil, **945.75C(p)**, and stir mag., **970.66B(c)**, 10 min. Place stirring rod in drain opening of percolator, **945.75B(h)**(*2*), and add ca 250 mL acid-alcohol soln. Quant. transfer beaker contents to percolator. Add acid-alcohol soln to ca 6 cm from top, let stand 5 min, resuspend solids with stirring rod, and let stand addnl 5 min. Drain to ca 250 mL mark, refill percolator to original vol. with acid-alcohol soln, and repeat previous steps until lower aq. phase is almost clear. Drain oil to 250 mL mark and transfer to original beaker. Wash percolator sides with 50 mL $H_2O$, and alcohol or isopropanol. If sides do not appear clean, follow with $H_2O$ and 5% detergent wash, **945.75C(i)**. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **58,** 441(1975).

## 969.41 Light Filth in Alimentary Pastes

**Flotation Method**

**First Action 1969**
**Final Action 1988**

Weigh 225 g sample into 1.5–2.0 L beaker. Add 1 L HCl (30 + 970) and 0.3 mL antifoam soln, **945.75C(e)**. (For spaghetti, break into lengths that will not lie flat on bottom of beaker.) Autoclave 30 min at 121° as in either **945.75B(b)**(*1*) or (*2*).

Wet sieve on No. 230 sieve, **945.75B(r)**, with hot tap $H_2O$ (50–70°) to remove all original liq. and major portion of fine material.

Return sieve retainings to original beaker with hot $H_2O$ (60–100°), dilg to ca 1 L. Add 30 mL HCl, mag. stirring bar, and 50 mL mineral oil, **945.75C(p)**.

Stir mag., **970.66B(c)**, 6 min. Promptly transfer to percolator, **945.75B(h)**(*2*), contg ca 250 mL $H_2O$. Rinse beaker into percolator with hot tap $H_2O$ to bring to 1700 mL. After 3 min, drain oil interface to 250 mL. Discard drainings and refill by pouring hot tap $H_2O$ down percolator sides to loosen adhering material and refill to 1700 mL mark. After 2–3 min, drain and refill for 2 more cycles. (Lower layer should be almost free of suspended material after last refill; if not, continue thru ≥1 recycles.) Finally, drain oil-$H_2O$ interface to 250 mL mark, change to original beaker, and drain. Promptly wash down sides successively with ≥50 mL portions hot tap $H_2O$, isopropanol or alcohol, and hot tap $H_2O$. Use 5% detergent soln, **945.75C(i)**, if necessary.

Transfer beaker contents to ruled filter paper with min. of 50 mL washes of hot $H_2O$, alcohol or isopropanol, and $H_2O$ or detergent, using rubber policeman if necessary to clean sides of beaker. Examine microscopically.

Ref.: JAOAC **52,** 463(1969).

# BREAKFAST CEREALS

## 970.71 Light Filth in Cereals (Corn and Rice) and Corn Chip Products

**Flotation Method**

**First Action 1970**
**Final Action 1988**

(*Caution:* See safety notes on flammable solvents.)

**(a)** *Cereals and food products containing no fats or oils.*—(Check ingredient label.) To 1–1.5 L beaker (depending on bulk of product), add 50 g sample, 500 mL hot (55–70°) tap $H_2O$, and 40 mL HCl. Bring mixt. to full boil on mag. stirrer-hot plate, **945.75B(n)**, using slow stirring speed. Boil 20 min and wet sieve immediately on No. 230 sieve, **945.75B(r)**, with forceful hot (55–70°) $H_2O$ spray until residue no longer passes thru sieve and $H_2O$ is clear. Wash sieve retainings either into 2 L Wildman trap flask, **945.75B(h)**(*4*), or back into original beaker if Kilborn, **(h)**(*1*), or percolator, **(h)**(*2*), is to be used, using 40% isopropanol.

(*1*) *Trap flask.*—Bring vol. to 800 mL with 40% isopropanol and add 30 mL HCl. Raise stirring rod plunger and secure above liq. with clamp. Add mag. stirring bar, **945.75B(n)**, and stir at slow speed while bringing mixt. to bp. Boil 5 min. Add 50 mL mineral oil, **945.75C(p)**, and stir mag., **970.66B(c)**, 3 min.

Remove from heat and fill with 40% isopropanol. Let stand

10 min and trap off, rinsing neck of flask and rod with isopropanol or alcohol. Filter trappings thru ruled paper. Examine microscopically.

(2) *Kilborn or percolator*.—Bring vol. in original beaker to 600 mL with 40% isopropanol and add 25 mL HCl. Bring to bp with slow stirring, boil 5 min, add 50 mL mineral oil, and stir mag. 3 min, **970.66B(c)**.

Transfer from beaker to separator, rinsing beaker into separator with 40% isopropanol. If residue in separator is heavy, resuspend with glass rod. Rinse rod into separator.

Let stand 3 min and drain contents to within 3 cm of bottom of oil layer. Refill with hot (55–70°) tap $H_2O$. Repeat drain and refill steps with 3 min intervals, until $H_2O$ phase is free of plant material. Discard drainings. Drain oil layer into original beaker, rinsing sides of separator alternately with isopropanol or hot $H_2O$ and alcohol, using rubber policeman to clean sides. Filter contents of beaker thru ruled paper. Examine microscopically.

**(b)** *Cereals and food products containing natural and synthetic fats or vegetable oils*.—Proceed as in **(a)**, beginning ". . . 500 mL hot (55–70°) tap $H_2O$, . . ." but also add to this mixt. 20 mL emulsifier, **945.75C(j)**(*2*); then proceed as in **(a)** with no further changes.

Ref.: JAOAC **53,** 558(1970).

### 971.32 Light Filth in Cereals (Whole Wheat)

**Flotation Method**

**First Action 1971**
**Final Action 1988**

Weigh 50 g sample into 1 L beaker. Add 500 mL $H_2O$ and 40 mL HCl. Boil 20 min with mag. stirring to keep solids from scorching. Immediately after boiling, wet-sieve on No. 230 sieve, **945.75B(r)**, with hot (55–70°) tap $H_2O$ until effluent is clear. Transfer sieve retainings with isopropanol to preshaped filter paper cup, using ≥24 cm paper and 150 and 250 mL beakers, **945.75B(j)**. Transfer paper and retainings to Hirsch funnel. Apply vac. to apparent dryness. Turn vac. off, add 150 mL isopropanol, and vac. dry. Repeat isopropanol washing and vac. drying 2 more times.

Transfer filter paper retainings with 40% isopropanol to 2 L trap flask contg 25 mL oleate soln (10 g Na oleate, **945.75C(t)**, dild to 100 mL with 40% isopropanol and stirred in at room temp.). Bring vol. of flask to 800 mL with 40% isopropanol. Boil 5 min with mag. stirring. Cool contents of flask to 23 ± 2° with either air or $H_2O$ cooling. Add 50 mL flotation liq., **945.75(k)**, and stir mag. 3 min, **970.66B(c)**. Fill flask with 40% isopropanol by slowly running isopropanol down rod onto top of stopper, held ca 3 mm above liq. Let stand 3 min and trap off. Add 50 mL flotation liq. and stir ca 15 sec. Add 40% isopropanol as needed to fill flask. Let stand 20 min and perform second trapping. Combine trappings. Filter thru ruled filter paper and examine microscopically.

Ref.: JAOAC **54,** 573(1971).

### 980.27 Light Filth in Barley, Oatmeal, and Mixed Dry Infant Cereal

**Flotation Method**

**First Action 1980**
**Final Action 1988**

Place 50 g sample in 2 L beaker and, with forceful stream hot (55–70°) $H_2O$ directed at side of beaker, add 1 L $H_2O$. Add 80 mL HCl. If product ingredient list includes vegetable oil, add 20 mL DM-710, **945.75C(j)**(*2*). Place mag. stirring bar, **945.75B(n)**, in beaker and put beaker on preheated mag. stirring hot plate set at max. heat. Initially stir vigorously to prevent scorching, then slowly as mixt. thins while heating 20 min. If product darkens, reduce heat to next lower step. Wet-sieve with forceful stream hot $H_2O$ on No. 230 sieve until $H_2O$ becomes clear. Wash residue to one side of sieve. Drench wet residue with isopropanol and let drain. Form paper cup by wrapping No. 588 (S&S) 32 cm filter paper, or fluted equiv., around 600 mL beaker and forcing it into 1 L beaker. Remove 600 mL beaker from paper cup. Leave paper cup in 1 L beaker. Using 6″ (15 cm) powder funnel to aid transfer, wash residue from sieve into paper cup with isopropanol. Add enough isopropanol to bring to 400 mL mark. Boil gently on preheated hot plate 10 min in hood. Drain isopropanol from paper cup by gravity or vac. (Do not let residue dry out.) Discard isopropanol. Moisten sides of 2 L trap flask with 40% isopropanol. Place powder funnel in neck of 2 L trap flask. Wash residue from filter paper cup into trap flask with 40% isopropanol.

Fill to 800 mL mark with 40% isopropanol. Add 50 mL mineral oil, **945.75C(p)**, and mag. stir 3 min. After stirring, add 50 mL 1 + 1 mixt. Tween-80 and 40% isopropanol soln, **945.75C(x)** and $Na_4EDTA$ plus 40% isopropanol soln, **945.75C(z)**, slowly down stirring rod. Do not mix. Let stand 5 min. Fill trap flask with 40% isopropanol by pouring slowly down stirring rod. Let stand 20 min and trap off. Add 35 mL mineral oil, stir gently by hand 30 sec, and let stand 10 min. Repeat trapping. Filter onto ruled paper and examine microscopically at 30×.

Ref.: JAOAC **63,** 187(1980).

## EGGS AND EGG PRODUCTS

(Eggs may be contaminated with chicken excrement, dirt, sand, metal fragments, hairs, and feathers, depending upon condition of the eggs, method of manufacture, and storage conditions. Method of isolation of contaminants depends upon nature of product (whole, whites, or yolks) and physical state (fresh, frozen, or dried).)

### 960.50 Filth in Eggs and Egg Products

**First Action 1960**
**Final Action 1989**

#### *A. Reagents*

**(a)** *Anionic surfactant*.—Na *N*-methyl-*N*-tall oil acid taurate, Igepon TK-32 (GAF Chemicals Corp, 1361 Alps Rd, Wayne, NJ 07470), or equiv.

**(b)** *Phenolphthalein soln*.—Prep. 5% soln in alcohol, dil. with equal vol. $H_2O$, and filter.

**(c)** *Disodium phosphate soln*.—Filtered satd soln (ca 100 g anhyd. salt/L), and filtered 6% (anhyd. basis) aq. soln.

**(d)** *Trisodium phosphate soln*.—Filtered satd soln.

**(e)** *Tetrasodium EDTA soln*.—10% filtered aq. soln of $Na_4EDTA$.

#### *B. Light and Heavy Filth and Other Extraneous Materials*

**(a)** *Whole eggs or yolks*.—Thaw frozen sample at room temp. or in cold running $H_2O$. Weigh 100 g thawed sample into 250 mL centrf. bottle. Add 30 mL 6% $Na_2HPO_4$ and stir. Shake vigorously 1.5–2.0 min, add addnl 30 mL 6% $Na_2HPO_4$, and shake ca 2 min. Dil. with 6% reagent to fill bottle and centrf.

whole eggs 5 min at 1500 rpm and yolks 5 min at 800 rpm. Decant ca $^2/_3$ liq. into 1.5 or 2.0 L beaker and isolate light filth as in (**b**). Add ca equal vol. 6% $Na_2HPO_4$ soln to residue in bottle, shake well, and recentrf. Decant closely. To sediment in bottle add ca $^1/_2$ vol. $H_3PO_4$ and warm on steam bath. Transfer to 250 mL beaker, boil 3–5 min, and filter while boiling. Examine at 30× for metal and glass fragments, and chicken excrement. Check amorphous white material for uric acid as in **962.20**.

(**b**) *Egg whites*.—Use decanted whole egg or yolk material from (**a**) or weigh 100 g thawed whites into 1.5 or 2.0 L beaker and add ca 300 mL 6% $Na_2HPO_4$ soln in small portions with thoro stirring. Add 16 mL $Na_4$EDTA soln, (**e**), then 12 mL phthln, (**b**). Let stand 10 min; then adjust to pH 7.6–8.0, using $H_3PO_4$ (1 + 9) or $Na_3PO_4$ soln as needed. Add 2 mL surfactant, (**a**), and readjust to pH 7.6–8.0, using short range pH paper. Add 200 mL pancreatin soln, **945.75C(q)**, and readjust to 7.6–8.0.

Place in 37–38° $H_2O$ bath to ca depth of digestion mixt.; stir, and adjust to pH 7.6–8.0 at ca 15 min intervals for ca 2 hr. Add 2 mL surfactant and dil. to 1.0–1.2 L with $H_2O$. Adjust to pH 8.0 and place in incubator at 37° overnight. Readjust to pH 8.0 and let stand 15–20 min without stirring. Decant in small portions onto ruled paper, using full suction, while washing paper with hot tap $H_2O$. Examine paper microscopically.

(**c**) *Dried egg yolks*.—Defat egg yolks as follows: Weigh 25 g sample into 150 mL tall-form beaker, add 50 mL pet ether, and stir thoroly (until smooth). While stirring, add solv. to almost fill beaker. Stir top again after 1 min, let stand 1 min, and decant solv. into larger beaker. Repeat defatting step twice more with pet ether. Filter combined washes thru smooth textured paper, air dry paper thoroly, and hold for pancreatin digestion. Discard solv. Place 150 mL beaker on steam bath and remove solv. completely from residue with continuous stirring to prevent bumping. Transfer dried residue in beaker and dried residue on paper, using spatula, to 600 mL beaker and proceed as in (**d**).

(**d**) *Dried whole eggs*.—Weigh 25 g sample into 600 mL beaker, or continue with dried yolk residue from (**c**). Add mixt. of 90 mL satd soln of $Na_2HPO_4$ and 10 mL alcohol in small portions with stirring. (Suspension must be smooth and finely divided at this point.) With stirring, add 12 mL $Na_4$EDTA soln, (**e**), then 5 mL phthln, (**b**). If intense red develops, discharge with $H_3PO_4$ (1 + 9). Adjust to pH 7.6–8.0 with satd $Na_3PO_4$ soln, using short range indicator paper. Add 200 mL pancreatin soln, **945.75C(q)**, to suspension. Continue as in (**b**), beginning "Place in 37–38° $H_2O$ bath . . ."

(**e**) *Dried whites*.—Weigh 25 g sample into 250 or 400 mL beaker. Dil. 4.5 mL surfactant, (**a**), to 35 mL with $H_2O$ and add to beaker in portions of 5 mL, rotating and shaking beaker until sample absorbs each portion. Let soak 10–15 min; then add 20 mL $H_2O$ in 4–5 portions with thoro stirring after each addn. Stir to smooth slurry. (Material must be finely dispersed before proceeding.) Add 7 mL $Na_4$EDTA soln, (**e**), then 3 mL phthln, (**b**). Transfer to 1.5 or 2 L beaker and dil. with $H_2O$ to 700–800 mL. Adjust to pH 7.2–7.6 and add 200 mL pancreatin soln, **945.75C(q)**. Continue as in (**b**), beginning "Place in 37–38° $H_2O$ bath . . ."

***C. Sedimentation Method for Chicken Excrement and Heavy Filth***

(**a**) *Frozen whole eggs or yolks*.—Examine by **960.50B(a)**.

(**b**) *Dried egg yolk*.—Add 25 g sample in small portions with continuous stirring to mixt. of 75 mL $H_3PO_4$ (1 + 9) and 5 mL surfactant, **960.50A(a)**, in 150 mL tall-form beaker. Stir to smooth paste and add $H_3PO_4$, few mL at time, to fill beaker while stirring. Stir top layer 1 min and let stand 5 min. Decant ca $^2/_3$ vol. into 250 mL beaker and add $H_3PO_4$ (1 + 9) to both beakers equal to vol. present. Stir contents of both beakers 1 min and let stand 5 min. Again stir top layers 1 min and slowly add $H_3PO_4$ with stirring to fill both beakers. Let stand 5 min and repeat stirring and standing. Decant both beakers closely into 1 L beaker.

Dil. material in 1 L beaker with $H_2O$, stirring continuously, until full. Stir top layer 1 min and let stand 5 min, and repeat stirring and standing. Decant closely, discarding supernates. Composite all residues in 250 mL beaker by transferring with $H_3PO_4$ (1 + 9) from wash bottle. Decant acid and floating egg material and transfer residue to ruled paper with $H_2O$, using min. suction. Wash residue with two 30 mL portions $H_2O$, using min. suction. Examine microscopically, keeping paper moist. Check amorphous white material for uric acid as in **962.20** or **986.29**.

Refs.: JAOAC **43,** 565(1960); **48,** 545(1965).

## POULTRY, MEAT, AND FISH AND OTHER MARINE PRODUCTS

### 950.87★ Filth and Sand in Chicken Giblet Paste

**Procedure**
**Surplus 1970**

*See* **40.063**, 11th ed.

### 950.88★ Glass in Meat Scraps

**Procedure**
**Surplus 1970**

*See* **40.065**, 11th ed.

### 968.36 Shell in Crabmeat (Canned)

**Digestion Method**
**First Action 1968**

Weigh 57 g (2 oz) representative sample into 400 mL beaker. Add 150 mL 1.5% NaOH soln and stir to break up lumps. Add 10 drops *1% aq. Alizarin Red S* indicator. Heat until meat has been digested (10 min at ca 80°), stirring 3 or 4 times. Pour on No. 12 sieve nested in No. 60 sieve, **945.75B(r)**, and wash with $H_2O$. Wash shell from both sieves onto weighed paper, dry at 100°, and cool to room temp. Weigh and count shell. Report shell as number of pieces and wt/lb.

Ref.: JAOAC **51,** 521(1968).

### 976.27 Light Filth in Crabmeat (Canned)

**Flotation Method**
**First Action 1976**
**Final Action 1988**

Transfer entire contents of ≤7 oz (200 g) can (or 7 oz portion of larger sample) to 2 L trap flask, **945.75B(h)**(*4*). Thoroly wash can (and parchment, if present) with tap $H_2O$ and add washings to flask. Add ca 800 mL hot (55–70°) tap $H_2O$.

With mag. stirring, **970.66B(c)**, heat to bp. Add 50 mL mineral oil, **945.75C(p)**, and stir mag. 3 min while continuing to boil. Remove flask from heat, fill with hot tap $H_2O$, and let stand 30 min, stirring gently by hand at 10 and 20 min. Trap off into 400 mL beaker. Add 30 mL mineral oil to trap flask. With stirring bar spinning at max. speed, disperse oil thruout aq. (lower) phase, stirring by hand. Stir mag. 5 min at max. speed. Fill flask with hot tap $H_2O$ and let stand 20 min, stirring gently by hand at 10 min. Trap off into same beaker. Wash mouth of trap flask with isopropanol and decant washings into beaker.

Transfer to 2 L percolator, **945.75B(h)**(*2*), contg ca 250 mL $H_2O$. Rinse beaker into percolator and bring vol. to ca 1700 mL with $H_2O$ at room temp., ca 20°. Let stand 3 min. Drain oil to 250 mL mark and discard drainings. Repeat fill and drain cycle ≥2 more times. Drain remaining oil and $H_2O$ into original 400 mL beaker. Wash percolator sides with 1% detergent soln, **945.75C(i)**, and isopropanol, and collect washings in beaker. Filter onto ruled paper and examine microscopically. If filtering action slows, use new filter paper.

Ref.: JAOAC **59,** 825(1976).

## 968.37 Shell in Clams and Oysters (Canned)

**Digestion Method**

**First Action 1968**

Weigh 57 g (2 oz) representative sample into 600 mL beaker. Continue as in **968.36**, except digest by boiling ca 15 min with mag stirring.

Ref.: JAOAC **51,** 521(1968).

## 972.38 Light Filth in Fish (Canned) and Fish Products

**Flotation Method**

**First Action 1972**
**Final Action 1988**

For ≤8 oz (225 g) samples, transfer entire contents of can (or 8 oz portion of larger sample) to 1.5 L beaker and break up lumps with spatula. Wash can thoroly with small amt of isopropanol and add washings to beaker. Add 50 mL HCl and $H_2O$ to make 800 mL. With mag. stirring, heat to bp and boil 20 min. (If product foams, add $H_2O$ occasionally.) Add 50 mL mineral oil, **945.75C(p)**, and stir mag., **970.66B(c)**, 5 min while continuing to boil.

Transfer to 2 L percolator, **945.75B(h)**(*2*), contg ca 250 mL $H_2O$. Retain the 1.5 L beaker to fill percolator with $H_2O$ during refill cycles. Fill percolator with hot tap $H_2O$ (55–70°) to within 3 cm of top. Let stand 3 min and drain contents to ca 3 cm of bottom of oil layer. (If large amt of suspended solids is present, let stand longer to permit sepn of oil.) Repeat drain and refill steps at 3 min intervals until aq. phase appears clear. Finally, slowly drain percolator to min. vol. of aq. phase without loss of oil phase. Drain oil layer into 600 mL beaker. Wash percolator with warm $H_2O$, 5% detergent soln, **945.75C(i)**, $H_2O$, and isopropanol in sequence, using ca 50 mL per wash, and collect washings in 600 mL beaker. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **55,** 69(1972).

## 974.32 Light Filth in Shrimp (Canned)

**Flotation Method**

**First Action 1974**
**Final Action 1988**

For shrimp <2.5 cm long, place entire contents of can into 2 L beaker contg mag. stirring bar. For larger shrimp, skewer on probe, and wash each shrimp with hot (55–70°) $H_2O$ from plastic squeeze bottle over 2 L beaker contg stirring bar. Discard shrimp. Wash can thoroly, pouring washings into beaker. Bring $H_2O$ level in beaker to ca 925 mL with hot tap $H_2O$. Add 25 mL HCl and 50 mL light mineral oil, **945.75C(p)**. Boil and stir mag., **970.66B(c)**, 3 min. Transfer promptly to percolator, **945.75B(h)**(*2*), which has its rubber hose fitting clamped shut as close to tubulation opening as possible and contg ca 200 mL hot tap $H_2O$. Reserve beaker. Add ca 800 mL addnl hot tap $H_2O$. Let stand 10 min. Drain oil layer to ca 7.5 cm from bottom, using rod to force shrimp tissue thru tubulator, if necessary. Remove rod, and wash with hot $H_2O$ into reserved beaker. Reserve rod for further washings. Drain percolator to 300 mL mark, let stand 1 min and slowly drain and discard remaining aq. phase until min. vol. remains. Do not let vortex form, as it may cause loss of oil. Drain oil layer into reserved beaker. Filter thru ruled paper. Wash paper with 55–70° hot tap $H_2O$. Alternately wash percolator and rod with 5% detergent soln, **945.75C(i)**, and hot tap $H_2O$. Filter onto the ruled filter paper and examine microscopically.

Ref.: JAOAC **57,** 691(1974).

## 973.60 Light Filth in Pork Sausage (Uncooked) and Ground Beef or Hamburger

**Enzyme Digestion Method**

**First Action 1973**
**Final Action 1988**

### *A. Determination*

Grind sausage, using meat grinder with end plate having $^3/_{16}$″ holes. Weigh 225 g sample into 1.5–2 L beaker. Add 980 mL warm $H_2O$ and 20 mL emulsifier, **945.75C(j)**(*1*), and stir 5 min. Add 20 mL HCl and stir 1 min. Proceed with overnight digestion, **(a)**, or rapid digestion, **(b)**.

With ground beef, use only overnight digestion with 5.0 g 1:10,000 or 10 g NF pepsin. In flotation, **(c)**, omit addn of 50 mL HCl and subsequent boiling. After addn of light mineral oil, let stand 20 min, instead of 10 min.

**(a)** *Overnight digestion.*—Add 0.5 g 1:10,000 pepsin (No. 0151, Difco Laboratories, or equiv.) or 2.0 g NF pepsin and stir 1 min. Digest in 50° $H_2O$ bath or incubator 18 hr. Add 5 mL Triton X-114, **945.75C(bb)**(*2*), and stir 1 min. Keep all samples at digestion temp. in bath until ready to sieve. Sieve portionwise on No. 230 sieve with hot $H_2O$ spray. Transfer to ruled filter paper if small amt residue remains on sieve or proceed with flotation, **(c)**.

**(b)** *Rapid digestion.*—Add 2.0 g 1:10,000 pepsin or 10 g NF pepsin and stir 1 min. Digest in 62° $H_2O$ bath 2 hr. Add 5 mL Triton X-114, **945.75C(bb)**(*2*), and stir 1 min. Keep all samples at digestion temp. until ready to sieve. Sieve portionwise on No. 230 sieve. Proceed with flotation, **(c)**.

**(c)** *Flotation.*—Wet residue on sieve with 40% isopropanol and immediately transfer quant. to 2 L trap flask, using 40% isopropanol. Bring vol. to 1 L with 40% isopropanol and add 50 mL HCl. Add mag. stirring bar, **945.75B(n)**, and, with gentle stirring, boil 10 min on mag. stirrer-hot plate. Cool to room temp. in cold $H_2O$ bath and add 40 mL flotation liq., **945.75C(k)**. Stir mag. 3 min, **970.66B(c)**. Let oil phase sep. 1 min, and slowly fill flask with 40% isopropanol by letting liq. flow down stoppered rod while top of stopper is maintained just above flask contents. After filling flask, gently stir settled plant material with stoppered rod 5–10 sec. Let stand undisturbed 5 min and immediately trap off. Add 25 mL light mineral oil, **945.75C(p)**, stir gently by hand 30 sec, and let stand 10 min. Repeat trapping. Wash flask neck thoroly with isopropanol and transfer washings to beaker contg trappings. Filter onto ruled paper and examine microscopically.

Refs.: JAOAC **56,** 631(1973); **59,** 51(1976).

### B. Alternative Method for Sausages

(*Caution:* See safety notes on chloroform.)

(**a**) *Bulk or link sausages that are easily teased apart.*—Weigh 225 g sample into 1.5–2 L beaker. Add 1 L 10% Tergitol soln, **945.75C(bb)**(*1*), and 75 × 12 mm stirring bar; stir mag., 5 min, or until thoroly dispersed. Sieve portionwise on No. 230 sieve, **945.75B(r)**. Form filter paper around 1 L beaker, **945.75B(j)**, moistening with $H_2O$ to make paper pliable. Insert paper into buchner, 91 mm id plate, wash with isopropanol, and aspirate to near dryness.

Wet residue on sieve with isopropanol and quant. transfer to filter paper cup with isopropanol. Add enough isopropanol to cover residue and, after 1 min, apply vac. until dripping ceases. Transfer paper cup to 1 L beaker and add 300 mL $CHCl_3$. Boil on steam bath 5 min. Let soln cool few min, lift paper, drain, and transfer to 300 mL fresh $CHCl_3$. Repeat 5 min boil and drain. Return paper cup to buchner and apply vac. until dripping ceases. Cover residue with isopropanol for 1 min, reapply vac., and continue to aspirate 5 min. Quant. transfer residue to 2 L trap flask, using 40% isopropanol, and proceed as in **973.60A(c)**, second sentence.

(**b**) *Link sausages compressed into casings so that product is not easily teased apart.*—Remove casing and weigh 225 g sample into 2 L beaker. Add 1 L 10% Tergitol soln, **945.75C(bb)**(*1*), and stir with mech. stirrer, **945.75B(e)**, at max. speed at which no splashing occurs, 15 min or until thoroly dispersed. Proceed as in (**a**), beginning, "Sieve portionwise on No. 230 sieve, . . ."

Ref.: JAOAC **55,** 66(1972).

# FRUITS AND FRUIT PRODUCTS

## 945.76 Filth in Apple Butter

**Flotation Method**

**Final Action 1988**

Weigh 100 g well mixed sample into 400 mL beaker, add enough cold $H_2O$ to obtain uniform dispersion, and transfer to 2 L trap flask, **945.75B(h)**(*4*). Add 35 mL heptane, **945.75C(l)**, and stir. Add cold $H_2O$ to bring heptane into neck of flask. Let stand 30 min with occasional stirring, and trap off. Transfer trapping to second flask contg ca 1 L $H_2O$, stir, fill flask with $H_2O$, and let stand 15 min with occasional stirring. Trap off, filter onto ruled paper, and examine paper microscopically.

Ref.: JAOAC **56,** 522 (1973).

## 945.77★ Filth in Apple Chops (Dried)

**First Action**

**Surplus 1969**

### A. Heavy Filth

*See* **40.069**, 11th ed.

### B. Insects and Light Filth

*See* **40.070**, 11th ed.

## 981.20 Thrips and Other Insects in Frozen Blackberries and Frozen Raspberries

**Flotation Method**

**First Action 1981**

**Final Action 1988**

(*Caution:* See safety notes on chloroform and carcinogens.)

### A. Sample Preparation

Place entire frozen contents of retail package(s) in 2–3 L beaker and add 500 mL hot tap $H_2O$ (55–70°). Gently sep. frozen mass during thawing. Nest 8 in. SS funnel in 3 L beaker. Place 8 in. No. 8 sieve over funnel. After berries are completely thawed, pour entire contents of beaker onto sieve and distribute berries evenly over sieve. Rinse both beaker used for thawing and retail package with 100 mL $H_2O$ each and pour rinses thru sieve contg berries.

*Do not wash berries once on the sieve*. Let berries drain 10 min (±30 s). Remove berries to 1 L tared beaker and record 10 min drained wt. Retain berries in tared beaker for analysis. Retain drainings and beaker washings for detn of amt filth present in proportionate liq.

### B. Determination

Add 100 g drained berries to 1 L beaker contg mag. stirring bar (**945.75B(n)**). Mash berries with fork. Add 300 mL tap $H_2O$ (55–70°) and 70 mL HCl. Fill beaker to 800 mL mark with $H_2O$ (55–70°). Place beaker on preheated hot plate adjusted to maintain berry-HCl mixture at 55–70°. Mag. stir 5 min while maintaining temp. Wet-sieve portionwise on 8 in. No. 25 sieve nested in No. 80 sieve, using 55–70° $H_2O$ until only seeds remain and drainings are clear. Discard seeds on No. 25 sieve. Gently rinse residue to edge of No. 80 sieve and wet thoroly with isopropanol. Let drain. Form paper cup (**945.75B(j)**) by forming 32 cm No. 558 filter paper (S&S) around 600 mL beaker and forcing it into 1 L beaker. Nest wide-mouth glass powder funnel in paper cup. Wash residue from sieve into paper cup using isopropanol initially and minimal amt of 40% isopropanol for final rinse. Add enough isopropanol to bring to 400 mL mark. Boil 1 min on preheated hot plate. Transfer paper cup into wide-mouth funnel and drain into original beaker. Discard drainings. Place paper cup in beaker, repeat boil in 400 mL fresh isopropanol, and save drainings. Place filter paper cup contg sample in clean 1 L beaker and add 400 mL $CHCl_3$. Cover beaker with watch glass contg ice cube to minimize loss of $CHCl_3$ during boiling. Boil 5 min on steam bath, and drain $CHCl_3$ from cup as in isopropanol drain above. Place filter paper cup contg sample in isopropanol saved from last isopropanol boil. Let cup soak 5 min with occasional up and down movement of cup. Wash residue from filter paper onto No. 230 sieve with hot $H_2O$, and then wet residue with 40% isopropanol and let drain. Wash residue from sieve with 40% isopropanol into 2 L trap, using powder funnel nested in neck of flask. Fill flask with 40% isopropanol to 800 mL mark. Add mag. stirring bar. Place trap flask on preheated mag. stirring hot plate and, with gentle stirring, let come to gentle boil. Turn off mag. stirrer. Boil 5 min. Turn off hot plate. Add 50 mL light mineral oil (**945.75C(p)**) and mag. stir 2 min. Remove trap flask from mag. stirrer/hot plate. Add 50 mL 1 + 1 mixt. Tween-80 and 40% isopropanol soln, **945.75C(x)** and $Na_4EDTA$ plus 40% isopropanol soln, **945.75C(z)** gently down stirring rod so that soln is just under oil layer. Stir in slowly 1 min. Let trap flask stand 4 min. Fill trap flask with 40% isopropanol gently down stirring rod. Let stand 10 min. Trap off into 250 mL beaker. Add 35 mL light mineral oil to trap flask. Stir in 1 min. Let stand 6 min. Trap off into second 250 mL beaker. Filter onto ruled filter papers (**945.75B(i)**) and examine papers under 10–30×.

Det. vol. of drained liq. to be filtered onto ruled paper, using following formula:

$$\text{mL drained liq. to be filtered} = (100\ \text{g}/10\ \text{min drained wt(g)}) \times \text{total mL drained liq.}$$

Mix drained liq. to resuspend all solids and immediately measure vol. calcd above.

Pour drained liq. (vol. calcd above) thru No. 25 sieve nested in No. 80 sieve, and then wash No. 80 sieve residue onto ruled filter paper and examine at 10–30× as required.

Ref.: JAOAC **64,** 194(1981).

## 945.78 Maggots in Blueberries and Cherries

### Sedimentation Method

### Procedure

Weigh 567 g (20 oz) fresh fruit or use No. 2 can of processed fruit. Add 100 mL $H_2O$ to fresh or frozen fruit and boil 5 min, with frequent stirring. (Omit this step with canned fruit.) Transfer 1 cm layer of fruit to No. 6 sieve immersed in pan of $H_2O$. Shake loose maggots and debris thru sieve. Carefully mash fruit under $H_2O$ to rub any remaining maggots thru sieve. Rinse and discard any pulp and seeds. Repeat process with another portion of fruit.

After all fruit is screened, transfer mixt. to black-bottom pan. (With cherries, transfer first to No. 6 sieve resting in ca 3 cm $H_2O$, shake sieve until maggots drop thru, and discard pulp on sieve.) Slowly decant $H_2O$ and pulp from pan. Add more $H_2O$ and repeat decantation. Pick out and count maggots by examination of contents of pan. Transfer contents of this pan to white-bottom pan and count maggots in pan.

## 964.23 Filth in Fig and Fruit Paste

### First Action 1964

### *A. Determination*

(**a**) *Light filth*.—Weigh 100 g paste into 1 L beaker. Add 400 mL boiling $H_2O$ and mag. stirring bar. Boil on mag. stirrer-hot plate, **945.75B(n)**, until all lumps are disintegrated. Wet sieve mixt. on 8″ No. 140 sieve, **945.75B(r)**, with hot tap $H_2O$ to remove fine and sol. material. Transfer residue from sieve to 2 L trap flask, **945.75B(h)**(*4*), with $H_2O$. Add $H_2O$ to bring vol. to ca 900 mL, and ext twice with 35 and 25 mL kerosene, **945.75C(o)**, as in **970.66B(b)**. Pour onto ruled filter paper. Examine papers microscopically as in **970.66B(f)**(*1*) and (**g**).

(**b**) *Heavy filth*.—Empty remaining trap flask contents and rinsings onto 8″ No. 140 sieve. Wet sieve with hot tap $H_2O$ to remove kerosene as completely as possible. Transfer material from sieve to 1 L beaker and add hot $H_2O$ to ca 400 mL. Boil vigorously 15 min, frequently adding 10% $Na_4EDTA$ soln to keep pH ca 8. (Check with pH paper.) Transfer hot mixt. to 8″ No. 140 sieve. Wet sieve until seeds are completely sepd from fig tissue. Return residue on sieve to the 1 L beaker. Add $H_2O$ to ca 300 mL, swirl, and quickly decant suspended fig tissue and filth elements onto ruled paper in Hirsch funnel, retaining seeds in beaker. Add $H_2O$ and repeat decanting, changing paper as necessary. Examine papers for heavy filth elements at ca 30×.

Ref.: JAOAC **47,** 897(1964).

### *B. Alternative Method for Light Filth*

Weigh 100 g paste into 1 L beaker. Break paste into small lumps. Add 400 mL boiling $H_2O$ and mag. stirring bar. Boil and stir on mag. stirrer-hot plate, **945.75B(n)**, until all lumps are disintegrated. Wet-sieve mixt. on 8″ No. 140 sieve, **945.75B(r)**, with hot tap $H_2O$ to remove fine and sol. material. Transfer residue from sieve to 2 L trap flask, **945.75B(h)**(*4*), with $H_2O$. Add $H_2O$ to bring vol. to ca 900 mL, add 35 mL kerosene, **945.75C(o)**, and ext as in **970.66B(b)**. Make second extn with 25 mL kerosene.

If trapped material is relatively free of plant tissue, filter on ruled paper. Examine microscopically for whole and equiv. insects only. Det. insect head count for fig paste from number of whole or equiv. forms of lepidoptera and coleoptera.

If trapped material contains excessive plant tissue, transfer with ca 150 mL $H_2O$ to percolator, **945.75B(h)**(*2*), contg ca 250 mL $H_2O$. Fill percolator to within 5 cm of top, let kerosene layer sep., and drain slowly to 250 mL mark. Repeat refill and drain cycle if necessary. Drain remaining liq. into original beaker, rinsing percolator alternately with $H_2O$ and acetone; filter and examine as above.

Ref.: JAOAC **58,** 443(1975).

## 950.89 Filth in Jam and Jelly

### Final Action 1988

(**a**) *Jam*.—Empty contents of jar into dish and mix thoroly. Weigh 100 g into beaker, add 200 mL $H_2O$ (ca 50°), transfer to 1 L trap flask, **945.75B(h)**(*4*), add 10 mL HCl, and boil ca 5 min. Cool to room temp., add 25 mL heptane, **945.75C(l)**, and stir thoroly. Trap off, filter, and examine microscopically.

(**b**) *Jelly*.—Empty contents of jar into dish and mix thoroly. Weigh 100 g into beaker and add 300–400 mL hot $H_2O$; warm beaker, with stirring, until jelly dissolves, filter, and examine microscopically.

When so-called "jellies" contg small amts of fruit tissue will not filter thru paper, proceed as in (**a**).

## 970.72 Filth in Citrus and Pineapple Juices (Canned)

### Final Action 1988

### *A. Fly Eggs and Maggots*

Filter 250 mL thoroly mixed sample thru buchner fitted with 10XX bolting cloth, **945.75B(d)** (wire mesh screen under bolting cloth facilitates filtration). Pour juice slowly to avoid accumulation of excess pulp on cloth (2 or 3 cloths may be necessary). Examine filters microscopically.

### *B. Light Filth*

To 250 mL juice in 2 L trap flask add 15 mL castor oil, USP, and fill with enough hot $H_2O$ (ca 70°), stirring vigorously, to bring oil layer into neck of flask. Let stand 30 min. Trap off, filter, and examine.

## 969.42 Light Filth in Raisins

**Microscopic Examination Method**

**First Action 1969**
**Final Action 1988**

(*Caution:* See safety notes on toxic solvents and chloroform.)

Add 500 mL $CHCl_3$ to 225 g sample in 1 L beaker and boil on steam bath 10 min, keeping $CHCl_3$ vol. at ca 500 mL. Decant $CHCl_3$, holding back raisins with glass rod, onto 7.5 cm ruled filter paper in Hirsch funnel. Retain paper. Repeat 10 min $CHCl_3$ boil and decant. Using $H_2O$, wash filter retainings from paper back into beaker contg raisin tissue. Bring vol. in beaker to 700 mL with hot $H_2O$ (55–70°) and rehydrate *in steam bath* 30 min. Sieve portionwise onto 8″ No. 8 sieve nested in 8″ No. 140 sieve, **945.75B(r)**. Thoroly wash each portion with stream of hot $H_2O$ while gently rubbing raisins over sieve with fingers. Microscopically examine any decomposed raisins for fly eggs and maggots.

Wet retainings on No. 140 sieve with 25% isopropanol, transfer to 2 L trap flask, **945.75B(h)**(*4*), with 25% isopropanol, and bring vol. to 1 L. Add 70 mL HCl and mag. stirring bar to flask, heat to boiling, and continue for 10 min, slowly stirring on mag. stirrer-hot plate, **945.75B(n)**. Cool to <25° in cold $H_2O$ bath. Add 40 mL flotation oil (mix kerosene, **945.75C(o)**, and mineral oil, **945.75C(p)**, (1 + 2)) and stir mag., **970.66B(c)**, 5 min. Let stand 1 min after gentle 10–15 sec stir with stoppered rod (*see* **970.66B(b)**). Fill with deaerated 25% isopropanol by slowly running alcohol down rod onto top of stopper maintained ca 3 mm above liq. Let stand 15 min, gently stirring mixt. 2–3 times during first 10 min. Trap; filter first and second extns sep. Add 25 mL flotation oil and gently hand stir 1 min. Let stand 1 min; gently disturb oil-alcohol interface with several up-and-down strokes of stoppered rod to cause fine plant material to settle. Let stand 10 min. Perform second trapping. Thoroly wash flask neck with isopropanol. Pour trappings onto ruled filter paper and examine at 30×. If second extn is difficult to filter, pour onto No. 230 sieve, **945.75B(r)**, and wash twice alternately with undild isopropanol and hot $H_2O$. Wash sieve retainings into 400 mL beaker with hot $H_2O$ and add 7 mL HCl/100 mL $H_2O$. Boil 10 min and pour onto ruled filter paper. Examine microscopically.

Ref.: JAOAC **52,** 19(1969).

## 971.33 Residue (Acid-Insoluble) (Soil) in Fruits and Vegetables (Frozen)

**Gravimetric Method**

**First Action 1971**
**Final Action 1973**

Remove frozen sample from container. Place in weighed plastic bag, reweigh, and seal tightly with rubber band. Thaw sample by immersing bag in hot $H_2O$ and transfer contents to high-speed blender, washing inside of bag. Blend until sample is disintegrated and transfer to 2 L beaker. Nearly fill beaker with $H_2O$ and mix contents thoroly by swirling. Let stand 10 min and decant supernate into second 2 L beaker. Refill first beaker with $H_2O$ and repeat mixing. Fill second beaker with $H_2O$ and mix by swirling. After 10 min, decant second beaker into third and first into second. Continue operation, decanting from third beaker into sink until vegetable material is washed from sample. If many seeds settle, float them off with hot 15% NaCl soln, increasing NaCl concn if necessary to complete flotation. Remove NaCl residue with hot $H_2O$. Collect mineral residue from the 3 beakers on ashless filter paper, and discard filtrate. Ignite paper in weighed porcelain crucible over medium Bunsen flame and place in furnace 1 hr at ca 600°. Cool, add 5 mL HCl, and heat to bp. Cool, add 10 mL $H_2O$, and reheat to bp. Filter and wash free from acid. Ignite, ash as before, and weigh to det. acid-insol. residue. Calc. % insol. residue = wt acid-insol. residue (g) × 100/net wt sample (g).

Ref.: JAOAC **54,** 581(1971).

# SNACK FOOD PRODUCTS

## 950.90 Filth in Unpopped Popcorn

**Procedure**

*See* **950.86**.

## 950.91 Filth in Popped Popcorn

**Procedure**

Weigh 50 g sample into 2 L trap flask. Add 500 mL hot $H_2O$, boil 15 min, and cool to room temp. Add 35 mL heptane, **945.75C(l)**, mix, and let stand 5 min. Fill with $H_2O$, trap off, filter, and examine microscopically.

## 955.44 Filth in Potato Chips

**Flotation Method**

**Final Action 1988**

Weigh 100 g sample into 1.5 L beaker. Crush chips into small pieces and cover with pet ether. Let stand ca 5 min and decant thru filter. Add pet ether and decant again thru filter. Let pet ether evap. from chips. Transfer to 2 L trap flask, add 500 mL 60% alcohol, and boil ca 30 min, replacing alcohol lost by evapn. Cool, add 35 mL heptane, **945.75C(l)**, mix, let stand ca 5 min, and fill with 60% alcohol. Let stand, trap off twice, and filter as usual. Examine papers microscopically.

## 984.28 Filth in Corn Chips

**Final Action 1988**

*See* **970.71**.

# SUGARS AND SUGAR PRODUCTS

## 971.34 Filth in Candy

**Flotation Method**

**Final Action 1988**

(**a**) *In hard candy, gum drops, gum, starch, or pectin-base candies.*—Dissolve in boiling HCl (1 + 70), filter thru rapid paper on Hirsch funnel, and examine microscopically.

(**b**) *In hard candy difficult to filter by* (**a**) (*e.g., licorice candy*).—Proceed as in (**c**).

(**c**) *All water-insoluble candy except those containing con-*

*fectioners corn flakes, wheat bran, or other cereal fillers, and those whose major constituent, excluding chocolate coating, consists primarily of finely ground nutmeats (e.g., peanut butter, almond paste, etc.).*—Weigh 225 g sample into 1.5–2 L beaker. Add 1 L 5% soln of Tergitol, **945.75C(bb)**(*1*), and heat *in* steam bath 10 min. Stir 5–10 min on mag. stirrer-hot plate. Sieve portionwise on No. 230 sieve, **945.75B(r)**. If residue on sieve is small, transfer directly to ruled filter paper; otherwise, transfer quant. to 2 L trap flask, using 40% isopropanol. Bring vol. to 1 L with 40% isopropanol and add 50 mL HCl. Gently stir on mag. stirrer-hot plate while heating *to full boil.* Immediately transfer flask to cool stirring unit and add 40 mL light mineral oil, **945.75C(p)**. Stir mag., **970.66B(c)**, 2 min. Let stand 1 min; then slowly fill flask with 40% isopropanol by running liq. down stoppered rod while top of stopper is maintained just above liq. After filling flask, gently stir settled plant material 5–10 sec with stoppered rod. Let stand undisturbed 2 min and immediately trap off. Add 25 mL light mineral oil, stir by hand gently 30 sec, and let stand 10 min. Repeat trapping. Wash flask neck thoroly with isopropanol and transfer washings to beaker contg trappings. Filter onto ruled paper and examine microscopically.

(**d**) *Water-insoluble candies containing confectioners corn flakes, wheat bran, or other cereal fillers, and those whose major constituent, excluding the chocolate coating, consists primarily of finely ground nutmeats (e.g., peanut butter, almond paste, etc.).*—(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.) Proceed as in (**c**) thru sieving on No. 230 sieve. Wash residue on sieve with isopropanol. Form filter paper around 600 mL beaker, **945.75B(j)**, moistening with $H_2O$ to make paper pliable. Insert paper into 91 mm buchner, wash with isopropanol, and aspirate to near dryness. Quant. transfer residue on sieve to filter paper cup with isopropanol and add enough isopropanol to cover residue. After 1 min, apply vac. until dripping ceases. Place paper cup contg sieved residue in 1 L beaker, add 200 mL $CHCl_3$, and boil 5 min on steam bath. After few min of cooling, lift paper, drain, and transfer to 200 mL fresh $CHCl_3$. Repeat 5 min boil and drain. Return paper cup to buchner and apply vac. until dripping ceases. Cover residue with isopropanol 1 min, reapply vac., and continue to aspirate 5 min after visible dripping ceases. Proceed as in (**c**), beginning with ". . . transfer quant. to 2 L trap flask, using 40% isopropanol." Continue as in (**c**), except after bringing contents of flask to full boil, cool to room temp. in cold $H_2O$ bath, and use flotation liq., **945.75C(k)**, in place of mineral oil.

(**e**) *In chocolate candy coating.*—Heat 400 mL $CH_2Cl_2$ in 800 mL beaker to 30–35° and keep at this temp. Place portion of candy in wire basket (ca 8 cm diam. × 3 cm high) made from No. 8 screen and with wire handles. Move basket up and down thru $CH_2Cl_2$ until chocolate coating dissolves. Rinse each candy center with fine stream of $CH_2Cl_2$ from wash bottle and save center. Repeat with balance of sample. Stir $CH_2Cl_2$-chocolate suspension and pour thru No. 140 sieve. Transfer residue from sieve to filter paper and examine microscopically. Examine candy centers by appropriate method, (**a**), (**b**), (**c**), or (**d**).

Ref.: JAOAC **54,** 568(1971).

## 950.92★ Filth in Chewing Gum

**Procedure**

**Surplus 1970**

*See* **40.082**, 11th ed.

## 945.79 Filth in Sirups, Molasses, and Honey

**Filtration Methods**

**Final Action 1988**

(**a**) Mix sample thoroly and dissolve 200 g in 200 mL hot $H_2O$ acidified with 5 mL $HNO_3$. Filter at once thru rapid paper in Hirsch funnel. Wash with min. amt of hot $H_2O$ and examine microscopically.

(**b**) *Alternative method.*—Dissolve 200 g in 500 mL hot $H_2O$. Filter at once thru 10XX bolting cloth in Hirsch funnel. Wash with min. amt of hot $H_2O$ and examine microscopically.

## 945.80 Filth in Sugars

**Filtration Method**

**Final Action 1988**

Dissolve 100 g sample in ca 200 mL hot $H_2O$. Boil, and filter at once thru rapid paper in Hirsch funnel. Examine microscopically.

# VEGETABLES AND VEGETABLE PRODUCTS

## 945.81 Weevils in Beans and Peas

**Flotation Method**

**First Action**

*Microscopic examination.*—If peas or beans are canned and of normal texture, pour on No. 8 sieve in pan filled with enough $H_2O$ to stand 2–3 cm above mesh of sieve. Mash peas thru sieve with fingers. After as much as possible of material has been worked thru, remove sieve from pan and shake excess $H_2O$ back into pan. Transfer material retained on sieve to 2 L beaker. Pour material that passed thru No. 8 sieve onto No. 40 sieve, discarding that which passes thru. Let material on sieve drain few min, and shake lightly to remove free $H_2O$ from solid material. (If peas are unusually hard, or have tough skins, pass contents of can thru meat or food chopper directly onto No. 40 sieve.) Discard any excess $H_2O$ passing thru this sieve. Cook dried or frozen peas before maceration.

Add material retained on the No. 40 sieve to the beaker. Add ca 130 mL heptane, **945.75C(l)**, to this material and mix thoroly with large spoon. Rinse any material remaining on sieve into beaker with $H_2O$. Stir material in beaker and pick out any insects that may rise to top of $H_2O$ layer. Repeat stirring and searching several times until no more larvae are recovered.

Add enough $H_2O$ to bring contents of beaker to within 1–2 cm of top. Pick out any larvae visible at surface. Stir again, and let mixt. stand ca 5 min; then skim off heptane and upper part of $H_2O$ layer with spoon and place in trap flask, **945.75B(h)**(*4*), previously filled ca 1/4 full of $H_2O$. Add 90–100 mL heptane to material remaining in beaker, and stir vigorously. Let stand ca 5 addnl min, skim off heptane and upper part of $H_2O$ layer as before, and add to material already in trap flask.

Fill flask with $H_2O$. Trap off as much heptane as possible and filter into Hirsch funnel. Lower stopper into flask, and, to rinse sides of trap flask, apply vac. ca 5 min by fitting large rubber stopper and glass tube over mouth of flask. (As ordinary erlenmeyer collapses under vac. of 20" of Hg (50 cm; 67.7 kPa), use either less vac. or heavy-wall flask.) Release vac., add $H_2O$, and trap off. Add trapped-off portion to that already on filter. Examine microscopically.

## 945.82 Light Filth in Broccoli (Canned)

### Flotation Method

### First Action

(**a**) *Insects*.—Transfer contents of can to pan of suitable size and chop up leaves into pieces 2–5 cm long. Weigh 100 g well mixed sample into 1 L beaker. Add 500–600 mL $H_2O$ and boil 5 min. Pour $H_2O$ and sample into 2 L trap flask, **945.75B(h)**(*4*). Add 35 mL heptane, **945.75C(l)**, and stir thoroly to ensure contact between heptane and all portions of leaves. Fill flask with deaerated $H_2O$, let stand 30 min, trap off heptane layer, filter, and examine microscopically. Add 40 mL heptane to flask and repeat extn.

If plant tissue rises to interface, place No. 8 sieve, 6–8″ diam., in suitable size evapg dish contg enough $H_2O$ to cover screen ca 1 cm. Pour entrapped heptane from trap flask onto sieve as it is held under the $H_2O$. Move sieve gently up and down to let insects pass thru into the $H_2O$. Remove screen and filter contents of dish. Repeat washing to free any insects left on greens on screen, and filter washings. Examine papers microscopically.

(**b**) *Aphids and Thrips*.—Det. drained wt of contents of canned greens as in **945.62**, reserving drained liquor. Chop drained leaves into pieces 2–5 cm long and weigh 100 g well mixed sample into 1 L beaker. Add $H_2O$ to cover adequately, followed by 25 g neut. $Pb(OAc)_2.3H_2O$ crystals (or equiv. soln of $Pb(OAc)_2$) and 10 mL HOAc. Boil on hot plate 5–10 min, cool, and transfer to 2 L trap flask, **945.75B(h)**(*4*). Add 35 mL heptane, **945.75C(l)**, and mix thoroly to ensure contact between heptane and all portions of leaves. Fill flask with deaerated $H_2O$. Let settle few min for most of vegetable matter to sink to bottom. To force any tissue that rises (probably held by entrapped globules of heptane) to sink, pivot lower end of trap-rod on bottom of flask, and rotate upper part of rod around neck of flask to knock globules from vegetable tissue without at same time breaking interface and thus rewetting tissue with heptane. Again let flask stand, trap off heptane layer, and filter.

Re-ext with 20 mL heptane, trap off, and filter (usually possible on same paper). Det. total number of aphids or other light filth in entire liquor drained from can by subjecting it to heptane flotation as usual. (Normally liquor does not present any difficulty and use of $Pb(OAc)_2$ is unnecessary.) Count total number of aphids and thrips including parts contg heads. Count cast skins and other insects sep. Calc. on basis of 100 g of drained material.

## 973.61 Foreign Matter in Corn (Canned)

### Flotation and Macroscopic Methods

### First Action 1973
### Final Action 1988

(Applicable to canned whole and cream-style corn)

Place 200 g well mixed sample in 1.5 L beaker and add 1 L 40% isopropanol. Bring to bp, stirring mag., **970.66B(c)**, add 50 mL mineral oil, **945.75C(p)**, and boil and stir 3 min more. Transfer immediately to percolator contg ca 100 mL 40% isopropanol and glass or metal rod for forcing corn thru spout. Retain stirring bar in beaker. Rinse bar with undild isopropanol. Add ca 900 mL 40% isopropanol to beaker, stir, and add to percolator, **945.75B(h)**(*3*). Reserve beaker. After ca 5 min standing, drain percolator to within 8 cm of bottom onto 8″ No. 20 sieve nested in large white enamel tray of ca 2 L capacity. Use rod to force corn thru percolator drain spout. Withdraw rod after removing corn from percolator and wash with small amt of undild isopropanol into reserved beaker. Discard isopropanol collected in tray. Leave sieve in place with retained corn material. Using reserved beaker, add ca 1.5 L hot tap $H_2O$ (50–70°) to percolator. Let phases sep. ca 3 min and make final drain. Discard all but last 5 cm oil-aq. phase. Drain into 600 mL beaker. Wash sides of percolator with alternate isopropanol and $H_2O$ rinses, and collect in same beaker. Add rest of corn from can to corn retained on sieve, sieve portionwise if necessary, and wash with tap $H_2O$ to remove starch and fine particles. Reverse sieve into white enamel tray. Wash corn into tray with forceful spray of $H_2O$ (ca 22°) to 3 cm depth in tray. Let corn settle and examine under $H_2O$ for worm-eaten or rotten kernels and whole worms, heads, or large fragments. Add these to trappings previously obtained from percolator. Tip tray and slowly decant $H_2O$ while carefully observing flowing $H_2O$ for insect fragments. Refill tray with 3 cm $H_2O$ (ca 22°) and repeat decantation, examining closely for objectionable material. Discard pan contents. Filter beaker contents thru ruled filter paper and examine microscopically. If filtration is impeded by excessive starch material, proceed as in **970.66B(d)**.

Ref.: JAOAC **56**, 634(1973).

## 974.33 Filth in Green Leafy Vegetables

### *A. Light Filth*

(Applicable to whole or chopped kale, turnip, mustard, and collard greens.)

(**a**) *Canned* (*First Action 1979; Final Action 1988*).—Drain can contents on No. 8 sieve 2 min and reserve brine. Immediately take 100 g from sieve. If pieces are ≤1 cm in length, place in 2 L trap flask. Cut larger pieces on small cutting board to this size to avoid interference with mag. stirring. Wash residue from cutting into trap flask with 40% isopropanol. Add 40% isopropanol to fill trap flask to 1 L mark. Add mag. stirring bar (13 × 76 mm). Add 50 mL light mineral oil, **945.75C(p)**. Place flask on mag. stirrer, **970.66B(c)**. Slowly bring stirrer to max. speed at which stirring bar is just visible at bottom of vortex, avoiding visible or audible splashing. Proper stirring will produce vertical rolling of contents. Stir 5 min, remove flask from stirrer, and let stand 1 min. Add 40% isopropanol down rod to fill flask. Manually stir material in bottom of flask with rotary motion ca 15 sec. Repeat stirring at two 5 min intervals. Raise stirring rod from bottom of flask and clamp. Let stand addnl 10 min and trap off into beaker. Repeat trapping with 35 mL oil, stirring manually 1 min. Filter onto ruled papers.

Filter reserved brine on sep. papers. Examine papers for aphids and other extraneous materials at 10–30X. Det. number of aphids, etc., in 100 g of drained greens and add to this number in proportionate amt of drained liq. calcd as follows:

$$\frac{100 \text{ g sample}}{\text{total g drained wt}} \times \text{total number aphids, etc. in liq.}$$

Ref.: JAOAC **62**, 600(1979).

(**b**) *Frozen* (*First Action 1974; Final Action 1976*).—Thaw and accurately weigh 100 g sample. Chop into ca 2.5 cm (1″) pieces and mix thoroly. Transfer to 2 L trap flask, **945.75B(h)**(*4*) for collard and mustard greens or beaker for other products. Add *1% anhyd. $Na_2SO_4$ soln* to cover product to depth of 2.5 cm, cover, and boil 30–40 min. Add $H_2O$ as required to maintain original vol. Remove from heat, add 1% $Na_2SO_4$ soln to ca 1.2–1.4 L, and add 35 mL light mineral oil, **945.75C(p)**. Stir mag. 10 min, **970.66B(c)**. Proceed as in **(a)** or **(b)**.

(*1*) *Percolator method*.—Quant. transfer contents to percolator, **945.75B(h)**(*2*), and reserve beaker. Stir gently with long glass stirring rod at 5 and 10 min. Let stand *undisturbed* 5 min; then drain to ca 250 mL. Add ca 1 L 1% $Na_2SO_4$ soln, and repeat drain and refill cycles until lower aq. phase is clear and free of suspended material.

After last cycle, drain oil-$H_2O$ interface into reserved beaker. Immediately wash sides of percolator with 100–200 mL portions hot tap $H_2O$, isopropanol or alcohol, and 5% detergent soln, **945.75C(i)**, if necessary. Filter thru ruled paper, washing sides of beaker as above and using rubber policeman if needed. Examine paper microscopically.

(*2*) *Trap flask method*.—After mixing, add trap flask rod and fill flask slowly with 1% $Na_2SO_4$ soln, letting soln run down rod. Let stand 10 min, stirring gently with rod several times during first 5 min. Secure rod above settled debris during last 5 min. Trap as in **970.66B(b)**, and rinse neck of flask with $H_2O$. Add 25 mL light mineral oil and clamp rod above settled debris. Stir mag. 30 sec and repeat trapping. Make final trap after 10 min, rinsing flask neck with alcohol or isopropanol. Filter thru ruled paper and examine microscopically.

Ref.: JAOAC **57**, 693(1974).

### *B. Heavy Filth*
### *—Final Action 1988*

*Canned*.—Recover heavy filth such as soil, maggots (especially those of spinach leaf miner), and rodent excreta, that sink to bottom of trap flask, as follows: Transfer contents of trap flask, **945.82(a)** or **(b)**, by rinsing with $H_2O$ into 4–6 L pail. Add $H_2O$ to pail until ca full. Stir, let stand short time, and decant ca half pail contents. Refill pail with $H_2O$ and repeat operation until most of floating greens are removed. Wash heavy filth left in pail into black shallow pan and examine visually for larvae, stones, and other debris, picking material out with forceps.

## 975.47 Soil in Spinach (Frozen)
### Acid-Insoluble Residue Method
### Final Action

*See* **971.33**.

## 970.73 Filth in Pureed Infant Food
### Final Action 1988

### *A. Light Filth*

Transfer contents of 2 cans or jars (ca 250 g) of food to 1 L trap flask, **945.75B(h)**(*4*), previously rinsed with $H_2O$. Thoroly mix in ca 20 mL of the oil. Fill with deaerated $H_2O$ either at room temp. or at 50–70°. Let mixt. stand 30 min, stirring 4–6 times during this period to release filth from layer of food. Trap off, filter, and examine microscopically.

Use type of oil and temp. indicated in following table:

| Food | Oil | Temp. |
|---|---|---|
| All fruits, Asparagus, Beets, Carrots, Green beans, Peas | Light mineral oil | Room |
| Spinach | Light mineral oil | 50–70° |
| Squash | Castor oil | 50–70° |

### *B. Fly Eggs and Maggots*

Transfer residue in trap flask, **970.73A**, to 2 L separator. Add ca 100 mL heptane, **945.75C(l)**, and shake vigorously. Let material settle ca 2 hr, occasionally stirring surface layer to permit any eggs and maggots to settle out. Withdraw ca 200 mL from bottom of separator and filter this material thru 10XX bolting cloth, **945.75B(d)**, using several cloths if there is large accumulation of sediment. Examine microscopically at 15–20×.

## 967.24 Filth in Mushrooms

(For maggots, mites, etc., in canned, fresh frozen, freeze-dried, and dehydrate products)

### *A. Insects*
### *—First Action 1967*
### *—Final Action 1989*

**(a)** *Canned mushrooms*.—Pour contents of can evenly over weighed No. 8 sieve. Use 8″ sieve, for containers of net wt <3 lb (1.4 kg) and 12″ sieve for larger containers. Drain 2 min, and reweigh sieve and mushrooms to det. drained wt mushrooms.

Rinse container, and use rinsings and several addnl portions $H_2O$ to rinse mushrooms on sieve (ca 500 mL total). Combine drained liq. with rinsings and filter thru ruled paper. Examine residue on paper microscopically and det. total number of maggots in liq.

Place 100 g drained mushrooms in high-speed blender, **945.75B(c)**. Add 300 mL $H_2O$ and blend 30–45 sec at ca 3000 rpm. Attain proper speed quickly by using setting of 1.5–2× final setting on variable transformer for few sec at start. Fragments of mushrooms after blending should be 3–5 mm long. Pour mixt. into nested set of 8″ Nos. 20, 40, and 140 sieves, **945.75B(r)**. Rinse tissue 2–3 min with spray of tap $H_2O$ from aerator, **945.75B(a)**. Discard material on No. 20 sieve. Transfer residue from No. 40 sieve to 600 mL beaker with $H_2O$ and bring total vol. to ca 100 mL. Add 5 mL *sat. aq. crystal violet soln* and heat to bp. Pour stained mixt. into No. 40 sieve. Wash mushroom tissue, and maggots, if any, to edge of sieve and remove excess stain with tap $H_2O$ from aerator. Using wash bottle contg com. 5.25% NaOCl soln, and gentle spray of tap $H_2O$ from aerator, alternately spray tissue with $H_2O$ and NaOCl soln until stain has been removed from mushroom tissue. Wash tissue into 600 mL beaker and transfer to ruled paper, using vac. Avoid obscuring maggots with mushroom tissues. (Not more than 2–3 papers should be necessary.)

Transfer residue from No. 140 sieve to 600 mL beaker with $H_2O$ and repeat staining, bleaching, and filtering as above.

Examine papers for maggots and other extraneous materials at 10–30×. Maggots are stained dark violet. Det. number of maggots in 100 g drained mushrooms and add to this value the number in proportionate amt of drained liq. calcd as follows:

(100/total g drained mushrooms) × total number of maggots in liq.

**(b)** *Fresh, frozen, freeze-dried, and dehydrated mushrooms*.—For fresh and frozen mushrooms weigh 170 g sample, and for dried mushrooms weigh 15 g sample, into suitable container, and add enough $H_2O$ to immerse mushrooms. Soften mushroom tissue by soaking several hours or, alternatively, by heating on steam bath or simmering $1\frac{1}{2}$–2 hr as necessary, followed by cooling 30–60 min to fully rehydrate. Quant. transfer contents to high-speed blender and proceed as in **(a)**, beginning ". . . blend 30–45 sec at ca 3000 rpm."

Refs.: JAOAC **49**, 576(1966); **50**, 514(1967); **59**, 353(1976).

(For dried (not powdered) products)

### B. Light Filth—Procedure

Thoroly mix sample and weigh 15 g portion. Transfer mushrooms to trap flask, **945.75B(h)**(*4*), add $H_2O$, and let soak several hr, preferably overnight on steam bath, or boil 30 min. Cool to room temp., add 30 mL heptane, **945.75C(l)**, and churn contents by hard, rapid pounding of mushrooms against bottom of flask, using vertical movement of rubber plunger. Trap off twice, filter, and examine microscopically.

## 972.39 Light Filth in Potato Products (Dehydrated)

**Flotation Method**

**First Action 1972**
**Final Action 1988**

Weigh 50 g sample into 1.5–2 L beaker. Add 1 L hot HCl (1 + 9) and mag. stirring bar. Boil 10 min with gentle stirring on mag. stirrer-hot plate, **945.75B(n)**. Sieve portionwise on No. 230 sieve, **945.75B(r)**. Wet residue on sieve with 40% isopropanol and transfer quant. to 2 L trap flask, **945.75B(h)**(*4*), using 40% isopropanol. Bring vol. to 1 L with 40% isopropanol and add 50 mL HCl. Add mag. stirring bar, heat, and boil 10 min with gentle mag. stirring. Immediately transfer flask to cool stirring unit and add 40 mL mineral oil, **945.75C(p)**. Stir mag., **970.66B(c)**, 3 min. Slowly fill flask with 40% isopropanol by letting liq. flow down stoppered rod while top of stopper is held just above liq. After filling flask, gently stir settled plant material by hand 5–10 sec with stoppered rod. Let stand undisturbed 5 min and immediately trap off. Add 25 mL mineral oil, gently stir by hand 30 sec, and let stand 10 min. Repeat trapping. Wash flask neck thoroly with undild isopropanol and transfer washings to beaker contg trappings. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **55,** 71(1972).

## 955.45 Filth in Sauerkraut

**Sieving Method**

**Final Action 1988**

Use entire contents of container of <2 lb (900 g). Use 24 oz (700 g) well mixed sample from larger containers. Wash small portion at time on nested 8″ Nos. 8, 20, and 140 sieves, **945.75B(r)**. Wash material remaining on No. 20 sieve with washings passing thru No. 140 sieve. Transfer material on No. 20 sieve to paper and examine at ca 10× for whole insects or large body parts. Transfer material remaining on No. 140 sieve to paper and examine microscopically.

## 955.46 Filth in Tomato Products

**Final Action 1988**

### A. Fly Eggs and Maggots

(**a**) *Comminuted products.*—Thoroly mix sample and transfer 100 g to 2 L separator. Add 20–30 mL heptane and shake thoroly, releasing pressure as necessary. Fill separator with $H_2O$ in such manner as to produce max. agitation. Place separator in ring stand and let settle; at 15 min intervals during 1 hr, drain 15–20 mL from separator, and gently shake separator with rotary motion to facilitate settling out of fly eggs and maggots. If drained liq. contains seeds, pass it thru No. 10 sieve, and thoroly rinse seeds and sieve, recovering both liq. portion and rinse $H_2O$ in beaker. Filter thru 10XX bolting cloth, pretreated and dyed as in **945.75B(d)**, in Hirsch funnel. Examine for eggs and maggots at ca 10×. If fly eggs or maggots are found in this examination, continue sepg and draining, as above, addnl hr.

(**b**) *Canned tomatoes.*—Pulp entire contents of can in such way that min. number of eggs and maggots are crushed or broken. (This may be done by passing material thru No. 6 or No. 8 sieve and adding seeds and residue remaining on sieve to pulp.)

Place 500 g of the well mixed pulped tomatoes in 6 L separator. Add 125–150 mL heptane, **945.75C(l)**, and ca 1 L $H_2O$ and shake vigorously, releasing pressure as necessary. Fill separator with $H_2O$. Place separator in ring stand and let layers sep. At 15 min intervals during 1 hr, drain 25–30 mL from bottom of separator, and gently shake separator with rotary motion to facilitate settling of fly eggs and maggots. Each portion may be examined at once or combined with subsequent portions. Pass drained portions thru No. 10 sieve and thoroly rinse seeds and sieve, recovering both liq. portion and rinse $H_2O$ in beaker. Filter thru 10XX bolting cloth in Hirsch funnel. Examine cloth for eggs and maggots at ca 10×. If fly eggs or maggots are found in this examination, continue sepg and draining, as above, addnl hr.

### B. Light Filth

(**a**) *Comminuted products.*—Place 200 g of any tomato product except paste (where 100 g is used) in trap flask, **945.75B(h)**(*4*), with 20 mL castor oil and mix well. Add enough hot tap $H_2O$ (ca 70°) to fill flask. (At first, bubbles of air tend to bring up tomato tissues, but after several stirrings these begin to settle out, leaving $H_2O$ layer near oil fairly clear.) Let stand with occasional gentle stirring 30 min; then trap off into beaker. Wash out neck of flask with heptane to remove adhering castor oil. Add little more hot $H_2O$ to flask, stir, let stand 10 min, and then trap off again. (Occasionally it may be necessary to transfer trapped-off material to another trap flask and rewash to eliminate tomato tissue.) Filter trapped-off portion; thoroly wash beaker, sides of funnel, and paper with heptane to dissolve oil and speed filtration. Examine paper at 20–30×.

(**b**) *Canned tomatoes* (*Procedure*).—Drain entire can on No. 6 sieve, saving drained juice. (For cans contg <3 lb use 8″ sieve; use larger sieve for larger cans or drain and rinse portionwise.) Rinse portion on sieve with hot $H_2O$ (ca 70°) from wash bottle and transfer drained juice, fragments, and washings to 1 or more 2 L Wildman trap flasks (max. 900 mL/flask; No. 10 cans require ≥2). Bring vol. in flasks to ca 900 mL with $H_2O$ (70°) and add 20–25 mL castor oil. Tilt flask to ca 45° and mix 1 min with brisk rotary motion (200–250 strokes/min). Avoid splashing thru surface with stopper. Add hot $H_2O$ to bring oil layer into neck and let stand 30 min with occasional stirring. Trap off into beaker oil-$H_2O$ layer and any debris that rises. Wash out oil in neck with heptane. Add ca 10 mL hot $H_2O$ to flask, stir, let stand 10 min, and trap into same beaker. Add 25–30 mL heptane to beaker and stir to dissolve oil. Filter thru paper (use hot $H_2O$ or heptane if necessary) and examine paper microscopically.

# SPICES AND OTHER CONDIMENTS

## 945.83★ Filth in Spices (Whole)

**Flotation Method**

**First Action**
**Surplus 1970**

(Applicable where no specific method exists)

*See* **40.115**, 11th ed.

## 965.40 Filth in Spices

### Flotation Method

### First Action

(Applicable to whole, cracked, or pieces of allspice, anise, caraway, celery seed, cloves, coriander, cumin, dill seed, fennel seed, fenugreek, ginger, mace, mixed pickling spice, mustard, nutmeg, black pepper, white pepper, poppy seed, and turmeric)

Weigh 25 g sample into 400 mL beaker and proceed as in **975.48(a)** and **(b)**, except use more reagent, and where necessary, 2 L trap flask, also use 35 mL heptane, instead of 25 mL. For whole, cracked, or pieces of marjoram, and savory, add 400 mL hot $H_2O$ and 20 mL HCl.

## 960.51 Foreign Matter in Spices and Condiments

### Sieving Method

### First Action

(Applicable to ground allspice, anise, curry powder, dill seed, fennel, fenugreek, poppy seed, savory, and condiments; heavy filth only: caraway seed, cardamon, celery seed, cloves, coriander, cumin, ginger, mace, marjoram, mustard, oregano, rosemary, sage, and thyme)

Sift 200–400 g ground spice thru No. 20 sieve. Transfer any insects or other filth retained on sieve to suitable dish and examine with widefield stereoscopic microscope.

## 975.48 Heavy and Light Filth in Spices and Condiments

### Flotation Method

### First Action 1975

(Heavy and light filth for products lacking specific method)

(*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, chloroform, and petroleum ether.)

**(a)** *Heavy filth and sand.*—Weigh 10 g sample into 250 mL beaker. Add 150 mL pet ether and boil gently 15 min on steam bath in hood. Occasionally add pet ether to keep vol. const. Decant pet ether onto smooth 7 cm paper in buchner. Add 150 mL $CHCl_3$ to beaker and let stand 30 min with occasional stirring. Decant spice and $CHCl_3$ onto funnel, leaving heavy residue of sand and soil, if any, in beaker. If appreciable spice tissue remains on bottom of beaker, add successive portions of $CHCl_3$ mixed with $CCl_4$ to give increasingly higher sp gr until practically all spice tissue is floated off. Transfer residue from beaker to ashless paper and examine microscopically. If there is appreciable amt of residue, place paper in weighed crucible, ignite, and weigh sand and soil.

**(b)** *Light filth.*—Thoroly dry material in buchner and transfer, including fine material that must be scraped from paper, to 1 L trap flask, **945.75B(h)**(*4*). Add ca 150 mL $H_2O$, heat to bp, and simmer 15 min, with stirring; wash down inside of flask with $H_2O$; and cool to <20°. Add 25 mL heptane, **945.75C(l)**, stir mag., and let stand 5 min; then fill flask with $H_2O$ and let stand 30 min. Stir every 5 min, trap off, and filter. Add ca 15 mL heptane and mix thoroly; trap off and filter second time after 15 min. If second extn yields appreciable amt of filth, decant most of liq. from flask, add 15 mL heptane, and make third extn. Examine papers microscopically.

## 975.49 Light Filth in Spices and Condiments

### Flotation Method

### Final Action 1988

(*See* Table **975.49** for applicability and parameters for specific spices.)

#### *A. Pretreatment*

(*Caution:* See safety notes on chloroform.)

Form filter paper cup, 400 mL–1 L, **945.75B(j)**, and weigh sample into cup.

**(a)** *Isopropanol extraction.*—Add 400 mL isopropanol to sample beaker contg cup, and boil gently on hot plate 10 min. Transfer cup to buchner and aspirate to slow drip. Repeat twice with 400 mL isopropanol. Proceed with isolation step specified in Table **975.49**.

**(b)** *Chloroform-isopropanol extraction.*—Add 400 mL $CHCl_3$ to cup in sample beaker, and boil gently on hot plate in fume hood 10 min. Transfer cup to buchner and aspirate to slow drip. Repeat twice with 400 mL $CHCl_3$. Turn off vac., cover sample with isopropanol, and let stand 1 min. Aspirate to slow drip. Repeat isopropanol extn. Proceed with isolation step specified in Table **975.49**.

**(c)** *For crushed red peppers.*—Weigh 25 g sample into filter paper cup formed in 250 mL beaker. Place in 80–85° $H_2O$ bath or on top of steam bath. Add 100 mL isopropanol and heat 5 min. Lift paper cup and let drain. Discard drainings, avoiding contact with liq.

Add 100 mL isopropanol and repeat extn and draining. Place cup in Hirsch funnel and wash with ca 100 mL isopropanol. Aspirate to near dryness.

**(d)** *For ground mace and ground caraway seed.*—Add 400 mL $CHCl_3$ to cup in sample beaker, and boil gently on hot plate in fume hood 10 min. Transfer cup to buchner and aspirate to slow drip. Return cup to empty beaker, add 400 mL isopropanol, and boil gently 5 min. Transfer cup to buchner and aspirate to slow drip.

**(e)** *Alternative solvent saver* (*reflux*) *technic for ground turmeric.*—Weigh sample into 1 L beaker. Add 400 mL isopropanol and boil gently on hot plate 30 min with solv. saver app. inserted into beaker top. Solv. saver app. consists of support stands with clamped 1 L r-b flasks which are stoppered with 2-hole rubber stoppers. Each hole has glass tube and rubber hose attached. One hose is connected to cold $H_2O$ source, the other to drain outlet. With cold $H_2O$ circulating thru flask, which is inserted into 1 L beaker contg sample, solv. is heated to boil and allowed to reflux back into sample for set period of time. Multiple units should be set up in parallel, using "T" connectors, rather than in series, because increased temp. at end of series may affect efficiency of solv. reflux process. See Fig. **975.49**.

Pour sample into No. 230 sieve and wash with gentle stream of hot tap $H_2O$. Proceed with extn as in **975.49B(b)**.

#### *B. Isolation*

**(a)** *Mineral oil–n-heptane* (*85 + 15*).—Quant. transfer sample to 2 L trap flask, **945.75B(h)**(*4*), with 40% isopropanol. Dil. to 400 mL with 40% isopropanol and boil gently 10 min with mag. stirring. Cool in $H_2O$ bath to 20–25°. Add mixt. 50 mL Tween 80-40% isopropanol soln, **945.75C(x)**, and 50 mL $Na_4EDTA$-40% isopropanol soln, **945.75C(z)**, slowly down stirring rod. (Omit for parsley, rosemary, and bay leaves.) Hand stir 1 min, using gentle rotary motion, and let stand 5 min. Dil. to 800 mL with 40% isopropanol, add

**Table 975.49 Methods for Spices, Herbs, and Botanicals; for Those Not Listed, Use 975.48(a) and (b) for Ground Form of Product**

| Spice | Form | Sample, g | Pretreatment **975.49A** | Isolation **975.49B** | Method Heavy | Method Light Filth |
|---|---|---|---|---|---|---|
| Alfalfa leaves | Whole | 10 | | | **975.48(a)** | **985.37** |
| Allspice | Ground | 10 | | | **975.48(a)** | **981.21** |
| Anise | " | 10 | | | **975.48(a)** | **975.48(b)** |
| Annato | " | 25 | | | | **978.20** |
| Basil | Whole (1) | 25 | **b** | **a** | | |
| Bay leaves | " (1) | 25 | **b** | **a** | | |
| Capsiciums[a] | Ground | 25 | | | **978.21** | **978.22** |
| Caraway seed | " (4) | 10 | **d** | **d** | | |
| Cardamon | " | 10 | | | **975.48(a)** | **977.24** |
| Celery leaves | Whole (1) | 25 | **b** | **a** | | |
| Seed | Ground | 10 | | | **975.48(a)** | **977.24** |
| Chervil | Whole (1) | 10 | **a** | **a** | | |
| Chives | " (1) | 5 | **a** | **a** | | |
| Cinnamon | Ground | see method | | | **968.38(a)** | **968.38(b)** |
| | Unground | 100 | | | | **969.43** |
| Cloves | Ground (1) | 10 | **a** | **b** | | |
| Condimental seeds | Whole | 200 | | | **945.84** (Excreta) | |
| Coriander | Ground | 10 | | | **975.48(a)** | **977.24** |
| Cumin | " (1) | 10 | **a** | **b** | | |
| Curry powder | Powder | 10 | | | **975.48(a)** | **975.48(b)** |
| Fennel | Ground | 10 | | | **975.48(a)** | **975.48(b)** |
| Fenugreek | " | 10 | | | **975.48(a)** | **975.48(b)** |
| Dill seed | " | 10 | | | **975.48(a)** | **975.48(b)** |
| Weed | Whole (1) | 25 | **b** | **a** | | |
| Garlic | Powder | 50 | | | **975.50(a)** | **975.50(b)** |
| Ginger | Ground | 10 | | | | **977.24** |
| Mace | Ground (4) | 10 | **d** | **d** | | |
| Marjoram | Ground (1) | 10 | **b** | **b** | | |
| | Unground | 10 | | | | **985.39** |
| Mint | Flakes (1) | 25 | **a** | **a** | | |
| Mustard seed | Ground (1) | 10 | **a** | **b** | | |
| Nutmeg | " | 10 | | | | **979.26** |
| | Reconditioned | see method | | | | **971.35** |
| Onion | Powder | 50 | | | **975.50(a)** | **975.50(b)** |
| Oregano | Ground (1) | 10 | **b** | **b** | | |
| | Unground | 10 | | | | **969.44** |
| Papaya leaves | Whole | 10 | | | | **985.37** |
| Paprika | Ground | 25 | | | | **977.25B** |
| Parsley | Whole (1) | 10 | **a** | **a** | | |
| Pepper | | | | | | |
| Black | Ground | see method | | | **972.40B** | **972.40A** |
| White | " | see method | | | **972.40B** | **977.24** |
| Red | Crushed (2) | 25 | **c** | **c** | | |
| Peppermint leaves | Whole | 5 | | | | **985.37** |
| Poppy seed | Ground | 10 | | | **975.48** | **975.48(b)** |
| Rosemary | " (1) | 10 | **a** | **b** | | |
| | Whole (1) | 25 | **b** | **a** | | |
| Sage | Ground (1) | 10 | **b** | **b** | | |
| | Rubbed | 25 | | | | **979.25** |
| | Rubbed and ground | 10 | | | | **985.38** |
| Savory | Ground | 10 | | | **975.48(a)** | **975.48(b)** |
| Spearmint leaves | Whole | 5 | | | | **985.37** |
| Tarragon | Whole (1) | 10 | **a** | **a** | | |
| Thyme | Ground (1) | 10 | **a** | **b** | | |
| | Whole (1) | 25 | **a** | **a** | | |
| Turmeric | Ground (3) | 10 | **a** or **e** | **b** | | |
| Vegetables | Flakes (1) | 25 | **a** | **a** | | |

[a] Excluding paprika.
Refs.: JAOAC (**1**): **58**, 447(1975); (**2**): **58**, 445(1975); (**3**): **58**, 451(1975); (**4**): **59**, 27(1976).

50 mL flotation liq., **945.75C(k)**, and stir mag., **970.66B(c)**, 5 min. Fill flask with 40% isopropanol, and let stand 30 min with intermittent stirring.

Trap off, rinsing neck of flask with 40% isopropanol. Add 35 mL flotation liq. Hand stir solids on bottom with vigorous rotary motion. Fill flask with 40% isopropanol. Let stand 20 min. Trap off, rinse neck with isopropanol, and filter onto ruled paper. Examine microscopically at 30×.

(**b**) *Mineral oil*.—Place filter cup with sample in No. 230 sieve, **945.75B(r)**, and wash sample into sieve with gentle stream of hot tap $H_2O$. Sieve with forceful spray of hot (55–70°) tap $H_2O$ until rinse is clear. Wet residue on sieve with 40% isopropanol and transfer quant. to 2 L trap flask, **945.75B(h)**(*4*), using 40% isopropanol. Dil. to 400 mL with 40% isopropanol and boil gently 10 min with mag. stirring. Remove from heat and immediately add mixt. 50 mL Tween 80-40% isopropanol soln, **945.75C(x)**, plus 50 mL $Na_4EDTA$-40% isopropanol soln, **945.75C(z)**, slowly down stirring rod. Hand stir 1 min with gentle rotary motion. Let stand undisturbed 5–10 min. Dil. to 800 mL with 40% isopropanol, add

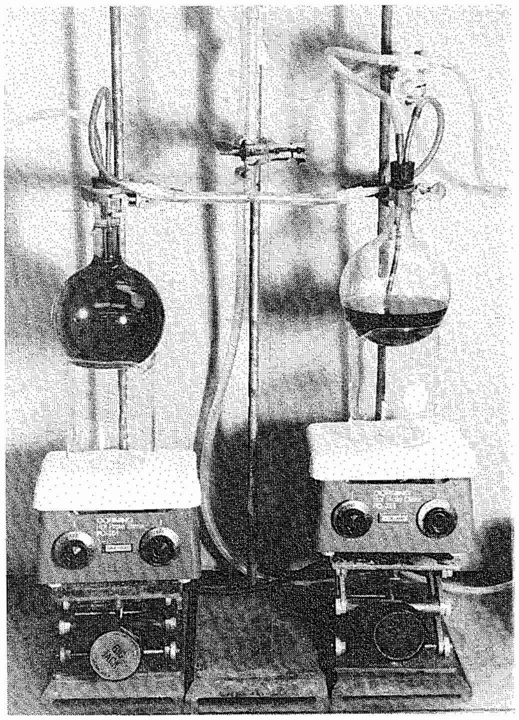


**FIG. 975.49—Solvent saver apparatus**

50 mL mineral oil, **945.75C(p)**, and stir mag., **970.66B(c)**, 3 min. Fill flask with 40% isopropanol, and let stand 30 min with intermittent stirring. Trap off, and repeat as in **(a)**, using 35 mL mineral oil for second trapping.

**(c)** *For crushed red peppers.*—Transfer bulk of sample directly to 2 L trap flask, **945.75B(h)**(*4*), by scraping from paper with spatula. Complete transfer by rinsing paper with 40% isopropanol, and finally dil. to ca 800 mL. Stir and heat to vigorous boil for ca 5 min (*Caution:* Watch for excessive foaming! Control with cold $H_2O$ from wash bottle.). Transfer to cooling bath until temp. drops to 20–25°.

Add 40 mL flotation liq., **945.75C(k)**, and stir mag., **970.66B(c)**, 5 min. Let stand 5 min while mixing 50 mL Tween 80-40% isopropanol soln, **945.75C(x)**, and 50 mL $Na_4$EDTA-40% isopropanol soln, **945.75C(z)**, with 200 mL of 40% isopropanol. Slowly add mixt. down rod with top of stopper held just *below* top of liq. Gently swirl upper portion of liq. using particular care not to disturb settlings at this time.

Let stand 5 min. Raise rod and spin stopper with gentle rotary motion to free suspended material. With top of stopper just *above* oil phase, slowly fill flask with 40% isopropanol. Swirl top portion of liq. gently, avoiding any disturbance of settled material.

Clamp rod and stopper about midpoint of flask. Let stand 5 min, spin stopper to dislodge material on it, and let stand 20–30 min undisturbed. Trap off and rinse neck with 40% isopropanol.

Add 35 mL flotation liq. and swirl rapidly to suspend plant material without incorporating air. Let stand ca 20 min. Trap off and filter onto ruled paper. Examine microscopically at 30×.

**(d)** *For ground mace and ground caraway seed.*—Proceed as in **(b)**, except after adding 50 mL mineral oil, stir mag. 5 min.

Refs.: JAOAC **58,** 445, 447, 451(1975); **59,** 827(1976); **62,** 419(1979); **65,** 1089(1982).

## 985.37 Light Fifth in Whole Leaves of Alfalfa, Papaya, Peppermint, and Spearmint

**Flotation Method**

**First Action 1985**

### *A. Apparatus and Reagents*

**(a)** *Reflux apparatus.*—**975.49A(e)**.
**(b)** *Tween 80–40% isopropanol.*—**945.75C(x)**.
**(c)** *Tetrasodium EDTA–40% isopropanol.*—**945.75C(z)**.

### *B. Pretreatment*

Add 5 g (peppermint, spearmint) or 10 g (alfalfa, papaya) sample and 500 mL isopropanol to 1 L beaker. Boil 10 min with mag. stirrer, using reflux app.

### *C. Isolation*

Wet-sieve product on No. 230 sieve with hot $H_2O$ until washings are clear. Quant. transfer residue to 2 L trap flask with 40% isopropanol. Fill flask to 400 mL with 40% isopropanol. Boil on hot plate for 10 min with slow mag. stirring, **945.75B(n)**. Cool to 20–25° in $H_2O$ bath. Add 100 mL premixed Tween 80–$Na_4$EDTA (1 + 1) down stirring rod. Hand-stir gently 1 min. Let stand 5 min. Fill flask to 800 mL with 40% isopropanol. Add 50 mL flotation liq., **945.75C(k)**, down stirring rod. Mag. stir, **970.66B(c)**, 5 min. Let stand 5 min. Fill flask successively to 1200 mL, 1600 mL, and into neck with 40% isopropanol down stirring rod, agitating contents vigorously (up-down) with rod and wafer for 3–4 s after each fill. Let stand 5 min. Again vigorously agitate flask contents. Let stand 25 min. Trap into 400 mL beaker, washing neck, wafer, and rod with 40% isopropanol and add rinse to beaker. Add 35 mL flotation liq. Hand-stir (up-down) for 30 s. Let stand 20 min. Trap into original beaker, washing neck, wafer, and rod with isopropanol and add rinse to beaker. Filter onto ruled filter paper, wash beaker with isopropanol, and filter washings. Examine papers microscopically at 30×.

Refs.: JAOAC **68,** 697(1985); **70,** 997(1987)

## 981.21 Light Filth in Ground Allspice

**Flotation Method**

**First Action 1981**
**Final Action 1989**

### *A. Determination*

Weigh 10 g sample into 1 L beaker. Add 500 mL 40% isopropanol-HCl (93 + 7). Simmer on steam bath 10 min. Quant. transfer to No. 230 plain weave sieve, **945.75B(r)**, using gentle stream of hot (50–70°) tap $H_2O$. Sieve with forceful stream of hot tap $H_2O$ until rinse is clear. Wet residue on sieve with 40% isopropanol and quant. transfer to 2 L Wildman trap flask, **945.75B(h)**(*4*), using 40% isopropanol. Bring vol. to 400 mL with 40% isopropanol and boil gently 10 min on hot plate, **945.75B(n)**, with mag. stirring. Remove from heat and immediately add, by pouring down stirring rod, 100 mL 1 + 1 mixture of Tween 80-40% isopropanol soln, **945.75C(x)**, and $Na_4$EDTA-40% isopropanol soln, **945.75C(z)**. Hand-stir 1 min, using gentle rotary motion. Let stand 10 min. Bring vol. to 800 mL with 40% isopropanol and add 50 mL mineral oil, **945.75C(p)**. Mag. stir 3 min, **970.66B(c)**, at speed that results in vortex where only upper surface of stirring bar is visible. Fill to top with 40% isopropanol, let stand 30 min with intermittent stirring, and trap off into beaker, rinsing neck of flask with isopropanol. Repeat extraction, using 35 mL mineral oil. Hand-stir bottom contents with gentle rotary motion. Add enough 40% isopropanol to bring oil into neck of flask. Let stand 30 min. Trap off, rinse, and filter entire beaker contents through ruled filter paper(s). Examine for hairs at 30×. If excessive plant tissue is present, bleach as in **965.38B(d)**. Examine microscopically.

Ref.: JAOAC **63,** 1266(1980).

## 978.20 Filth in Ground Annatto

**First Action 1978**
**Final Action 1988**

*See* **978.22**.

## 978.21 Heavy Filth in Capsicums (Ground)

**Sedimentation Method**

**First Action 1978**
**Final Action 1988**

(Applicable to red and cayenne pepper, chili powder, etc.)

(*Caution:* See safety notes on toxic solvents and carbon tetrachloride.)

*For heavy filth and sand.*—Isolate gross filth such as large larvae, adult insects, clumps of webbing, and insect and rodent excreta pellets by sifting pepper thru No. 10 sieve.

Weigh 50 g sifted sample into 600 mL beaker and add 400 mL pet ether. Boil gently 30 min, occasionally adding pet ether to keep vol. constant. Decant pet ether onto smooth 15 cm paper in buchner. Add 400 mL $CCl_4$ and let stand 30 min with occasional stirring. Decant pepper and solv. onto same 15 cm paper in buchner, leaving heavy residue of sand and soil in beaker. Repeat decantation with $CCl_4$ if necessary to secure practically complete sepn of spice tissues from heavy residue. Transfer residue from beaker to ashless paper and examine for filth. If there is appreciable residue, place paper in weighed crucible, ignite, and det. sand and soil.

## 978.22 Light Filth in Capsicums (Ground)

**Flotation Method**

**First Action 1978**
**Final Action 1988**

(Not applicable to paprika. Complete analysis without overnight interruptions.)

***A. Pretreatment***

Proceed as in **975.49A(a)**, using 25 g sample.

***B. Isolation***

Wet-sieve on No. 230 sieve with warm tap $H_2O$ until drainings are clear. Transfer sample with spoon thru wide-stem funnel into 2 L trap flask, **945.75B(h)(*4*)**. Rinse remaining material to edge of sieve and transfer quant. to trap flask with 60% alcohol. Dil. to 600 mL with 60% alcohol.

Simmer 10 min on hot plate. Alternatively, place on preheated hot plate, bring to boil, and then transfer to 3–4 cm deep boiling $H_2O$ bath for 10 min and simmer. This technic avoids severe frothing encountered in hot plate boiling. Cool to ≥20° but <25°. Remove from bath and add 40 mL flotation liq., **945.75C(k)**. Dil. to 800 mL with 60% alcohol and stir mag. 5 min, **970.66B(c)**. Set aside, add 100 mL 1 + 1 mixt. Tween-80 plus 60% alcohol, **945.75C(w)**, and $Na_4EDTA$-alcohol soln, **945.75C(y)**, and mix thru liq. by gently swirling stopper (wafer) 1 min. Let stand 3 min. Slowly add 60% alcohol down trap rod, maintaining stopper above oil layer, until oil just reaches neck of flask. Swirl stopper thru lower portion of trap flask to suspend settlings (dark material may rise halfway up in trap flask). Add 60% alcohol down rod to bring bottom of oil layer to level 1 cm above fully raised stopper.

Clamp rod with stopper at ca midpoint of flask. Let stand 15 min; then gently swirl stopper thru upper half of liq. to hasten rising of oil droplets. Let stand 15 min undisturbed and trap off into beaker, rinsing neck of flask with 60% alcohol. Filter onto labeled ruled paper.

Add 30 mL flotation liq. and stir manually 1 min. Clamp rod at midpoint and let stand 10 min. Swirl gently thru upper half of liq. and adjust oil level. Let stand 15 min and trap off. Rinse neck of flask with 95% alcohol or isopropanol. Filter onto second ruled paper and examine papers at 30×.

Ref.: JAOAC **61,** 900(1978).

## 977.24 Light Filth in Cardamon, Celery Seed, Coriander, Ginger, and Pepper (White)

**Flotation Method**

**First Action 1977**

(Applicable to the ground spices)

Weigh 10 g sample (25 g for white pepper) into 800 mL beaker contg 400 mL isopropanol. Add mag. stirring bar, place on stirrer, **945.75B(n)**, and stir mag. 6 min, keeping all solids in motion. Pour mixt. onto No. 230 sieve, **945.75B(r)**, and wash residue with $H_2O$ until washings are clear. Transfer residue from sieve with 40% isopropanol into 2 L trap flask, **945.75C(h)(*4*)**. Add 760 mL 40% isopropanol and 40 mL HCl. Bring to vigorous boil on hot plate with mag. stirring. Cool to 20–25° in cold $H_2O$ bath. Add 40 mL flotation liq., **945.75C(k)**, and stir mag., **970.66B(c)**, 5 min. Let stand 5 min and then slowly fill flask with 40% isopropanol by letting liq. flow down stirring rod with top of stopper just below oil layer. Resuspend material at bottom of flask without disturbing oil (upper) layer. Let stand 20 min, stirring bottom occasionally, and trap off. Add 30 mL flotation liq., and stir mag. 30 sec while pushing oil into aq. (lower) layer; continue stirring 4.5 min. Let stand 15 min, trap off, filter onto ruled paper, and examine microscopically at 30×. If filtering action slows, use new filter paper.

Ref.: JAOAC **60,** 117(1977).

## 968.38 Filth in Cinnamon (Ground)

**Flotation Method**

**First Action 1968**

**(a)** *Heavy filth and sand.*—Weigh 2 g sample into 50 mL centrf. tube and add ca 45 mL $CCl_4$. Centrf. 5 min at 800 rpm. Stir layer at top of liq. and repeat centrfg. Decant ca $^2/_3$ of liq. and floating layer, and add fresh $CCl_4$ up to 45 mL. Mix thoroly and again centrf. Decant as much of liq. and floating layer as possible without disturbing residue in centrf. tube. Wash residue onto 11 cm ashless paper with $CCl_4$. Examine under low-power microscope for filth. If there is appreciable residue, place paper in weighed crucible, ignite, and weigh sand and soil.

**(b)** *Light filth.*—(Where alcohol and 60% alcohol are specified, isopropanol and 40% isopropanol, resp., may be substituted. Use same alcohol thruout method.) Weigh 50 g sample into 800 mL beaker. Add 500 mL hot (55–70°) tap $H_2O$ and 50 mL HCl. Stir several min with stirring bar at high speed on mag. stirrer-hot plate, **970.66B(c)**, holding temp. without boiling until gel is dispersed (suspension will become less viscous and vortex will become more pronounced). Sieve portionwise onto No. 230 sieve, **945.75B(r)**, with forceful stream of hot tap $H_2O$, using aerator, **945.75B(a)**. After fine material has passed thru sieve, wash residue alternately with alcohol and hot tap $H_2O$ until most foam and color have passed thru.

Transfer residue to 1.5 L beaker with 60% alcohol, using spoon to transfer bulk of material. Dil. to 1 L with 60% alcohol. Add 50 mL HCl and heat (do not boil) while stirring with mag. stirrer to prevent charring. When mixt. is hot (ca 55°), add 50 mL mineral oil, **945.75C(p)**, and stir mag. 4 min. Transfer beaker contents to 2 L percolator, **945.75B(h)**(*2*), rinse beaker well with 60% alcohol, and add rinsings to percolator. Bring vol. in percolator to ca 1.7 L with 60% alcohol. Resuspend material in percolator by vigorously stirring with glass rod, and rinse rod into percolator with 60% alcohol. Let settle 3 min, and immediately drain material in percolator to within several cm of bottom of mineral oil layer. Refill percolator with hot tap $H_2O$, adding $H_2O$ rapidly to thoroly resuspend material in percolator. Let settle 3 min, and drain again. Repeat hot $H_2O$ rinses until aq. medium is practically free of suspended matter with max. of 7 rinses. Discard hot $H_2O$ rinses. Drain mineral oil layer into 800 mL beaker and rinse sides of percolator with alternate rinses of 95% alcohol and hot (55–70°) tap $H_2O$ (use rubber policeman if necessary). Pour mineral oil and final rinses onto ruled paper and examine microscopically.

Ref.: JAOAC **51,** 518(1968).

## 969.43 Light Filth in Cinnamon (Unground) (Crude and Reconditioned)

**Sieving Method**

**First Action 1969**

If sample is reconditioned or if pieces are not rolled and are 8 cm (3″) long, weigh 100 g sample directly into 1.5 L beaker. If sample consists of quills, break open quills into lengths of ≤8 cm and transfer broken pieces, including dust and small particles, to 1.5 L beaker. Add 1 L hot tap $H_2O$ and 50 mL HCl. Heat on hot plate to ca 60°. Pour portionwise onto No. 6 over No. 230 sieve, **945.75B(r)**, and rinse well with forcible stream of hot tap $H_2O$, using aerator, **945.75B(a)**, while turning larger pieces with glass rod. Discard material on No. 6 sieve and transfer residue on No. 230 sieve to 2 L trap flask, **945.75B(h)**(*4*), with hot tap $H_2O$, using spoon if necessary. Fill trap flask to 1 L with $H_2O$ and add 50 mL HCl. Heat with stirring to ca 60–70°. Add 50 mL mineral oil, **945.75C(p)**, and stir mag., **970.66B(c)**, 2 min. Fill with $H_2O$, let stand 5 min, and trap. Add 25 mL mineral oil, gently stir with stopper 1 min, let stand 5 min, and again trap. Rinse neck of flask with alcohol or isopropanol. Filter trappings onto ruled filter paper and examine for insects and other arthropods, hairs, excreta, etc.

Ref.: JAOAC **52,** 469(1969).

## 945.84 Excreta (Rodent and Insect) in Condiment Seeds

**Sedimentation Method**

**First Action**

Prep. liq. with sp gr of 1.16–1.19 by mixing $CHCl_3$ or $CCl_4$ with alcohol or pet ether. Mix 200 g sample with 500–700 mL of the liq. in 1 qt (1 L) drug percolator. Let stand 30 min, stirring at ca 5 min intervals. Trap sediment in lower end of percolator with cork plug and remove lower cork so as to deliver all sediment into beaker. Lift upper cork slightly and rinse tube and cork by letting small amt of liq. pass. After stirring top layer, make 2 more sepns at 5 min intervals. Transfer contents of beaker to filter paper, drain liq., and examine. Sep. rodent excreta and insect excreta, air dry, and weigh each sep. to nearest mg.

## 975.50 Filth in Garlic Powder and Ground Onion

**Sedimentation/Flotation Method**

**First Action**

(*Caution:* See safety notes on toxic solvents and carbon tetrachloride.)

(**a**) *Heavy filth and sand.*—Weigh 50 g sample into 250 mL hook-lip beaker. Add 200 mL $CCl_4$, stir thoroly, and let stand 30 min with occasional stirring. Decant plant tissue onto 15 cm paper in buchner, add 100 mL $CCl_4$, and repeat decantation until practically no plant tissue remains with sand and soil on bottom of beaker. Transfer residue in beaker to ashless paper with stream of $CCl_4$ from wash bottle and examine for filth. If there is appreciable residue, place paper in weighed crucible, ignite, and weigh sand and soil.

(**b**) *Light filth.*—Dry residue of plant tissue from buchner, (**a**), overnight or in oven 1 hr at 80°, and transfer to 2 L trap flask, **945.75B(h)**(*4*). Add 250 mL Tween 80–60% alcohol soln, **945.75C(w)**, mix well, and let stand 15–30 min. Add 60% alcohol to 800 mL and trap off twice in 60% alcohol with 75 and 35 mL heptane, **945.75C(l)**, resp., as in **970.66B(b)**. Let stand 1–1.5 hr for each extn and avoid stirring except for few circular upward strokes immediately after filling flask with 60% alcohol. Filter, and examine microscopically.

## 979.25 Light Filth in Sage (Rubbed)

**Flotation Method**

**First Action 1979**

### *A. Pretreatment*

Form 32 cm filter paper cup over 400 mL beaker and insert into 1 L beaker, **945.75B(j)**. Place 25 g sample into cup. Add 400 mL $CHCl_3$, ca equally between cup and beaker. Boil gently 10 min in hood. (Alternatively, bring to bp on hot plate and continue heating 10 min total on steam bath.) Transfer cup to buchner and aspirate to slow drip. Return cup to beaker and repeat extn with two 400 mL portions $CHCl_3$. After third $CHCl_3$ aspiration, turn off vac., cover sample with isopropanol, let stand 1 min, and aspirate to slow drip. Repeat isopropanol extn once.

### *B. Isolation*

Transfer sample from paper cup to No. 230 sieve, **945.75B(r)**, with gentle stream of hot tap $H_2O$. Sieve with forceful spray of *hot* (55–70°) tap $H_2O$ until rinse is clear. Wash residue to edge of sieve and let drain momentarily. Rinse sieve with ca 100 mL of alcohol delivered from wash bottle. Let stand 1 min. Again wash sieve residue with hot tap $H_2O$ until drainings are colorless; then wet well with 40% isopropanol.

Add mag. stirring bar, **945.75B(n)**, to 2 L trap flask, **945.75B(h)**(*4*). Place wide stem funnel in flask opening and transfer bulk of sample with spoon. Rinse remaining material to edge of sieve with aerator spray and quant. transfer to flask with total of 400 mL 40% isopropanol. Stir gently while boiling 10 min on hot plate. (Alternatively, bring to boil on hot plate and continue heating 10 min in ca 40 mm boiling water bath.) Remove from heat and immediately add 100 mL 1 + 1 mixt. Tween 80 plus isopropanol soln, **945.75C(x)**, and $Na_4EDTA$, plus 40% isopropanol soln, **945.75C(z)**, slowly down rod and mix by gentle swirling 1 min.

Cool in $H_2O$ bath at 20–25°. Add 50 mL mineral oil, **945.75C(p)**. Dil. to 800 mL with 40% isopropanol added slowly down stirring rod to avoid mixing or agitation of flask contents.

Stir mag., **970.66B(c)**, 5 min, and let stand 3 min. Add 100 mL premixed Tween 80-$Na_4EDTA$ and very gently swirl thru top of liq. 1 min.

Fill flask with 40% isopropanol, added slowly down stirring rod to minimize agitation of liq. Let stand 20 min *undisturbed*. Trap off, rinsing neck of flask with 40% isopropanol, and add to trappings in beaker.

Add 35 mL mineral oil and hand stir 1 min. Let stand ca 1 min. Slowly fill flask with 40% isopropanol. Let stand 7 min, spin stopper to free of settlings, adjust oil level to ca 1 cm above fully raised stopper and let stand 8 min. Trap off into beaker, rinsing neck of flask well with isopropanol. Transfer trappings to ruled filter paper, rinsing beaker well with isopropanol. Examine papers at 30X.

Ref.: JAOAC **62,** 597(1979).

## 985.38 Light Filth in Sage (Rubbed and Ground)

### Brine Saturation Method

### First Action 1985

#### A. Reagents

(**a**) *Brine.*—Prep. by dissolving ca 360 g NaCl/L $H_2O$. Less expensive sources of salt which were found by use and by comparative study to be equiv. to AR grade NaCl were: *(1)* Morton Rock Salt for making ice cream, Morton Salt Div. of Morton Thiokol, Inc., Chicago, IL 60606; or, *(2)* Sterling salt crystals for water softeners, International Salt Co., AKZO, Abington Executive Park, Clarks Summit, PA 18411. Filter brine before use.

(**b**) *Olive Oil, NF.*—Less expensive sources of olive oil which were found to be equiv. to NF grade oil by use and by comparative study and which were more readily com. available were: *(1)* James Plagnoil Pure Olive Oil, Marseille, France; *(2)* Bertolli Pure Olive Oil, Lucca, Italy; *(3)* Vigo Spanish Virgin Olive Oil imported by Vigo Import Co., Tampa, FL; and *(4)* Flag Brand Pure Sicilian Olive Oil, Product of Italy, packed for Progresso Foods Div., Imasco Corp., Harahan, LA.

#### B. Extraction

*Rubbed and ground sage.*—Weigh 10 g sample into 600 mL beaker. Add mag. stirring bar, **945.75B(n)**, and 300 mL isopropanol. Cover with watch glass and boil ca 3 min with const stirring on mag. stirrer hot plate. Transfer to 230 mesh sieve, **945.75B(r)**, with isopropanol, and wash with ca 100 mL isopropanol from wash bottle, then with hot tap $H_2O$ to remove isopropanol, and then with brine to remove tap $H_2O$. Transfer to original 600 mL beaker and bring vol. to ca 350 mL with brine. Cover and bring to boil with const stirring. (*Note:* Do not boil. Product loss will occur from boil-over.) Transfer beaker to cold $H_2O$ bath (use plastic or glass bowl filled with cold $H_2O$ on mag. stirrer). Uncover, wash down insides of beaker, and cool to touch with const stirring. Repeat heating and cooling to complete brine satn, and then transfer to 2 L trap flask, **945.75B(h)**(*4*), with ca 100 mL brine, fill flask ca $^3/_4$ full with 32° (± 2°) tap $H_2O$, and insert trap rod. (*Note:* Temp. variation below that specified could result in loss of oil trapped due to adhesion to sides of flask.) Add 50 mL olive oil, and then fill flask to normal (neck) level with tap $H_2O$. Place on mag. stirrer with trap rod to one side of flask bottom to avoid interfering with stirring bar. Stir rapidly for 5 min so that no significant oil layer is visible in neck of flask but not so rapidly that vortex extends more than 1 in. into flask. Remove from stirrer and let oil layer rise. Stir bottom of flask to release any trapped oil; repeat after 5 min. Wait ca 20 min, and then briefly and gently stir entire flask contents, particularly at the neck, to release and sink any product that has accumulated at interface. After 10 min, stir interface only with pivoting rotary motion of trap rod, keeping plunger relatively stationary at bottom of flask. Wait 10 min and repeat previous stirring if product has accumulated at interface. Then wait 10 more min and trap oil layer into 250 mL beaker, rinsing neck of flask and trap thoroly several times with isopropanol and finally with $H_2O$. Filter on ruled paper and examine microscopically, **970.66B(g)**.

Ref.: JAOAC **68,** 894(1985).

## 985.39 Light Filth in Unground Marjoram

### Flotation Method

### First Action 1985

#### A. Sample Preparation

Form filter paper cup, 400 mL into 1 L, **945.75B(j)**, and weigh 10 g sample into cup.

Place cup into 1 L beaker contg 400 mL 95% EtOH. Boil gently on hot plate 10 min, with mag. stirring. **970.66B(c)**. Place cup in buchner funnel. Draw off excess EtOH with vac. Discard EtOH in beaker. Repeat operation twice more; then proceed with extn.

Quant. transfer material to 2 L Wildman trap flask. **945.75B(h)**(*4*). Bring to 600 mL with 60% EtOH. Add mag. stir bar, **945.75B(n)**. Boil gently 10 min with mag. stirring at minimal rate. If material accumulates on walls of flask, rinse side with minimal amt of 60% EtOH. Remove from heat and add 100 mL Tween 80–EDTA mixt. [**945.75C(w)**–(**y**)] (1 + 1). Hand-stir contents, using gentle rotary motion; then cool in $H_2O$ bath to 25 ± 2°. Bring vol. to 800 mL with 15% EtOH. Add 50 mL mineral oil, **945.75C(p)**. Stir mag. 3 min at speed resulting in vortex where only the upper surface of stirring bar is visible. Add enough 15% EtOH to bring interface into neck of trap flask and let stand. Stir bottom of flask gently at 10 and 20 min intervals. Let stand addnl 10 min. Trap off, rinsing neck of flask with 15% EtOH. Repeat extn using 35 mL mineral oil. Vigorously hand-stir trap flask contents to mix oil with aq. phase. Add enough 15% EtOH to bring oil to neck of flask. Trap off, and rinse with 95% EtOH followed by 60% EtOH. Filter entire beaker contents thru ruled filter paper(s). Examine microscopically at 30×.

Ref.: JAOAC **68,** 699(1985).

## 979.26 Light Filth in Nutmeg (Ground)

### Flotation Method

### First Action 1979

### Final Action 1988

#### A. Pretreatment

Form 32 cm filter paper defatting cup, 400–800 mL, **945.75B(j)**. Weigh 10 g sample into cup, add 400 mL $CHCl_3$, and simmer 10 min. Drain or aspirate and discard $CHCl_3$. Return cup to beaker, add 400 mL isopropanol, and heat to vigorous boil.

### B. Isolation

Immediately wet-sieve on No. 230 sieve, **945.75B(r)**, until washings are clear. Rinse material on sieve with 40% isopropanol and let drain. Quant. transfer to 2 L trap flask, **945.75B(h)**(*4*), with 40% isopropanol and dil. to 800 mL. Add stirring bar, **945.75B(n)**, and heat to vigorous boil while stirring. Add 40 mL mineral oil, **945.75C(p)**, and continue heating to vigorous boil.

Transfer flask to cool stirrer, and mag. stir, **970.66B(c)**, 5 min. Set aside and fill with 40% isopropanol down stirring rod to bring oil just into neck of flask. Stir to suspend settlings. Stir after 5 min and clamp wafer at ca midpoint of flask. After 5 min adjust oil level to 1 cm above fully raised stopper, and swirl interface. Let stand 5 min and trap off into beaker. Rinse neck with 40% isopropanol and add rinse to trappings.

Add 30 mL mineral oil and hand stir 1 min. Let stand 10 min and trap off. Rinse neck of flask with isopropanol and add rinse to beaker. Filter onto ruled paper(s). Examine papers at 30X.

Ref.: JAOAC **62,** 595(1979).

## 971.35 Light Filth in Nutmeg (Reconditioned)

**Flotation Method**

**First Action 1971**
**Final Action 1988**

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Weigh 100 g sample into 1.5 L beaker (50 g if finely ground product is used). Add 400 mL $CHCl_3$ and boil 5 min. Prep. 32 cm folded S&S 588 filter paper, by moistening with $H_2O$ and forming around base of 1 L beaker. Place 7 cm disk of bolting cloth (mesh size not critical) in 10 cm plate diam. buchner, insert paper, apply vac., and press moistened paper until good seal is obtained. Rinse paper with isopropanol, and aspirate until nearly dry. Quant. transfer nutmeg to paper, and aspirate off $CHCl_3$. Transfer paper contg spice tissue back to original beaker, add 400 mL fresh $CHCl_3$, and boil 5 min. After cooling few min, lift paper, and drain and discard $CHCl_3$. Replace paper in beaker, add 400 mL $CHCl_3$, and repeat 5 min boiling third time.

Replace paper in buchner and aspirate off $CHCl_3$, maintaining suction ca 5 min after visible dripping ceases. Release vac.; add isopropanol until spice is covered, let stand few min, and reapply vac. until visible dripping ceases. Repeat isopropanol wash step and aspirate ca 5 min after visible dripping ceases.

Quant. transfer retained spice to 8" diam. No. 230 sieve, **945.75B(r)**, with copious rinses of hot tap $H_2O$. Wash material on sieve with forceful stream of hot $H_2O$ from aerator, **945.75B(a)**, until no more spice tissue passes sieve. Transfer most of sieve contents with spoon and quant. transfer remaining material to 2 L trap flask with 60% alcohol-$CaCl_2$ soln, **945.75C(c)**, from wash bottle. Add mag. stirring bar, bring vol. to 1 L with 60% alcohol-$CaCl_2$ soln, add 50 mL HCl, and place flask on mag. stirrer-hot plate, **945.75B(n)**. Heat to *full boil* with gentle stirring. Immediately transfer flask to cool stirring unit and add 40 mL light mineral oil, **945.75C(p)**, by pouring down stirring rod. Stir 2 min with mag. stirrer, **970.66B(c)**. Fill with 60% alcohol-$CaCl_2$ soln and gently stir 5–10 sec with stirring rod. Let stand 2 min and trap off. Add 35 mL light mineral oil, stir by hand gently 30 sec, and let stand 10 min. Repeat trappings. Wash flask neck thoroly with isopropanol and transfer washings to beaker with trappings. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **54,** 575(1971).

## 969.44 Light Filth in Oregano (Unground)

**Flotation Method**

**First Action 1969**

### A. Reagent

*15% Alcohol.*—Prep. ca 1700 mL/sample prior to analysis.

### B. Determination

Weigh 10 g sample into 2 L trap flask, **945.75B(h)**(*4*), add 400 mL 60% alcohol, and boil gently 10 min, occasionally swirling flask gently and/or using plunger to prevent material from accumulating on wall of flask above surface of liq. Immediately add 100 mL 1 + 1 mixt. Tween-80 plus 60% alcohol soln, **945.75C(w)**, and $Na_4EDTA$ plus alcohol soln, **945.75C(y)**, and swirl few sec, again using plunger to clear material from wall of flask. Let stand 10 min in cold $H_2O$ bath. Dil. to 800 mL with 15% alcohol. Add 50 mL mineral oil, **945.75C(p)**, and stir mag., **970.66B(c)**, 2 min. Fill with 15% alcohol and hand stir every 2–3 min for 20 min. Clamp stirring rod in place so that plunger is held above sediment at bottom of flask. Leave flask undisturbed 10 min. Trap, filter onto ruled paper, and examine microscopically.

Ref.: JAOAC **52,** 21(1969).

## 977.25 Filth in Paprika

**First Action 1977**
**Final Action 1988**

### A. Gross Contamination

*See* **960.51**.

#### *Light Filth in Ground Paprika*

### B. Pretreatment

Form 32 cm filter paper (rapid flow) cup around 400 mL beaker, as in **945.75B(j)**. Remove paper, place in 1 L beaker, and add 25 g sample.

Pour 400 mL isopropanol into the 1 L beaker, distributing liq. equally inside and outside cup. Place on preheated hot plate and boil gently exactly 10 min. (Alternatively, bring to boil on hotter hot plate; then set aside *in* steam bath opening for 10 min boil.) Remove cup from beaker without delay, and let drain or place on buchner and aspirate to slow drip. Discard liq. Replace cup in 1 L beaker and repeat twice with 400 mL isopropanol.

### C. Isolation

Wash sample from cup into No. 230 sieve, **945.75B(r)**, with gentle $H_2O$ stream, avoiding splashing and loss of sample. Wet sieve with forceful stream of warm $H_2O$ from aerator until washings are clear. (Ignore foam or froth produced by action of strong spray on paprika.)

Add 400 mL 40% isopropanol to wash bottle. Place wide-stem powder funnel in trap flask. Transfer bulk of sieved sample to trap flask, using portion of 40% isopropanol. Wash remaining material on sieve to edge with warm $H_2O$ and complete quant. transfer to trap flask with 40% isopropanol. Wash walls of flask with 40% isopropanol. Pour remainder of 400 mL 40% isopropanol into trap flask.

Place on hot plate and boil gently 10 min; swirl to rinse material from walls of flask. (Do not allow material to accumulate and dry on flask walls.) Remove from hot plate and immediately add 100 mL 1 + 1 mixt. Tween-80 plus 40% isopropanol soln, **945.75C(x)**, and $Na_4EDTA$ plus 40% isopropanol soln, **945.75C(z)**. Stir gently ca 1 min. Let stand 10 min. Dil. to 800 mL with 40% isopropanol added slowly down stirring rod, positioned with stopper just above liq. level. Add 50 mL mineral oil, **945.75C(p)**, and stir mag., **970.66B(c)**, 3 min, with stopper located above liq. level.

Add 40% isopropanol slowly down stirring rod to bring oil into neck of flask. Let stand ca 10 min. Raise stopper to middle of flask and swirl gently to cause movement of upper liq. and hasten rising of oil droplets. Rinse rod with 40% isopropanol and clamp so that stopper is at midpoint of flask. Add 40% isopropanol down rod to bring bottom of oil layer to level 1 cm above fully raised stopper. Let stand 10 min and swirl gently again. Let stand 10 min undisturbed and trap off into beaker.

Add ca 35 mL mineral oil and hand stir 1 min at speed sufficient to keep oil moving thru trap flask. Add ca 20 mL 40% isopropanol, stir gently at ca 5 min intervals for 20–25 min, then let stand undisturbed 5–10 min. Trap off into second beaker and rinse neck of trap flask with alcohol or undild isopropanol.

Filter solns from both beakers onto sep. ruled filter papers in Hirsch funnel, rinsing each beaker carefully with isopropanol. Examine microscopically at 30×.

Ref.: JAOAC **60,** 114(1977).

## 972.40 Light Filth in Pepper

**First Action 1972**
**Final Action 1988**

### *A. Light Filth (Ground Black Pepper Only)*

Weigh 50 g sample (or use floated material from **972.40B** before ignition) into 400 mL beaker and add enough hot $H_2O$ (55–70°) to make thin slurry. Pour slurry onto No. 230 sieve, **945.74B(r)**, and wash residue with forceful stream of hot $H_2O$, using aerator, **945.75B(a)**, until effluent is clear. Wash sieve residue with ca 100 mL isopropanol and let drain. Transfer residue from sieve with 40% isopropanol into 2 L trap flask. Dil. to 800 mL with 40% isopropanol. Bring to rolling boil with mag. stirring. Cool to room temp. in $H_2O$ bath. Add 40 mL flotation liq., **945.75C(k)**, and stir mag. 3 min, **970.66B(c)**. Let oil phase sep. 5 min and then fill flask with 40% isopropanol by letting liq. flow down stirring rod. Let stand 20 min, with gentle stirring at 5 min intervals, and trap off. Repeat trapping with 20 mL flotation liq.; stir gently after addn to avoid disturbing bottom layer. Let stand 10 min and trap off. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **55,** 83(1972).

### *B. Heavy Filth and Sand (Ground Black and White Pepper)*

(*Caution:* See safety notes on toxic solvents and carbon tetrachloride.)

Weigh 50 g sample into 600 mL beaker. Add 400 mL $CCl_4$ and let beaker stand ≥1 hr with occasional stirring. Decant pepper and solv. onto 15 cm paper in buchner, leaving heavy residue of sand and soil in beaker. Repeat decantation with $CCl_4$ if necessary to secure practically complete sepn of spice materials from any heavy residue. Transfer residue from beaker to ashless paper and examine for filth. If there is appreciable residue, place paper in weighed crucible, ignite, and weigh sand and soil.

## 972.58★ Light and Heavy Filth in Peppers (Unground, Fermented, Crushed)

**First Action**
**Surplus 1970**

*See* **40.121**, 11th ed.

## 972.59★ Light and Heavy Filth in Pepper Sauce

**First Action**
**Surplus 1970**

*See* **40.122**, 11th ed.

## 945.85 Filth in Pickles

**First Action**

### *A. Whole Pickles*

Pour entire contents of jar onto No. 8 sieve nested in No. 140 sieve, **945.75B(r)**. Wash jar thoroly to remove any filth adhering to sides, and pour washings thru sieves. Wash pickles thoroly with stream of hot $H_2O$, turning from time to time. Transfer material on No. 140 sieve directly to ruled paper and examine microscopically.

### *B. Chopped Pickles and Relish*

Add 200 mL $H_2O$ to 100 g sample in trap flask or beaker, boil 15 min, and cool. If boiling is done in beaker, transfer to trap flask, **945.75B(h)**(*4*). Trap off twice, using 25 and 15 mL heptane, **945.75C(l)**. Filter, and examine microscopically.

## 945.86 Filth in Dressings for Food

**Filtration Method**
**First Action**

(Applicable to salad dressing, french dressing, and related products)

Weigh 200 g sample into 800 mL beaker, stir in 50 mL $H_3PO_4$, and mix thoroly. Thin with ca 600 mL $H_2O$, and again mix thoroly. If possible, filter thru S&S No. 8 ruled paper with suction; otherwise thru No. 140 sieve, **945.75B(r)**, and transfer to ruled paper. Examine papers microscopically.

## 973.62 Filth in Horseradish (Prepared)

**Flotation Method**
**First Action 1973**
**Final Action 1988**

Weigh 100 g sample into 600 mL beaker. Add 200 mL $H_2O$ and transfer to 2 L trap flask with $H_2O$. Dil. to 1 L, add 50 mL HCl, and stir few sec. Add mag. stirring bar, **945.75B(n)**, and 50 mL flotation liq., **945.75C(k)**, and stir mag., **970.66B(c)**, 3 min. Slowly fill flask with distd $H_2O$ by running liq. down stoppered rod while stopper is maintained just above liq. After filling flask, gently stir settled material 5–10 sec with stoppered rod. Let stand undisturbed 5 min; then trap off. Add 35 mL flotation liq., stir gently by hand 30 sec, and let stand 10 min. Repeat trapping. Wash flask neck thoroly with isopropanol and transfer washings to beaker contg trappings. Filter onto ruled paper and examine microscopically.

Ref.: JAOAC **56,** 629(1973).

## 968.39 Light Filth in Mustard (Prepared)

**Flotation Method**

**First Action 1968**
**Final Action 1988**

Weigh 100 g well mixed sample into 1 L beaker, and slowly add 400 mL HCl (3 + 97) and 20 mL mineral oil, **945.75C(p)**, with constant stirring until smooth slurry forms. Place on hot plate and bring to *rolling boil;* hold at *rolling boil* ca 10 min. Transfer quant. to Kilborn funnel, **945.75B(h)**(*1*); retain beaker and stirring rod for rinsing. Fill separator to ca 1 cm from top with cold $H_2O$.

After 1.5–2 min, gently stir contents of separator; let oil layer sep. again ca 1.5–2 min, and slowly drain and discard lower layer until interface is ca 5 cm above constriction. Fill separator with cold $H_2O$ to ca 1 cm from top; let oil sep. 1.5–2 min, and slowly drain and discard lower aq. layer until interface is ca 5 cm above constriction. Repeat $H_2O$ wash until lower layer is clear.

Filter mineral oil and $H_2O$ retained in separator thru ruled paper, **945.75B(i)**, using Hirsch funnel. After mineral oil layer has passed thru paper, rinse all glassware thoroly with alcohol, followed by $H_2O$, then 5% detergent soln, **945.75C(i)**, and cold $H_2O$. Filter each rinse sep. thru same paper. Rinse final papers with enough alcohol to remove yellow color. Examine papers at 30×.

Ref.: JAOAC **51,** 522(1968).

## 945.87★ Filth in Whole Tamarind Pulp

**First Action**
**Surplus 1970**

*See* **40.125**, 11th ed.

## MISCELLANEOUS

## 969.45 Light Filth in Gums (Plant, Crude)

**Flotation Method**

**First Action 1969**
**Final Action 1988**

(If av. particle is ≤5 mm, proceed with method. If particle size is >5 mm, break into pieces by hand or by dropping small amts at a time into high-speed blender until desired size is reached. Where 95% and 40% alcohol are specified, isopropanol and 30% isopropanol, resp., can be substituted.)

Weigh 50 g sample into 2 L beaker, add 1.2 L $H_2O$ and 15 mL HCl, and stir well. Autoclave 1 hr at 121°. Slow vent. (Arabic and guar gums will completely dissolve in 15–30 min in 1.2 L $H_2O$ + 25 mL HCl when placed on mag. stirrer-hot plate or in steam bath.) Sieve portionwise on No. 230 sieve, **945.75B(r)**, using forcible stream of hot (55–70°) tap $H_2O$ from aerator, **945.75B(a)**, until all gum has passed thru. Transfer directly to ruled filter paper if negligible amts of plant tissue remain on sieve. If large amts of plant debris remain on sieve, transfer to 1 L trap flask with 40% alcohol. Bring vol. to 500 mL with 40% alcohol and add 25 mL HCl. Heat to ca 60° on mag. stirrer-hot plate with stirring. Add 25 mL mineral oil, **945.75C(p)**, and stir mag. 2 min, **970.66B(c)**. Fill flask with 40% alcohol and gently swirl contents with stopper. Let stand 10 min and perform first trapping. Add 25 mL mineral oil, and gently stir with stopper 1 min. Let stand 5 min and perform second trapping. Rinse neck of flask with 95% alcohol and pour trappings onto ruled filter paper. Examine at 30×.

Ref.: JAOAC **52,** 17(1969).

## 970.74 Foreign Matter in Drugs (Leafy, Crude)

**First Action**

***A. Gross Contamination***

*See* **960.51**.

***B. Heavy and Light Filth***

*See* **975.48**.

## 971.36 Light Filth in Papain (Crude and Refined)

**Flotation Method**

**First Action 1971**
**Final Action 1988**

Weigh 50 g sample into 1.5 L beaker. (*Caution:* Weigh under effective fume removal device to avoid breathing dust. Avoid skin contact.) Add 1 L hot $H_2O$ and small amt of Antifoam A spray. Boil 20 min with stirring. Wet sieve forcefully on No. 230 sieve until all whitish material passes thru sieve and only plant debris and extraneous materials remain. If small amt of material remains on sieve, transfer directly to ruled filter paper. If large amt of plant tissue remains, transfer to 1 L trap flask with 40% isopropanol or 55% alcohol (use same alcohol thruout method). Bring vol. to 500 mL with alcohol and add 25 mL HCl. Boil 5 min, add 25 mL mineral oil, **945.75C(p)**, stir mag. 2 min, **970.66B(c)**, fill with alcohol, and trap off after 10 min. Perform second trapping, using 15 mL mineral oil, stir by hand 1 min, and trap off after 10 min. Filter trappings thru ruled paper and examine microscopically.

Ref.: JAOAC **54,** 565(1971).

## 973.63 Insect Penetration thru Packaging Materials

**Microscopic Examination Method**

**First Action 1973**
**Final Action 1988**

**(a) Entrance Characteristics**

(*1*) *Kraft paper, paper box.*

(*a*) *Surface fraying.*—Consists of paper fibers cut and lifted from surface of packaging material by mandibular activity. Represents first activity of hole formation. May occur at random on "entrance" surface of packaging materials. *See* Fig. **973.63(B)**.

(*b*) *Terraced depression.*—Consists of "step effect" formed when *secondary depression* is superimposed on *initial depression; see* Fig. **973.63(D)**. This terracing may be present around entire perimeter of final hole or at one or more points around it.

(*c*) *Tapered hole.*—Diam. of hole is greater on entrance side than exit side. This feature is most obvious on thicker packaging materials. *See* Fig. **973.63(E)**.

(*2*) *Foil, Cellophane, polyethylene plastic, waxed paper.*

(*a*) *Mandibular scratches.*—Found on Al foil, Cellophane, and polyethylene plastic. Consists of small, short surface scratches or grooves formed by pincerlike action of mandibles.

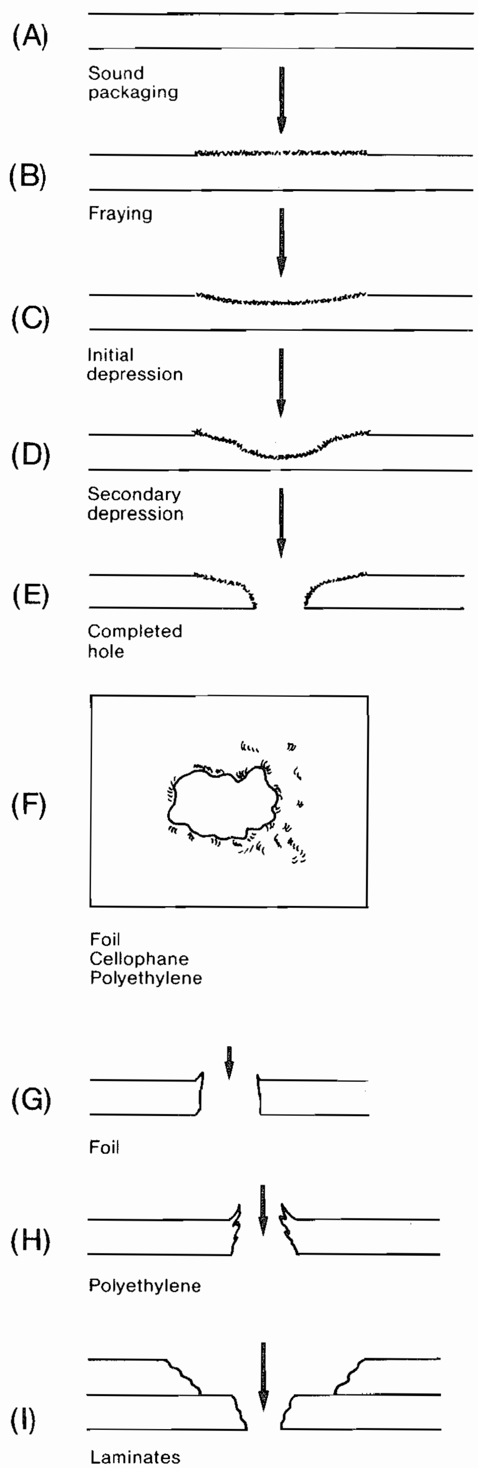



**FIG. 973.63—Insect penetration of packaging**

Frequently observed around perimeter of hole or in localized groups at random on entrance surface. *See* Fig. **973.63(F)**.

(*b*) *Upturned edges.*—Present around perimeter of holes in Al foil and polyethylene plastic materials. Appear as continuous irregularly upturned edge in foil (Fig. **973.63(G)**) and generally as upturned fraying of plastic (Fig. **973.63(H)**) in polyethylene materials. Not observed on waxed paper and Cellophane materials.

(*c*) *Roughened surface.*—Observed around perimeter of holes or randomly on surface of polyethylene plastic and waxed paper. Consists of surface fraying or pulled up tufts resulting from mandibular action on material. Distinct mandibular scratches may be observed around or in roughened areas.

(*3*) *Foil/paper, foil/plastic, or other laminates.*

(*a*) *Entrance characteristics.*—*See* specific materials.

(*b*) *Terracing of laminates.*—Quite common on entrance side of these materials. Observed as larger hole bored in laminate material on entrance surface and smaller hole in exit side of material. *See* Fig. **973.63(I)**.

**(b) Exit Characteristics**

All types of packaging materials.

(*1*) Clean-cut hole perimeter.

(*2*) Diam. of hole smaller than on entrance side.

(*3*) No surface fraying, scratches, or depressions (*see* Fig. **973.63(E)**).

Ref.: JAOAC **56,** 640(1973).

## ANIMAL EXCRETIONS

(Liquid Excretions: *See* Fig. **945.88**.)

(Solid Excretions: *See* Fig. **981.22**.)

### 945.88 Urine Stains on Foods and Containers
**Ultraviolet Light Examination**
**First Action**

(*Caution:* See safety notes on hazardous radiations.)

(Applicable to suspect urine stains on all materials except seeds)

Examine suspected stains in dark room under long wave UV light (366 nm). (Dried urine on textiles usually fluoresces blue-white, but color varies somewhat, depending upon natural color of textile and type of lamp and filter used.) Run check patches with known types of urine. For microchem. analysis, outline stained area with pencil under the UV light. When odor of urine is detected, report this.

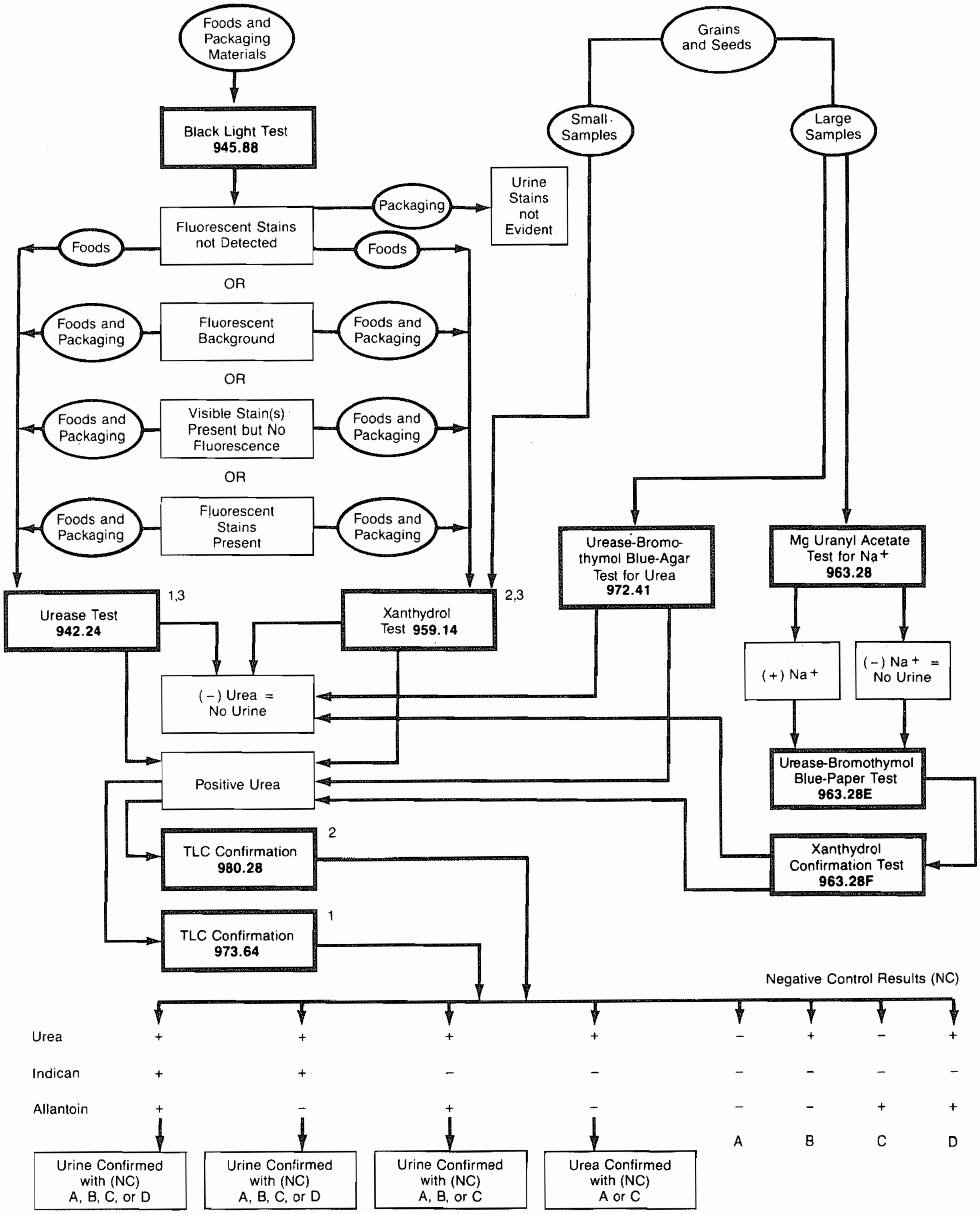
### 942.24 Urine Stains on Foods and Containers
**Urease Test for Urea**
**First Action**

(Applicable to urine residues on materials with significant amts of interfering substances, i.e., fats and oils)

Cut out portion of stained area and transfer 1 or 2 threads to 5 mL crucible or beaker. Save balance of cloth to confirm urine by **973.64**. Leach 10 min in just enough warm $H_2O$ to cover material. Remove threads and squeeze out as much liq. as possible with clean, flat-tip forceps.

Transfer 2 or 3 drops to microculture slide with deep cylindrical depression. Add small drop *urease mixt.* (suspension of $^1/_4$ of 25 mg urease tablet in 0.5–0.7 mL $H_2O$). Place small drop *10% $H_2PtCl_6$ soln* on cover slip and invert over the depression, with hanging drop at center of depression opening. (Cover slip may be sealed on with petrolatum if only minute amts of urea are suspected.)

With evolution of $NH_3$, brilliant, highly refractive, octahedral crystals of $(NH_4)_2PtCl_6$ are formed in hanging drop. Time




1) Use this test if fats, oils, or other interferences are expected to be present.
2) Use this test if interfering substances *are not* expected to be present.
3) If only minimal sample is available, proceed directly to TLC confirmation.

**FIG. 945.88—Methods for urine: method selection and results interpretation guide**

**Mammalian Feces → 981.22 and 988.17**
**Bird Droppings and Insect Excreta:**

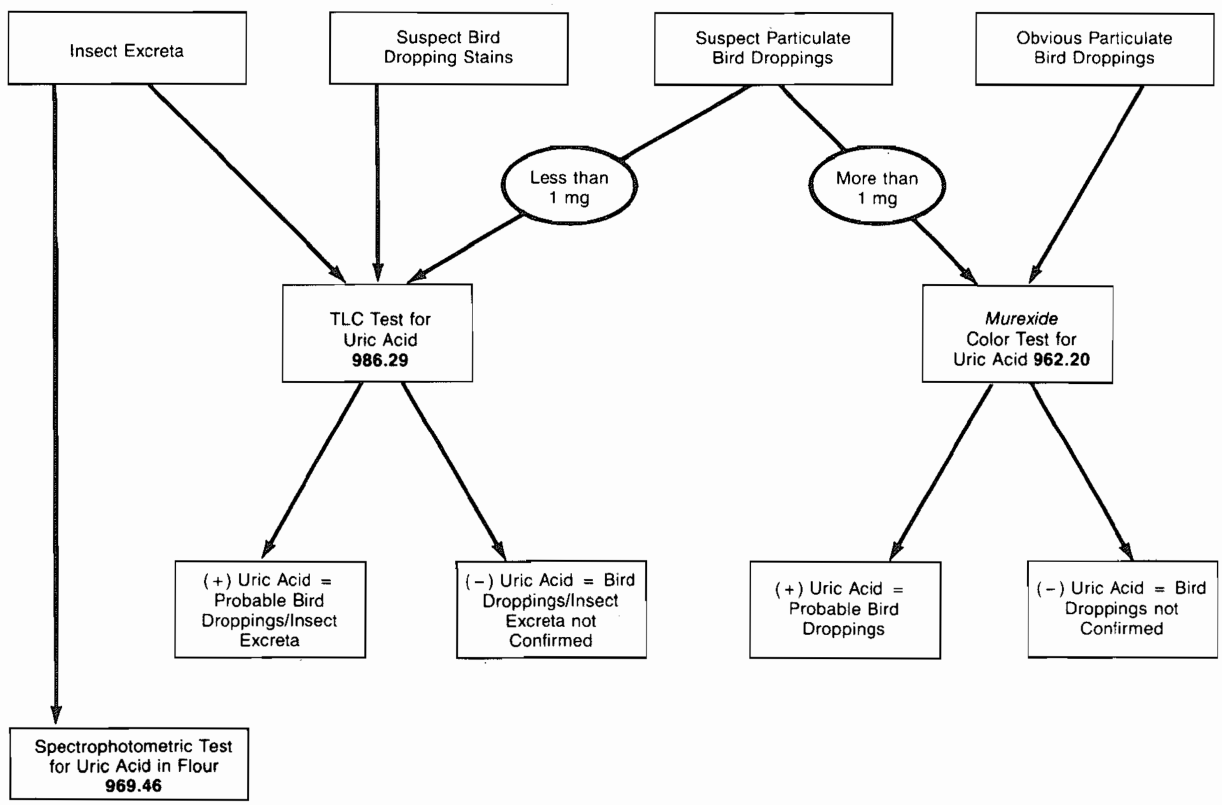



**FIG. 981.22—Method selection guide: methods for solid excretory materials**

required for crystals to form varies from few sec to 30 min, or even longer in some instances, according to conditions. Crystals may be visible to naked eye and are readily detected under microscope at 100×. Certain org. compds that are volatile and $H_2O$-sol. may yield crystals in the hanging drop, and if reagent soln is too concd, $H_2PtCl_6$ may crystallize. However, crystal habits of these substances are different from those of $(NH_4)_2PtCl_6$. (Stained patches of the food material can be tested by method similar to above.)

Ref.: JAOAC **25,** 772(1942).

CAS-57-13-6 (urea)

## 959.14 Urine Stains on Foods and Containers

### Xanthydrol Test for Urea

#### First Action

(Not applicable in presence of dried skim milk. Applicable to fluorescing urine residues on materials without significant amts of interfering substances.)

Place portion of stained cloth, ca 3 mm sq (stain located by fluorescence) on microscope slide. Add drop of HOAc (2 + 1) and stir. (Or instead of cutting out a patch of cloth, rinse stained material with $H_2O$ or other suitable solv. such as HOAc, acetone, or hot alcohol, evap. soln to dryness, dissolve residue in little HOAc (2 + 1), and place drop on slide.)

Transfer droplet with stirring rod to another place on slide and dil. with drop of HOAc (2 + 1). To both drops add very small amt of *xanthydrol* and stir into soln. If urea is present, crystals of dixanthylurea form very shortly. Examine with magnification of ca 100–120× (higher power may be used for closer examination if crystals formed are quite small). Use of polarizing microscope is desirable but not essential.

Crystals may be either or both of 2 kinds, depending on concn of urea present: (*a*) most prevalent are clusters of narrow feather-blades of low birefringence which form thruout soln at ca 1:200 to 1:25,000 concn (under low power they may appear to be needles or threads); (*b*) straight needles, often in sheaves or clusters, of much greater birefringence, forming chiefly at or near edge as drop evaps, at concns from 1:50 to 1:1,000. Both kinds have neg. elongation (observed with polarizing microscope, using red plate). Crystals should be noted before drop dries, but remain when it dries. Response is given by fresh urine solids content of ≥4 μg in drop. Test material from portion of sample other than fluorescent spot as blank.

Ref.: JAOAC **42,** 473(1959).

CAS-57-13-6 (urea)

## 963.28 Urine on Grain

### Magnesium Uranyl Acetate Test

### First Action 1963

(Applicable to grains and seeds)

#### *A. Principle*

Grain is sprayed with Mg uranyl acetate soln. If rodent urine is present, its Na content reacts to cause greenish fluorescence on kernel when sample is viewed under short-wave UV light.

#### *B. Reagents*

(**a**) *Magnesium uranyl acetate soln.*—Prep. reagents **929.03A(a)** and (**b**) in $^1/_{10}$ amts, mix, add 22 mL glycerol, mix, and filter thru washed, dried paper.

(**b**) *Urease soln.*—Wet 0.2 g urease powder with small amt of $H_2O$, stir into paste, and dil. to 10 mL with $H_2O$.

(**c**) *Bromothymol blue soln.*—Rub 0.15 g indicator powder in mortar with 2.4 mL 0.1*N* NaOH soln. After indicator dissolves, wash mortar and pestle with $H_2O$, and dil. to 50 mL with $H_2O$. Soln should be green; pH ca 7.0.

(**d**) *Urease-bromothymol blue test paper.*—Mix 10 mL indicator soln, (**c**), with 10 mL urease soln. Pour mixt. into watch glass. Using clean tweezers, dip pieces of heavy filter paper (Whatman No. 5, S&S No. 598, or 589 green ribbon have been found satisfactory) in soln. (To avoid uneven distribution of indicator and enzyme, wet entire paper at once by laying it on surface of soln.) Hang paper to dry in place free from $NH_3$ fumes, strong air currents, or heat. Paper should be orange when dry. Store dry paper in well-stoppered, dark glass bottle in cool place.

(**e**) *Xanthydrol.*—Eastman Kodak Co. No. 1559, crystals.

#### *C. Apparatus*

(**a**) *Ultraviolet lamp.*—Short wave, 253.7 nm, with filters to eliminate most visible light.

(**b**) *Chromatographic sprayer.*—250 mL, to deliver fine spray from air supply (Kontes Glass Co., K-422500, or equiv.). Hand-operated atomizer is satisfactory if it delivers fine spray.

#### *D. Ultraviolet Test*

Spread 50 g grain in shallow tray, or on sheet of waxed paper on tray. Place in hood or well ventilated area, and spray evenly with Mg uranyl acetate reagent, making several sweeps horizontally and vertically across sample. Let stand 1–3 min, and examine under short-wave UV light. With clean tweezers, transfer kernels showing greenish fluorescent areas to spot plate. (Avoid prolonged exposure to UV light and do not touch grains with bare fingers (use gloves). Perspiration may cause false fluorescence.)

Use as blanks 1 or 2 kernels showing no green fluorescence under UV light.

#### *E. Urease-Bromothymol Blue–Paper Test*

Add 1–4 drops $H_2O$ to each suspect kernel on spot plate. Let stand 3–5 min. Place strip of test paper, (**d**), on glass microscope slide, transfer drop of ext to paper with stirring rod, and cover with second slide. Blue spots, slowly developing over 2–4 min, indicate urea. (As reagent is slightly acid, color may not appear for several min, depending on how heavily grain was sprayed.)

#### *F. Confirmatory Test*

Transfer 1–2 drops aq. ext of suspect kernels to microscope slide and evap. to dryness. Add drop of HOAc (2 + 1) and very small amt of xanthydrol crystals. If urea is present, characteristic crystals of dixanthylurea form quickly, and are visible at 60× or lower with wide-field stereoscopic microscope.

Ref.: JAOAC **46,** 685(1963).

## 972.41 Urine on Grain

### Urease-Bromothymol Blue-Agar Test

### First Action 1972

(Applicable to grains, seeds, and packaging materials that do not show fluorescent residues or where background fluorescence is present)

#### *A. Apparatus*

(**a**) *Disposable trays.*—Microtiter® plates, flexible vinyl, flat-bottom (Dynatech Laboratories Inc., 14340 Sullyfield Circle, Chantilly, VA 22021), or equiv.

(**b**) *Culture tubes.*—6 (od) × 50 mm.

#### *B. Reagents*

(**a**) *Bromothymol blue* (*BTB*) *indicator soln.*—Sol. form (Fisher Scientific Co., B-100, or equiv.). Transfer 50 mg BTB to 20 mL g-s test tube and add 10 mL $H_2O$. Add 1 drop $H_3PO_4$ (1 + 9) and dissolve completely by stirring. Add ca 0.1*N* NaOH dropwise to dark green (pH 5.8–6.0). (This prepn is enough for 1 batch of test agar.)

(**b**) *Urease suspension.*—Grind 0.30 g urease in small mortar, add few mL $H_2O$, and continue grinding. Slowly dil. to 15 mL with stirring.

(**c**) *Test agar.*—Add 0.75 g bacteriological grade agar and 0.30 g Na benzoate to 300 mL cold $H_2O$ with vigorous stirring, and place on mag. stirrer-hot plate, **945.75B(n)**; heat with stirring and boil gently 1 min. Cool to 45–48°, add BTB soln, (**a**), and adjust to pH 5.5 (yellow-green), using 0.9–1.1*N* NaOH or $H_2SO_4$. Add urease suspension to pH-adjusted, 45–48° agar and readjust pH, if necessary. Divide agar into 2 equal portions in beakers. To one portion add 0.5 mL 0.1*N* $AgNO_3$. To other portion add 0.1*N* $H_2SO_4$ (ca 0.2 mL) to adjust to match color shade or pH of first portion. Mix each portion thoroly and let stand ca 5 min.

*Agar sensitivity test.*—Prep. known urea-contaminated seeds by spotting each seed with 0.5 μL 0.25% aq. soln of urea and let dry. Test these known samples as in **972.41C**. If no color response is obtained with known grains, either obtain new lot of urease or increase urease content after checking activity as in **941.04A(b)** and **941.04B** so that suspension contains enough urease to convert 0.80 g urea, and repeat sensitivity test.

#### *C. Test*

Initially check sample for free alkali, using test agar contg $AgNO_3$ soln (45–48°), by (**a**) or (**b**) below:

(**a**) *For immediate use.*—Add grain, or unknown sample, to wells of disposable tray, **972.41A(a)**, and, using dropping pipet, add agar until object is covered.

(**b**) *For storage and/or intermittent use.*—Add agar to culture tubes, **972.41A(b)**, to $^1/_2$ ht. Transfer tubes to heat-sealable plastic bags, seal, and store at 4 ± 1°. Agar can be stored ≤120 days. To use, let stand 1 hr at room temp. Use small glass rod to force test object below surface of agar. Remove rod and shake down agar. Observe frequently for color change near surface of object.

If test for free alkali is neg. (no color change of indicator), proceed with $AgNO_3$-free test agar, analyzing sample in similar manner. (Grains must be totally immersed during test.) Color change of indicator is yellow → green → blue, depending on concn of $NH_3$ produced. Reaction usually requires 1–3 min to give detectable color. Time varies inversely with urea concn. Spots from higher levels of urea continue to develop and enlarge for 10–12 min and then fade gradually.

Ref.: JAOAC **55,** 76(1972).

## 980.28 Urine Stains on Foods and Containers

### Thin Layer Chromatographic Method I

### First Action 1980

(Applicable to fluorescing urine residues on materials not expected to have significant amts of extractable interfering substances)

### *A. Apparatus and Reagent*

(**a**) *Thin layer apparatus.*—*See* **945.75B(s)**.

(**b**) *Sandwich chamber.*—With 1.4–2.0 mm spacer. *See* Fig. **980.28**.

(**c**) *Blender.*—High speed with stainless steel semimicro jar (Thomas Scientific, No. 3392-G15, or equiv.). *See* **945.75B(c)**.

(**d**) *Tube heater.*—Nine tube heater with control and circular gas manifold (Kontes Glass Co., Cat. Nos. K-720000 and K-655800, or equiv.).

(**e**) *Surface thermometer.*—Range −10° to 150°.

(**f**) *Developing solvent.*—*n*-BuOH-MeOH-$H_2O$ (2 + 2 + 1).

### *B. Preparation of Thin Layer Plates*

(**a**) *Quickfit plate leveler and spreader.*—Add 12 g cellulose powder, **945.75C(g)**, to 75 mL $H_2O$ in blender operated at 0.75 × line voltage. Work powder into $H_2O$, using small spatula. Apply line voltage, add 15 mL $H_2O$, and blend ≥1.5 min. Apply slurry to 5 plates, using 0.50 mm slot, and air dry overnight.

(**b**) *Brinkmann-Desga.*—Prep. slurry as in (**a**), using 15 g cellulose powder and 100 mL $H_2O$ (total). Apply slurry to 5 plates, using 0.375 mm layer, and air dry overnight.

(**c**) *Commercial plates.*—250 µm, precoated with MN 300 cellulose. Plates from Analtech, Inc., have been found satisfactory. Use 500 µm plates for "dirty" extns.

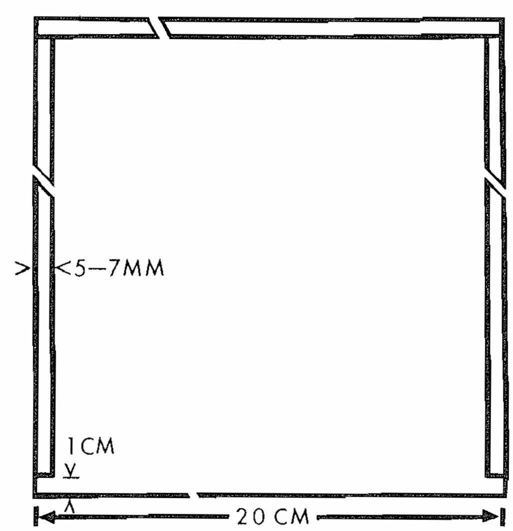



**FIG. 980.28—Sandwich chamber with three spacer strips along three edges. Snug fit is necessary at top corners. (Not to scale.)**

(**d**) *Pre-cleaning Analtech MN 300 cellulose plates.*—Develop unused plate in either std developing tank or sandwich chamber using developing solv. **980.28A**. Develop to ht of 15 cm above lower edge of plate. Thoroly dry plate using either hair dryer, forced draft oven at ≤80° for about 15 min, or overnight in fume hood. Plate must be at room temp. and completely free of solv. odor before use.

### *C. Determination*

(**a**) *Preliminary examination.*—Check samples as in **945.60** for fluorescent areas. Mark fluorescent spots and nearby equiv. nonfluorescent areas as controls. Transfer selected spots to 30 mL beakers. For intense fluorescent spot, use ca 8 mm diam.

(**b**) *Extraction and concentration.*—Add 10 mL acetone to selected spots in beakers and simmer 5 min on steam bath, avoiding excessive loss of solv. Preheat 13–15 mL conical tubes in tube heater or beaker of warm $H_2O$, add portion of acetone ext, and evap. to dryness before very slowly adding next portion. (Tubes must be preheated and acetone added very slowly to avoid bumping.) To hasten evapn, use air manifold or 4–6 SiC No. 60 chips per tube. Repeat extn and evapn twice, keeping tubes hot to avoid bumping. Chromatograph final residue without delay. If chromatgy must be delayed, add 50–75 µL acetone, stopper, and store in dark.

(**c**) *Preheating plates.*—Scrape ca 1 cm layer from 3 sides of plate. Place plate on heated surface, reading ca 87° on surface thermometer or 70° on 3″ (76 mm) immersion thermometer inserted thru hole in stopper until tip touches bottom of 250 mL conical flask contg 125 mL glycerol.

(**d**) *Spotting plate.*—Along unscraped side, 2 cm up from edge of plate and ca 1 cm from each scraped edge, spot at single point, drying between applications, 1 µL urea, **945.75C(aa)**, 20 µL allantoin, **945.75C(d)**, and 1 µL indoxyl sulfate, **945.75C(m)**, std solns. Spot individual stds near center of plate and spot sample exts between stds ca 10–12 mm apart. Rinse sample tubes with two 50 µL portions acetone and transfer to sample spots, keeping spot size to min.

(**e**) *Cleanup.*—If there is appreciable color and/or other extraneous material present, clean up with benzene as follows: Line TLC tank with heavy blotting paper, or equiv. Add 40–60 mL benzene to bottom of tank, add glass solv. trough, and cover tank. (For frequent use, coat ground glass surfaces with high viscosity silicone grease.) Add 25–30 mL benzene to trough in tank. Place plate in trough and let front rise ca 25 mm above spotting line, remove plate, and fan dry. Repeat benzene development and fan dry. Place plate in trough third time with tank closed and let benzene rise >13 cm. Dry plate in forced draft oven 5 min at 80°.

(**f**) *Development of plate.*—Scribe line across plate 10 cm above spotted stds. Form sandwich chamber with spotted plate, spacer, and uncoated plate. If com. spacers are not available, fabricate sandwich by cutting 3 strips cardboard or Teflon 5–7 mm wide, 1.4–2.0 mm thick, one strip 20 cm long and two strips ca 18.5 cm long. Position strips as in Fig. **980.28**, leaving ca 1 cm gap at bottom. Snug fit at top corners is necessary. Clamp, place in trough, add developing solv. to ca 1 cm of spotting line, cover with opaque material, and develop 10 cm. Dry plate 5 min in 80° forced draft oven.

(**g**) *Color development.*—Spray plate with pDMAB soln, **945.75C(r)**, until urea and allantoin stds appear. Heat 5 min in forced draft oven at 80°. Strong yellow to orange area, $R_f$ ca 0.65, is urea. Pale yellow smaller spot, $R_f$ ca 0.43, is allantoin. Using soft (No. 1) pencil, lightly outline each spot as color develops. Place under long wave UV light in well darkened room, check for pale yellow indican fluorescent area, $R_f$ ca 0.79, and outline as above. Protect urea and allantoin spots from fading due to NaOAc spray, **945.75C(s)**, by masking with plate glass. Spray satd NaOAc soln into indican area until damp. Let air dry in hood ca 10 min (do not heat).

High level of indican gives deep pink to red in room light. If no visual color is present, check with longwave UV light for fluorescent pink to orange on very pale blue background. This color is stable several days in dark.

Refs.: JAOAC **63,** 189(1980); **66,** 394(1983).

## 973.64 Urine Stains on Foods and Containers

### Thin Layer Chromatographic Method II

**First Action 1973**
**Final Action 1977**

(Applicable to residues on materials with significant amts of extractable interfering substances)

### *A. Apparatus*

(**a**) *Soxhlet extractor.*—250 mL extn flask; extn chamber 39 (id) × 115 mm with top joint ₮ 45/50 and bottom joint ₮24/40; 35 × 90 mm thimbles; condenser joint ₮45/50 (Thomas Scientific, No. 4406-E34, or equiv.).

(**b**) *Kuderna-Danish concentrator.*

(**c**) *Thin layer apparatus.—See* **945.75B(s)**.

### *B. Reagents*

(**a**) *Tryptophan soln.*—1 mg/mL 50% aq. acetone (used as longwave fluorescent marker with $R_f$ approx. that of urea in developing solv.).

(**b**) *Developing solvent.*—*n*-BuOH-HOAc-$H_2O$ (10 + 5 + 5); prep. fresh daily.

### *C. Determination*

(*Caution,* See safety notes on distillation, flammable solvents, and acetone.)

On previous day prep. plates for overnight drying. Equilibrate tanks ca 1 hr before extg samples.

(**a**) *Soxhlet extraction.*—Ext 18 g sample with 60 mL acetone 1 hr at 3–4 min/siphon. Transfer ext to 100 mL g-s graduate, dil. to vol., and take aliquot equiv. to 9 g sample. Conc. as in (**c**).

(**b**) *Alternative extraction.*—Place 18 g sample in 50 mL beaker or erlenmeyer. Add 1 mL acetone/g sample and boil gently 5 min, avoiding excessive loss of acetone. Decant thru glass wool pad into 100 mL g-s graduate. Repeat acetone boil and decanting 3 addnl times, and dil. to 100 mL. Conc. aliquot equiv. to 9 g as in (**c**).

(**c**) *Concentration.*—Conc. aliquot to ca 0.2 mL in Kuderna-Danish concentrator as follows: Evap. to ca 5 mL on steam bath in Kuderna-Danish concentrator fitted with 3-ball Snyder column and vol. flask or graduated collection tube; 20 mesh boiling chip is necessary. Remove collection tube from concentrator and fit tube with 2-ball micro-Snyder or micro-Vigreux column. Evap. to slightly less than desired vol., permit condensate to drain into tube, and remove column. Alternatively, place empty 13 mL graduated conical centrf. tube in beaker of boiling $H_2O$. When tube is hot, slowly transfer portions ext, using syringe fitted with long needle, to evapn tube. Let each portion evap. before next is added. Evap. to ca 0.20 mL. Chromatograph without appreciable delay.

(**d**) *Preparation of TLC tank.*—Add ca 150 mL satd $NaHSO_4$ soln to lined tank; then add ca 15 g addnl solid $NaHSO_4$ to bottom of tank. Place empty solv. trough in bottom of tank and cover tank.

(**e**) *Preparation of thin layer plates.*—(*1*) *Brinkmann-DeSaga.*—Add 15 g cellulose, **945.75C(g)**, to 100 mL $H_2O$ in blender operating at ca 90 v setting of variable transformer. Use small spatula to work powder into $H_2O$. Turn variable transformer to 120 v (line voltage) and blend ≥1.5 min. Apply slurry as 0.375 mm layer to 5 plates and air dry plates overnight.

(**f**) *Spotting of plates.*—Spot sample conc. as band ca 25 mm long on line 15 mm up and 15 mm in from edge of plate. Wash sides of evapn tube with ca 50 μL acetone and transfer wash to sample band area. Repeat 50 μL washings and transfers until last transfer is colorless (ca 4 transfers). Spot 1 μL each of urea std soln, **945.75C(aa)**, allantoin std soln, **945.75C(d)**, and indoxyl sulfate std soln, **945.75C(m)**, and tryptophan soln, **973.64B(a)**, ca 10 mm apart along line 15 mm to left of center of plate and 15 mm from bottom of plate.

(**g**) *Development of plates.*—Place plate in trough contg ether in lined tank presatd with ether. Let ether travel to top of plate. Remove plate and let air dry. Immediately draw intersecting lines to divide plate into 4 equal sqs. Dry plate 5 min in 80° forced-draft oven. Remove plate from oven and promptly place in dry solv. trough in TLC tank with spotted band down. Close tank and let stand 20 min. Slide top aside just enough to introduce long-stem funnel into solv. trough. Slowly add 20 mL developing solv., (**b**), to trough. Close lid and develop in dark to line of first direction. Dry plate 5 min in 80° forced-draft oven.

Rotate warm plate to place chromatographed stds in upper left quarter of plate and promptly place in dry trough in tank. Let stand 20 min without touching any liq. in closed tank. Then slide cover aside just enough to introduce long-stem funnel into solv. trough and slowly add 20 mL developing solv. Let front move to line in this second dimension. Dry plate 5 min in 80° forced-draft oven.

(**h**) *Color development.*—Spray plate with pDMAB reagent, **945.75C(r)**, until distinctly moist but not shiny wet and again heat 5 min in 80° forced-draft oven. Strong yellow-to-orange area at $R_f$ 0.75–0.80 is urea. Pale yellow smaller spot at $R_f$ 0.45–0.50 is allantoin. Mark each area as color develops, since colors fade from one step to next. Place under longwave black light in darkened room and check for pale yellow fluorescent area between urea and allantoin. Spray satd NaOAc soln, **945.75C(s)**, (ca 1–2 mL/plate) in space between urea and allantoin until yellow of *both* has faded. Let plate air dry ca 10 min in hood (do not heat), and check plate under longwave black light. Weak fluorescent pink-to-orange color against very pale blue fluorescent background confirms presence of urinary indican.

Refs.: JAOAC **56,** 637(1973); **57,** 689(1974).

## 981.22 Mammalian Feces

### Alkaline Phosphatase Test

**First Action 1981**

### *A. Principle*

Intestinal tract of most mammals contains alk. phosphatase isoenzyme. Isoenzyme, at test pH and temp., splits phosphate radical from phthln diphosphate to produce reddish free phthln.

### *B. Apparatus*

(**a**) *Cups.*—Plastic, 4 mL (Technicon Instruments Corp., S127-0018-01, or equiv.).

(**b**) *Plates.*—Tissue culture, 4 × 6 wells, 3 mL capacity (Thomas Scientific, No. 9383-C15, or equiv.).

(**c**) *Pipet.*—Cornwall, adjustable to deliver 1 mL (Fisher Scientific Co., No. 13-689, or equiv.).

(**d**) *Spatula*.—Curved on one end, knob on the other end (Thomas Scientific, No. 8340-H10, or equiv.).

### C. Reagents

(**a**) *Magnesium chloride soln*.—Dissolve 0.406 g $MgCl_2.6H_2O$ in $H_2O$ and dil. to 1 L.

(**b**) *Stock test reagent*.—Dissolve 9.5 g borax ($Na_2B_4O_7 \cdot 10 H_2O$) and 3.14 g anhyd. $Na_2CO_3$ in 500 mL $H_2O$ with stirring. Add 0.47 g phthln diphosphate and stir while adding 1 mL $MgCl_2$ soln. Prepn is stable.

(**c**) *Work test media (WTM)*.—It is recommended that only small amts of this reagent be prepd. Measure equal vols stock test reagent and $H_2O$ into sep. beakers. Place beaker of cold $H_2O$ on stirring hot plate, add stirring bar, and, with rapid stirring, add sufficient agar to yield 2% agar dispersion. Continue to stir while heating to boil (watch for foam-over). When agar foams, add stock test reagent and stir rapidly ca 1 min.

Add ca 1 mL portions of WTM to cups before cooling to 40–41°. WTM *must* be cooled to 40–41° before contacting samples.

Short term storage: WTM may be held ca 48 h at 40–41° if covered snugly with foil or plastic.

Long term storage: Gelled plugs, in cups, may be stored up to 4 months if sealed in plastic bag, held at room temp., and protected from direct sunlight. Discard any gells showing pink color and/or vol. loss.

### D. Determination

Transfer suspect feces, ca 1–3 mm diam. × 3–5 mm long, to moistened spot of filter paper in petri dish. Moisten with min. amt of addnl $H_2O$ if needed. Cover with small piece of clean Al foil and crush. Check for hairs or other diagnostic indicators. If no such evidence is seen, proceed as follows: Air dry paper and crushed particles. Cut out stained area and transfer paper and adhering particles to cup contg 1 mL gelled WTM. Cover with addnl 1 mL cool (40–41°) WTM or, alternatively, plug of gelled WTM. Use clean spatula to manipulate covering plug of WTM and to press plug into close contact with sample; place in 40–41° $H_2O$ bath. Check for development of red color near particles.

*Blank preparation*.—Autoclave crushed particle 15 min at 15 psi. Alternatively, place 100 mL beaker in 800 mL or 1 L beaker and add ca 25 mm depth $H_2O$ to each. Place small test tube with 1 mL WTM in smaller beaker. Heat $H_2O$ to boiling ca 2 min. Remove small tube and quickly transfer crushed pellet material to tube. Return tube to rapidly boiling $H_2O$ ca 2 min. Remove tube and with small glass rod work all particles from sidewall of tube down into liq. Replace tube in small beaker, cover large beaker with watch glass, and continue to boil ca 5 min. Remove small tube, mix contents quickly, and transfer to test cup position designated as blank (negative test) for test plate.

*Positive control preparation*.—Using calf intestine alkaline phosphatase (AKP) (Calbiochem Corp. No. 52457 or equiv.), prep. 1 mg/mL soln in borate buffer (Stock test reagent **981.22C(b)**) without phthln diphosphate. Add 20 μL AKP soln to 1 cm diam. filter paper disks (Whatman No. 1, or equiv.). Use pos. control disks with either liq. WTM or alternative gelled plug WTM. *Note:* Pos. control disks may be stored up to 4 months if held at room temp. and protected from light.

*Test response*.—Time of color development varies widely, depending in part on species variation in levels of alk. phosphatase and on temp. of WTM. Time varies from 2 to 3 min for most mouse samples, up to 4 h for samples from some grass eaters, such as deer or rabbit.

Refs.: JAOAC **64**, 196(1981); **66**, 394(1983).

## 988.17 Mammalian Feces

### Thin Layer Chromatographic Method for Coprostanol

**First Action 1988**

(Applicability includes identification of feces in heat-processed materials.)

### A. Principle

Suspected fecal material is extd with hexane. Coprostanol, characteristic sterol of mammalian feces, is resolved from other sterols in ext by TLC, and produces blue spot when heated with phosphomolybdic acid.

### B. Apparatus

(**a**) *Thin layer plates*.—Glass, 20 × 20 cm, precoated with 250 μm layer of silica gel. Prechanneled, with preadsorbent zone. (Whatman, or equiv. plates.)

(**b**) *Dipping tank and accessories*.—Glass (Kontes Cat. No. K416160, or equiv.).

(**c**) *Chromatographic tank with lid*.—Glass (Kontes Cat. No. K416180, or equiv.).

(**d**) *Spotting pipets*.—20 μL, glass (Drummond Scientific Co., 500 Parkway, PO Box 700, Broomall, PA 19008, or equiv.).

### C. Reagents

(**a**) *Alcoholic phosphomolybdic acid (PMA)*.—Dissolve 50 g PMA (Fisher Certified ACS, or equiv.) in 500 mL alcohol, filter soln, and dil. to 1 L with alcohol. Store in dark. Discard if greenish tinge appears.

(**b**) *Developing solvent*.—Ether–heptane (55 + 45).

(**c**) *Coprostanol std soln*.—5 μg coprostanol (Supelco std)/mL hexane.

(**d**) *Cholesterol std soln*.—5 μg cholesterol (Supelco std)/mL hexane.

### D. Preparation of Sample

Weigh sample of suspected fecal material to nearest 0.1 mg. If origin of feces is unknown or if herbivore dung is suspected, use ≥5 mg sample to reduce possibility of false neg. conclusions. Analyses may be made on much smaller test portions of samples, such as those from reconditioning operations of rodent-contaminated products, where more is known about sample.

Transfer sample to glass vial (ca 1–2 mL). Gently crush sample with glass rod if particle is >3–4 mm diam. Add hexane at ratio of 10 μL/mg feces, but not less than 30 μL per sample. Cap vial; let stand 1 h.

### E. Preparation of TLC Plates

Prep. plates ≥24 h before use to ensure evapn of alcohol. To prevent contamination, wear vinyl gloves when handling plates. Dip plates, top edge leading, into 5% alc. PMA until soln is 2–3 mm below preadsorbent-silica gel juncture. Do not let PMA diffuse into preadsorbent zone. Hold plate 10–15 s, then remove plate from soln and let it stand vertically ca ½ h, preadsorbent edge up, on paper toweling. Do not let dust settle on damp surface. Store plates in clean, dark container with gel surface free of any contact. Store plates ≤3 months.

### F. Determination

Use No. 1 (soft) pencil to lightly draw solv. front line 10 cm from preadsorbent-silica gel line. Spot 20 μL vols of stds and samples and at least 1 hexane reagent blank individually onto preadsorbent regions of plates. After std or sample has been applied, number lane with soft pencil and record lane no.

and sample. If all lanes are not required, avoid lanes nearest edges. Let hexane evap. 10 min.

Work in hood. Apply thin bead of silicone grease around chromatgc tank top to seal lid. Pour 55 mL ether and 45 mL heptane into 100 mL mixing cylinder. Invert cylinder 3–4 times to mix and pour contents into TLC tank. Immediately cover tank and let system equilibrate 10 min. Place TLC plate in tank and cover tank. Develop to solv. front line (ca 20 min). Remove plate and air-dry in hood ca 5 min. Place plate in 120° forced-draft oven 20 min. Remove plate and circle spots with soft pencil. Record $R_f$ for coprostanol and cholesterol stds. Record $R_f$ for any spots that appear in coprostanol and cholesterol regions. Cholesterol and coprostanol should be completely sepd.

Feces are indicated by presence of 2 spots: at $R_f$ ca 0.30 (cholesterol std) and at $R_f$ ca 0.40 (coprostanol std). For small samples ($\leq$2 mg) all spots may be faint and effort should be made to detect indicator spots. For larger samples, spots will be distinct. In addn to distinct cholesterol spot, cockroach excreta may present trace reaction in coprostanol region. Therefore, coprostanol spot should be approx. same or even greater in intensity than cholesterol spot to conclude that material is mammalian or bird feces rather than insect excreta. Coprostanol may occur in feces of some birds; therefore, if there is no evidence to eliminate possibility that material could be bird feces, pos. results should be reported as "fecal material from mammal or bird."

Colors fade in light. Photocopy machine may be used to make permanent record of plate.

Ref.: JAOAC **70,** 499(1987).

## 962.20 Excrement (Bird) on Food and Containers

### Microchemical Test for Uric Acid

### Final Action

(Not suitable for minute residues from suspect stained areas of food containers)

Transfer white, amorphous, grainy particles to depression of spot plate preheated to ca 100° on hot plate or in oven. Add small drop of $HNO_3$ (1 + 1) to sides of depression so that it will run down to wet particles; then evap. to dryness in 0.5–1.0 min. Heat 1–3 min. If particles turn orange-red to deep red with heat, uric acid and/or its salts may be present.

*To confirm:* Cool plate until there is no perceptible heat to back of hand; then streak across colored area with small glass rod wetted with 50% NaOH soln. Intense purple will develop almost immediately.

*Modification for particles about 1 mg.*—Position microscope or strong magnifying glass to observe 18 mm No. 2 cover glass placed on metal surface heated to ca 110–120°. Place suspect particle on glass, add 10 µL $HNO_3$, evap. to dryness, and heat in oven 5–7 min at 135–140°. Remove to cool white surface under magnifier and observe baked reaction residue. Pos. reaction shows yellow-orange to orange-red ring.

*To confirm:* With 1 mm glass rod place small drop 50% NaOH soln on edge of cover glass. Wipe rod and transfer small portion of drop to edge of baked residue. *Do not flood.* Purple-violet color develops promptly with uric acid or its salts.

Refs.: JAOAC **45,** 659(1962); **47,** 516(1964).

CAS-69-93-2 (uric acid)

## 986.29 Excrement (Bird and Insect) on Food and Containers

### Thin Layer Chromatographic Method for Uric Acid

### First Action 1986
### Final Action 1989

(Applicable to suspect material not suitable for detn by **962.20** and/or to confirmation of **962.20** when adequate material is available.)

#### *A. Apparatus and Reagents*

**(a)** *Thin layer cellulose plates.—See* **980.28B**. E. Merck cellulose plates, 0.10 mm (EM Science No. 5716-7) have also been found satisfactory.

**(b)** *Cellulose powder.—See* **945.75C(g)**.

**(c)** *Detection spray.—(1) Soln A.*—1% $K_3Fe(CN)_6$. *(2) Soln B.*—2% $FeCl_3$ (calcd as anhyd.). Refrigerate both solns. Protect soln A from light. Solns are stable ca 2 weeks. *(3) Spray reagent.*—To 18 mL $H_2O$, add 1 mL each of solns A and B; mix. Prep. *immediately* before use.

**(d)** *Developing solvent.*—*n*-BuOH-MeOH-$H_2O$ (4 + 4 + 3). Measure vols sep. and mix well to form stable single phase. To 30 mL of this soln, add 1 mL HOAc; mix well. Prep. fresh daily.

**(e)** *Dye mixture.*—Dissolve 16 mg amaranth (formerly FD&C Red No. 2) and 32 mg FD&C Yellow No. 6 in 50 mL $H_2O$; mix well.

**(f)** *Lithium carbonate soln.*—1 mg/mL.

**(g)** *Uric acid std soln.—(1) Stock soln.*—1 mg/mL. Dry 105 mg uric acid in 100° oven overnight and cool to room temp. in desiccator. Accurately weigh 60 mg $Li_2CO_3$ and transfer to 100 mL vol. flask. Accurately weigh 100 mg cool uric acid and transfer quant. to the 100 mL flask with ca 50 mL $H_2O$. Place in 60° $H_2O$ bath and agitate until soln clears. Cool immediately under tap $H_2O$ to room temp. and dil. to vol. with $H_2O$. For short term use (<3 days), store in refrigerator; for extended use, place portions in small containers and store hard-frozen. *(2) Working soln.*—100 µg/mL. Pipet 10 mL stock soln into 100 mL vol. flask and dil. to vol. with $H_2O$. Prep. fresh daily.

#### *B. Preparation of Sample*

**(a)** *Insect excreta.*—Transfer material to small test tube, crush with glass rod, and add 0.05–0.10 mL $Li_2CO_3$ soln, **(f)**. Let soak ca 10 min and centrf. Obtain clear supernate and proceed as in **986.29C**.

**(b)** *Paper bags or cartons.*—Cut 5–6 mm diam. portion from suspect area. Cut another 5–6 mm portion from nearby unstained area as neg. control. Place individually in small test tubes. Add ca 0.1 mL $Li_2CO_3$ soln, **(f)**, to each tube; agitate with small stirring rod. Let soak ca 10 min and proceed as in **986.29C**.

**(c)** *Other suspect material.*—Transfer small portion to test tube, add ca 0.1 mL $Li_2CO_3$ soln, **(f)**, and stir with glass rod. Let soak ca 10 min; centrf. Obtain clear supernate and proceed as in **986.29C**.

#### *C. Determination*

**(a)** *Spotting of plates.*—Place coated plate on heated metal slab reading ca 87° on surface thermometer or 70° on 3 in. (76 mm) immersion thermometer inserted through hole in stopper until tip touches bottom of 250 mL conical flask contg 125 mL glycerol. (*Note:* Plates tend to crack, particularly prescored plates, unless heated evenly.) Place infrared lamp or forced hot air source (e.g., hair dryer) above plate to speed drying of spots. Spot 1 µL uric acid working std soln, (**g**)(*2*), at each edge and at center of plate ca 15–20 mm up from bottom. Spot 1 µL dye mixt., (**e**), to side of each working std spot. These dyes serve as visual markers during development, with $R_f$ for amaranth at 0.38–0.40; uric acid, 0.41–0.43; and Yellow No. 6, 0.65, using Analtech plate and sandwich chamber. $R_f$ values are lower on Merck plates, with $R_f$ for amaranth approx. equal to that of uric acid. Spot samples and neg. controls along same line at ≥10 mm intervals. Keep spots at min. size by drying well between successive small addns.

(**b**) *Development of plates.*—Scribe horizontal line, ca 1 mm wide, across plate exactly 10 cm above origin, completely removing cellulose layer. Develop to this line in conventional satd tank without pre-equilibration or, alternatively, form sandwich chamber with uncoated plate [see **980.28A(b)** and **980.28C(c)** and (**f**)] and develop. Dry plate on heated metal slab or in forced draft oven ca 5 min at 75–80°.

(**c**) *Examination of UV light.*—Observe plate under shortwave (254 nm) UV light in darkened room, marking each quenching (dark) spot with penciled dots at top, bottom, left, and right edges. Shortwave lamps in fluorescent tube-style have integral filters with transmission characteristics that change with use. Some UV viewing cabinets have label attached calling attention to this fall-off of transmittance of 254 nm. High levels of uric acid should appear as dark spots at $R_f$ = 0.40 ± 0.05, depending on conditions of development.

(**d**) *Color development.*—Spray plate evenly in hood, concentrating on horizontal zone between upper (yellow) dye spots and ca 2 cm below lower (red) dye spots, only until blue uric acid spots clearly appear at $R_f$ stated in (**c**). Immediately outline spots with soft (No. 1) pencil, marking weakest spots first. Continue spraying *only until background begins to darken.* Immediately outline any addnl spots that appear (again, weakest ones first). (*Note:* Excessive spraying accelerates plate darkening.)

Refs.: JAOAC **61,** 903(1978); **66,** 394(1983); **69,** 499(1986).

CAS-69-93-2 (uric acid)

## 969.46 Excrement (Insect) in Flour

### Spectrophotometric Method for Uric Acid

**First Action 1969**
**Final Action 1970**

(Applicable to levels ≥4 mg/100 g)

### *A. Apparatus*

(**a**) *Spectrophotometer.*—Beckman Model DU, or equiv.

(**b**) *Centrifuge.*—Desk centrf. with multiple head to hold 15 mL polyethylene test tubes.

(**c**) *Incubator or water bath.*—Capable of maintaining temp. of 37 ± 1°.

### *B. Reagents*

(**a**) *Uric acid std soln.*—100 µg/mL. Dissolve 100 mg uric acid in 1 L 5% NaOAc soln. (If necessary, warm in $H_2O$ bath at 60–70°.) Filter and store in brown bottle; discard after 1 week. (Do not use com. uric acid std solns, as they may contain uricase inhibitors.)

(**b**) *Sodium borate buffer.*—0.01*M*, pH 9.2. Dissolve 3.8 g $Na_2B_4O_7.10H_2O$ in $H_2O$ and dil. to 1 L.

(**c**) *Sodium acetate soln.*—5%. Dissolve 100 g anhyd. NaOAc in $H_2O$ and dil. to 2 L. If necessary, adjust pH to 8.8–9.2 with HOAc or NaOH.

(**d**) *Glutathione soln.*—10 mg/mL in $H_2O$. Use within 30 min.

(**e**) *Uricase soln.*—Prep. suspension of 10 mg dried uricase in 50 mL 0.01*M* Na borate buffer. Use within 1 hr. (Clean all glassware that comes in contact with uricase enzyme with chromic acid soln; adsorbed uricase on glass surface produces low results.)

### *C. Preliminary Tests*

(**a**) *Test for purity of reagents.*—Dil. 5.0 mL uric acid std soln to 25 mL with 5% NaOAc soln. Place 5 mL in each of 3 test tubes. To 1 tube add 5 mL Na borate buffer, invert several times, and measure *A* at 292 nm. *A* should be ≥0.72, which corresponds to 0.072 *A* unit/µg uric acid/mL final soln. Test std uric acid soln daily.

(**b**) *Test for efficiency of uricase soln.*—Label remaining 2 tubes in (**a**) as No. 1 and No. 2; label a third test tube No. 3. Add 5 mL uricase soln to tubes No. 1 and No. 3. Close mouth of tube No. 1 with piece of cellophane sheet under thumb and invert. Stopper all 3 tubes with clean rubber stoppers and incubate 2 hr at 37°. After incubation, mix contents of tubes No. 2 and No. 3 by repeatedly pouring (6 times) from one tube to other, and immediately (within 60 sec) read *A* of combined solns at 292 nm, using soln in tube No. 1 as blank. *A* should be ≥0.648 for ≥90% of theoretical efficiency of uricase. If efficiency is <90%, incubate 4 hr. If increased incubation does not increase efficiency to 90%, discard uricase sample.

### *D. Preparation of Standard Curve*

Pipet 0.0, 2.5, 5.0, 10.0, and 15.0 mL uric acid std soln into 5 beakers (corresponds to 0.0, 1.0, 2.0, 4.0, and 6.0 µg uric acid/mL in final soln, resp.), and perform all steps as in **969.46E**, except omit flour.

### *E. Determination*

Add 25 mL 1*N* HCl and 5 mL glutathione soln to 4 g flour in 250 mL beaker. Mix well with glass rod and let stand overnight (≥16 hr). Add 25 mL 1*N* NaOH with stirring and adjust pH to 9.0–9.3 with 1*N* NaOH or 1*N* HCl. Transfer to 100 mL g-s graduate, carefully scraping all material sticking to sides of beaker with glass rod. Rinse beaker with 6 small portions 5% NaOAc and dil. to 100 mL with 5% NaOAc. Shake *gently* by inverting graduate several times every 10 min for 1 hr. (Vigorous shaking tends to produce turbid soln.) Transfer aliquot to 15 mL polyethylene test tube and centrf. 30 min at 3000 rpm. Decant supernate into small erlenmeyer, mix well, and pipet 4 mL into each of 2 test tubes, No. 1 and No. 2. To each tube, add 1 mL Na borate buffer and mix by rotating between palms of hands. (Mix soln with Na borate buffer within 15 min to avoid turbid soln.) Label third tube as No. 3. Add 5 mL uricase soln to tubes No. 1 and No. 3. Mix contents of tube No. 1 as in **969.46C(b)**. Stopper all 3 tubes with rubber stoppers and incubate 2 hr at 37°. Combine solns in tubes No. 2 and No. 3, as in **969.46C(b)**, and read *A* immediately (within 60 sec) at 292 nm against soln No. 1 (blank). (If flour ext appears very turbid after centrfg, dil. centrfd ext 1 + 4 with Na borate buffer and pipet 5.0 mL into each of 2 test tubes, No. 1 and No. 2. Add 5 mL uricase to each tube (No. 1 and No. 3) and proceed with detn as above.)

Reading, *A*, corresponds to amt of uric acid present in 4 mL portions of centrfd soln; amt of uric acid obtained from std curve × diln factor = amt of uric acid in sample.

Refs.: JAOAC **49,** 899(1966); **50,** 776(1967); **52,** 833(1969).

CAS-69-93-2 (uric acid)

## MOLD AND ROT FRAGMENTS

(*See* Fig. **984.29A**.)

### 984.29 Howard Mold Counting

#### General Instructions

***A. Diagnostic Characteristics of Mold***

Before attempting to make mold count, analysts should be familiar with cellular structure of product. They can do this thru direct microscopic examination of healthy tissue excised from raw product or thru study of ref. books. Analysts should assume that any product can be contaminated with a variety of vegetable, animal, and synthetic fibers. Presence of such materials greatly increases probability for misidentification of molds.

It is essential that analyst be able to distinguish hyphae and look alikes with which they might be confused. Although many filaments can be easily and accurately recognized as mold, others require more careful appraisal before they can be reported as mold. Only rarely are all mold characteristics given here present at one time; usually 2 or 3 are absent. Features mentioned below are observed at 100–450×.

(**a**) *Parallel walls.*—Mold hyphae are tubular. In most instances, diam. of filament is uniform throughout its length. Thus, hyphal walls usually look like parallel lines under microscope. This is one of the most useful characters for recognizing mold and for differentiating it from other fibers. Exceptions do occur, however. In some larger molds, walls may be collapsed or twisted. In certain molds, hyphae may have swellings along sides so that walls are not parallel. Hyphae of *Mucor* and *Geotrichum* are often tapered.

(**b**) *Septation.*—Many types of mold filaments are divided into segments or sections by cross walls. Some plant hairs also look segmented, but their walls are frequently convergent, forming sharp-pointed apex. *Mucor* and a few other molds generally have no cross walls.

(**c**) *Granulation.*—Thin-walled, tubular hypha contains protoplasm which shows thru cell walls and appears granular or stippled under high magnification. This is most clearly seen in some large mucors. In some fine molds, such as the one causing anthracnose, granulation of protoplasm is not evident. It disappears in some molds, such as those occasionally found in butter, leaving thin-walled, clear, almost invisible tubes. This empty mold is extremely difficult to count. At times it may become twisted, resembling a cotton fiber. Often the protoplasm separates intermittently, forming line of short links or chains connected by almost invisible hyphal walls.

(**d**) *Branching.*—If mold fragments are not too short, many of them may show an abundance of branching. Branches and main trunk are almost always same diam. When present, branching is one of the most reliable characteristics for recognizing mold.

(**e**) *Ends of filaments.*—Natural end of filament is usually bluntly rounded, much like a fingertip. Filaments are rarely sharply pointed, except in fertile (reproductive) hyphae. Occasionally, they are expanded into a ball or head, especially when mold is forming a fruiting body. Broken end of a fila-

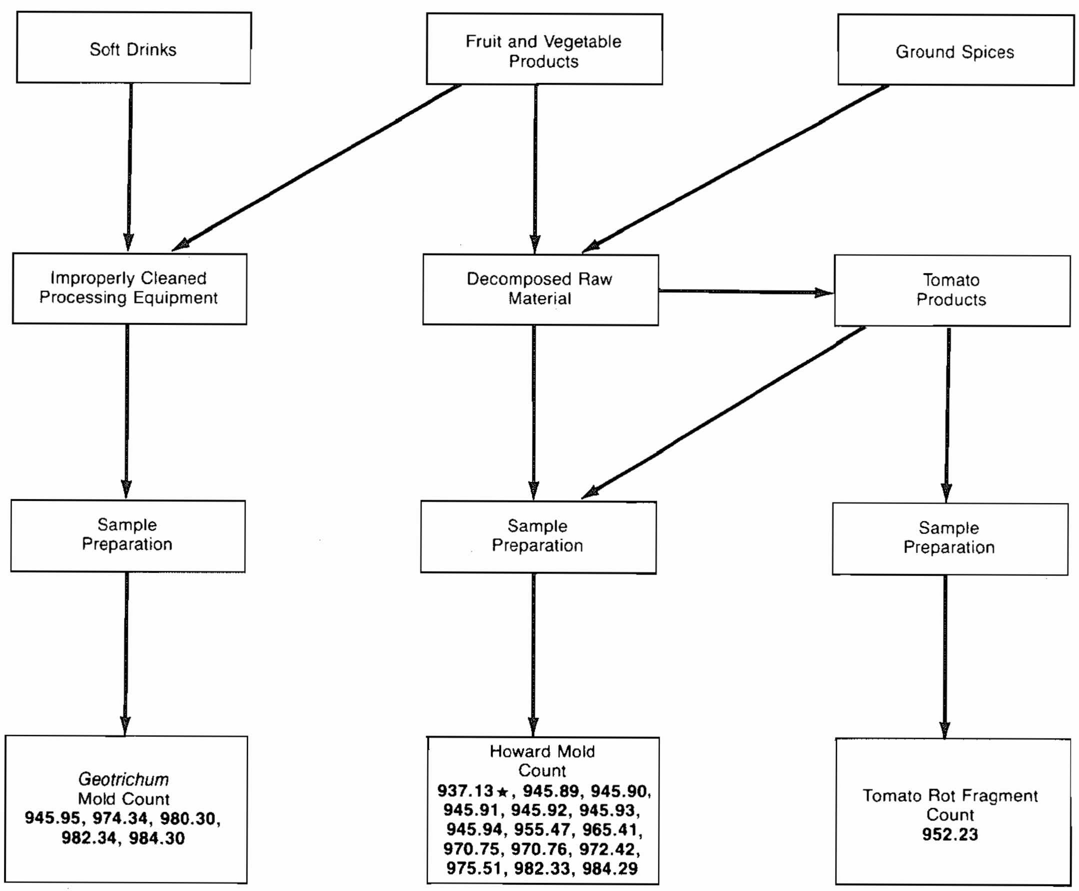



**FIG. 984.29A—Method selection guide: methods for mold and rot.**

ment is normally square. That portion of hypha adjacent to broken end may be collapsed and may contain no protoplasm.

(f) *Nonrefractile appearance*.—Hyphae do not strongly refract light. Some objects seen in mold preparation may resemble hyphae but have highly refractile appearance, such as unrolled spiral thickenings from walls of plant vessels. These refract light as a solid glass or plastic rod might do.

### B. Determination

Clean Howard cell, **945.75B(m)**(*1*), so that Newton's rings are produced between slide and cover glass. Remove cover and with knife blade or scalpel, place portion of well mixed sample on central disk; with same instrument, spread evenly over disk, and cover with glass so as to give uniform distribution. Use only enough sample to bring material to edge of disk. (It is of utmost importance that portion be taken from thoroly mixed sample and spread evenly over slide disk. Otherwise, when cover slip is put in place, insol. material, and consequently molds, may be more abundant at center of mount.) Discard any mount showing uneven distribution or absence of Newton's rings, or liq. that has been drawn across moat and between cover glass and shoulder.

Place slide under microscope **945.75B(o)**(*1*) and examine with such adjustment that each field of view covers 1.5 sq mm. (This area, which is essential, may frequently be obtained by so adjusting draw-tube that diam. of field becomes 1.382 mm. When such adjustment is not possible, make accessory drop-in ocular diaphragm with aperture accurately cut to necessary size. Diam. of area of field of view can be detd by use of stage micrometer. When instrument is properly adjusted, vol. of liq. examined per field is 0.15 cu mm.) Use magnification of 90–125×. In those instances where identifying characteristics of mold filaments are not clearly discernible in std field, use magnification of ca 200× (8 mm objective) to confirm identity of mold filaments previously observed in std field. *See* Fig. **984.29B**.

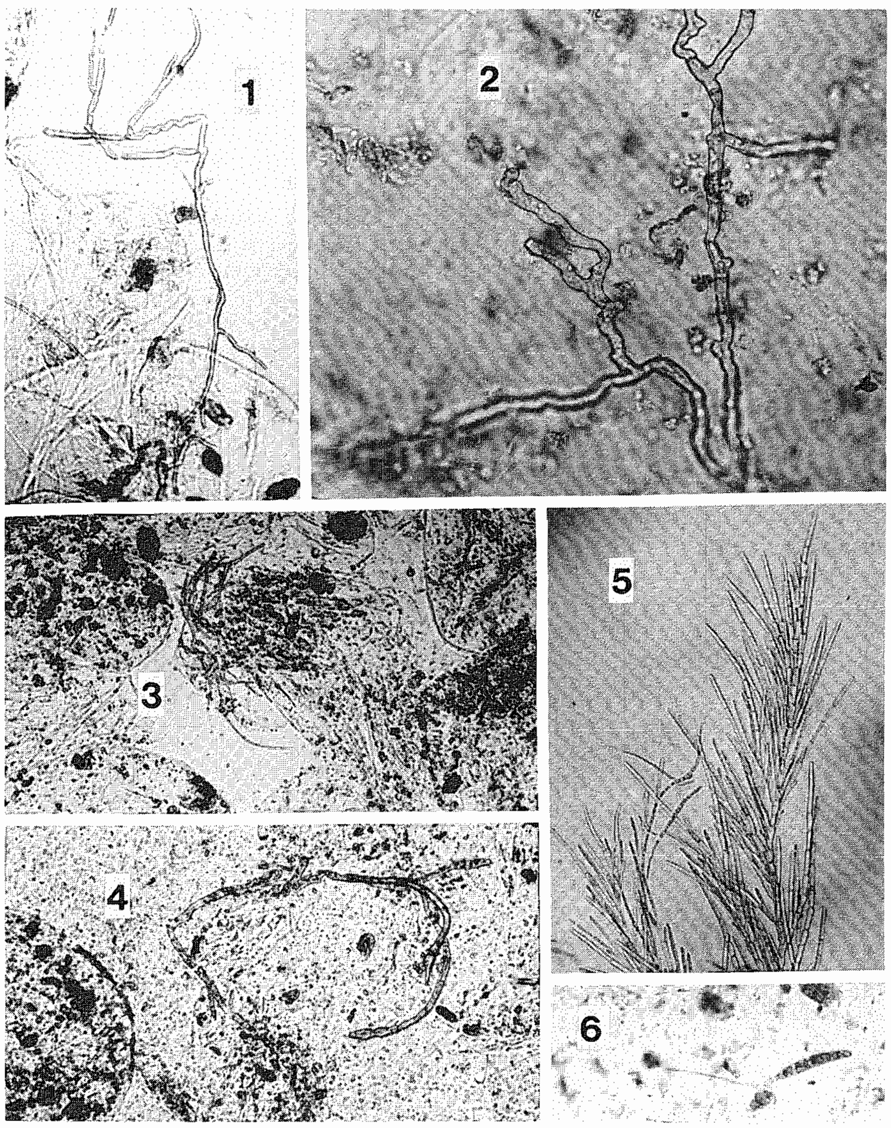



**FIG. 984.29B—Mold filaments in tomato products (100×). 1, branching mold and tomato cells. 2, coarse mold showing nonparallel and parallel walls, branching, granulation, and blunt tips. 3, very fine mold. 4, mold showing beginning of sporulation at end of hypha. 5, *Geotrichum* mold showing cross walls and feathery appearance characteristic of slimy machinery. 6, *Alternaria* spore with attached hypha.**

From each of ≥2 mounts examine ≥25 fields taken in such manner as to be representative of all sections of mount.

A field is scored either pos. or neg. No field can be scored pos. more than once. Method requires that field be counted as pos. when aggregate lengths of not >3 filaments of mold present exceed one-sixth diam. of field. One-sixth diam. of field is not enough to be counted as pos.; aggregate length must *exceed* one-sixth diam. of field.

Analyst must decide whether field is pos. Most pos. fields qualify as such on basis of single mold filament which, including length of branches, exceeds one-sixth of field diam. Field may be qualified as pos. if any one of following lengths exceeds one-sixth of field diam.

- Length of single unbranched filament.
- Length of single filament plus lengths of branches (aggregate length).
- Aggregate length of 2 mold filaments.
- Aggregate length of 3 mold filaments (no more than aggregate lengths of 3 filaments of mold can be counted).
- Aggregate length of all filaments in a *clump* of mold (a clump of mold is considered a single piece, and aggregate lengths of all filaments are counted).

***C. Calculations***

Calc. proportion of pos. fields from results of examination of all observed fields and report as % pos. fields.

## FRUIT AND FRUIT PRODUCTS

### 975.51 Mold in Apple Butter

**Howard Mold Count**

**Final Action**

Dil. 50 mL well-mixed sample with 50 mL stabilizer soln, **945.75C(v)**. Make Howard mold count as in **984.29**.

### 955.47 Mold in Drupelet Berries

**Howard Mold Count**

**Final Action 1974**

(Applicable to blackberries, loganberries, raspberries, and other drupelets; fresh, canned, and frozen)

(**a**) *Frozen with or without sugar*.—Pulp berries thru cyclone, **945.75B(g)**, and mix thoroly. Mix 25 g pulp with 50 mL stabilizer soln, **945.75C(v)**. Proceed as in **984.29**.

(**b**) *Frozen in sirup, canned in sirup or water*.— Drain berries 2 min on No. 20 sieve. Pulp, dil., as in (**a**). Make Howard mold count as in **984.29**.

### 970.75 Mold in Citrus and Pineapple Juices (Canned)

**Howard Mold Count**

**Final Action 1974**

(Applicable to single strength juices)

Pour contents of can into beaker and mix thoroly by pouring back and forth between beaker and can ≥12 times. After mixing, transfer 50 mL juice to graduated 50 mL conical-bottom centrf. tube. Centrf. 10 min at 2200 rpm, **945.75B(f)**. Check speed with tachometer, since rheostat does not necessarily indicate speed in rpm.

Without braking, let centrf. come to complete stop before removing tubes and read vol. sediment in centrf. tube. Remove tube and decant supernate without disturbing sediment. With pineapple juice, add 0.5 mL HCl (to dissolve oxalate crystals). Add $H_2O$ to tube to bring level to 10 mL mark and then add 5 mL stabilizer soln, **945.75C(v)**. Thoroly mix sediment, $H_2O$, and stabilizer soln and pour into small beaker. Mix by pouring back and forth between beaker and tube ≥6 times. Stir mixt. thoroly in beaker. Make Howard Mold count as in **984.29**. Sep. record those fields pos. due to *Geotrichum candidum; see* **984.30A**.

Ref.: JAOAC **66,** 393(1983).

### 970.76 Mold in Cranberry Sauce

**Howard Mold Count**

**Final Action 1974**

(**a**) *Strained sauce*.—Immerse unopened can of sauce in boiling $H_2O$ bath 30–45 min to facilitate breaking gel. Remove can from bath and open carefully to avoid loss of sauce thru sudden release of pressure. Transfer contents into beaker (1 L for No. 2 can). Stir sauce to break gel. (Slow-speed mech. mixer (350–450 rpm) may be used.) Thoroly mix 50 g stirred sauce with 50 g stabilizer soln, **945.75C(v)**. Proceed as in **984.29**.

(**b**) *Whole sauce* (*seeds and skins included*).—Pulp contents of container (if considerably >1 lb (500 g), such as No. 10 can, remove well-mixed aliquot of 1 lb) thru cyclone to remove skins and seeds, and prep. homogeneous pulp. Mix 50 g of this pulp with 50 g stabilizer soln, **945.75C(v)**. Proceed as in **984.29**.

### 982.33 Mold in Fruit Nectars, Purees, and Pastes

**Howard Mold Count**

**First Action 1982**
**Final Action 1988**

***A. Sample Preparation***

(**a**) *Fruit nectars*.—Measure 40 mL well mixed sample into 40 mL graduated, thick-wall centrf. tube (Corning, Pyrex No. 8340, or equiv.) and proceed as in **982.33B**.

(**b**) *Fruit purees with no added starch*.—Dil. sample 1 + 1 with $H_2O$, measure 40 mL well mixed sample into 40 mL graduated, thick-wall centrf. tube, and proceed as in **982.33B**.

(**c**) *Fruit purees with added starch*.—Weigh 50 g fruit puree into beaker and add 50 mL HCl soln (5 + 45). Mix well and heat on steam bath 15 min. Measure 40 mL well mixed, hydrolyzed sample into 40 mL graduated, thick-wall centrf. tube and proceed as in **982.33B**.

(**d**) *Fruit pastes*.—Disperse 1 part paste in 3 parts $H_2O$. If necessary, warm gently to break gel. Measure 40 mL well mixed sample into 40 mL graduated, thick-wall centrf. tube and proceed as in **982.33B**.

***B. Centrifugation and Concentration Adjustment***

Centrf. 10 min at 2200 rpm as in **970.75**. Gradually let centrf. come to complete stop. Remove tubes and immediately decant supernate without disturbing sediment. Gently tap centrf. tube to level top of sediment. Dil. sediment with stabilizer soln, **945.75C(v)**, as follows: (*1*) peach, apricot, mango, and papaya: 1 + 1; (*2*) pear and guava: 1 + 3; (*3*) strawberries, blackberries, raspberries, and blueberries: 1 + 6.

Proceed with Howard mold count as in **984.29**. For products dild 1 + 1 in **982.33B(*1*)**, divide number of pos. fields by 2 before calcg % mold count.

Refs.: JAOAC **65,** 1093(1982); **66,** 393(1983).

## 945.89 Mold in Pureed Infant Food

**Howard Mold Count**

**Final Action 1974**

Proceed as follows: Add ca 0.2 g NaOH to ca 6 g product before counting, and stir thoroly until NaOH is dissolved. Proceed as in **984.29**.

## 952.22 Mold in Strawberries (Frozen)

**Howard Mold Count**

**Final Action**

Pulp thawed berries thru cyclone and mix thoroly. (Pour juice thru cyclone last.) If necessary, remove air bubbles with suction or by mixing ca 100 g pulp with 3–5 drops *2-octanol*. Again mix thoroly and make mold count as in **984.29**.

# VEGETABLES AND VEGETABLE PRODUCTS

## 945.90 Mold in Tomatoes (Canned)

**Howard Mold Count**

**Final Action 1974**

**(a)** *Packing medium*.—Drain contents of can 2 min on No. 2 sieve. For containers of <3 lb net wt, use 8″ diam. sieve; for containers of ≥3 lb net wt, use 12″ sieve. Make Howard mold count, as is, as in **984.29**.

**(b)** *Whole drained tomatoes*.—Examine drained tomatoes and record number and size of any rotten portions present. Pass drained tomatoes thru laboratory cyclone, **945.75B(g)**. Make mold counts on pulped tomatoes, as for juice, as in **984.29**.

## 965.41 Mold in Tomato Products

**Howard Mold Count**

**Final Action**

(Applicable to tomato juice, sauce, catsup, paste, and puree. Not applicable to dehydrated products)

In making mold counts of tomato products, use juice and sauce as they come from container. For catsup, place 50 mL stabilizer soln, **945.75C(v)**, in 100 mL graduate, add 50 mL well mixed catsup sample by displacement, and mix thoroly. In case of puree and paste, add $H_2O$ to make mixt. with tomato sol. solids content that gives refractive index of 1.3448–1.3454 at 20° (1.3442–1.3448 at 25°). Add 2–6 drops 2-octanol to each 100 mL mold count prepn to reduce or eliminate air bubbles on Howard mold counting slide. Proceed as in **984.29**.

Refs.: Bur. Chem. Circ. **68** (1911). Food and Drug Adm. Leaflet, July 1942. Am. Can Co. Bull. (1954). Natl. Canners Assoc. Tomato Products Tables, 2nd rev. (Feb. 1966). JAOAC **49,** 572(1966); **53,** 366(1970).

## 945.91 Mold in Tomato Soup

**Howard Mold Count**

**Final Action 1974**

Place the unopened, punctured can in hot $H_2O$ and heat until contents are thoroly warmed; then open. Transfer 10 mL thoroly mixed soup to 50 mL centrf. tube and add 3 mL NaOH soln (1+1). If starch is absent, omit the NaOH. Stir until starch dissolves and tissues clear. Add enough $H_2O$ to fill tube, and centrf. (Time required to centrf. sample varies greatly. With centrf. arm length of $5^1/_4$″ and speed of ca 1600 rpm, ca 20 min is required for av. sample. In heavy soups, gelatinizing of much starch sometimes interferes with proper settling out of solids during centrfg. If liq. remains cloudy, it may be necessary to discard sample and start again by adding 3 mL NaOH soln to only 5 mL soup.) When supernate is clear, pour off; if not entirely clear, check supernate for mold before discarding. Add enough $H_2O$ to residue in tube to bring to original vol. of soup, mix, and count mold as in **984.29**.

## 945.92 Mold in Tomato Sauce

**Howard Mold Count**

**Final Action 1974**

(Applicable to sauce in pork and beans, spaghetti, ravioli, chili con carne, tamales, etc.)

Place unopened, punctured can in hot $H_2O$ and heat until contents are thoroly warmed. Open can and transfer contents onto No. 6 sieve. Drain until major portion of liq. passes thru. (With some products, sauce runs thru at once, but in case of some beans and spaghetti, ≥10 min may be required.) Mix sauce thoroly, place 10 mL in centrf. tube, and proceed as in **945.91**, beginning "add 3 mL NaOH." *Note:* Use care in counting products contg meat so as not to confuse mold filaments and muscle fibers that superficially resemble each other; muscle fibers are usually much thicker and striations are often visible.

## 945.93 Mold in Tomato Sauce Packing Medium on Fish

**Howard Mold Count**

**Final Action 1974**

Place unopened, punctured can in hot $H_2O$ (ca 90–95°) until contents are thoroly warmed. Open can and drain contents on No. 6 sieve until major portion of sauce and oil passes thru. Mix liq., place up to 50 mL in 50 mL centrf. tube, and centrf. as in **945.91**. Record vol. of lower oil-free sauce layer, and discard oil and part of mold-free aq. layer. Add $H_2O$ to bring to recorded vol., mix, and count mold as in **984.29A**, removing bits of fish tissue from slide, if necessary, before counting. See caution in **945.92**.

## 972.42 Mold in Tomato Powder (Dehydrated)

**Howard Mold Count**

**First Action 1972**
**Final Action 1988**

(Tomato powder is produced by dehydrating concd tomato pulp. In prepg powder for mold counting, moisture content is disregarded and diln with $H_2O$ is made to give mixt. with approx. tomato solids content of stdzd prepn for mold count of tomato puree or paste, i.e., 8.5%.)

### A. Microscopic Identification as Spray-Dried Product

Mold counts of spray-dried tomato powder show significantly higher counts than paste from which it is made because of breakage of mold hyphae aggregates. Use following procedure to det. whether powder represents spray-dried product.

Suitably mount a small portion of product on microscope slide in mineral oil or other non-aqueous mounting medium and examine microscopically at 100–200×. Spray-dried particles are translucent and contain air bubbles and numerous small granules within the particles. Shape of particles ranges from spherical to elongate to irregular with rounded outlines and essentially no sharp angles. In rehydrated powder, practically no intact tomato cells are evident. Drum-dried or similarly processed powder or flakes are characterized by irregular-shaped particles with angular outlines and practically no embedded air bubbles.

### B. Determination

Weigh 17.0 g thoroly mixed sample into high-speed blender, **945.75B(c)**, contg 150 mL $H_2O$ to produce mixt. equiv. to tomato puree. Blend 30 sec at ca 3200 rpm and, with rubber policeman, rub down any material adhering to walls. Rinse walls with 50.0 mL $H_2O$ to bring total vol. to 200 mL, and blend 1 min. Add 2 drops 2-octanol to break foam and count mold as in **984.29**.

Refs.: JAOAC **55,** 73(1972); **61,** 992(1978).

## 937.13★ Mold in Butter

**Final Action**
**Surplus 1970**

*See* **40.024**, 11th ed.

## 945.94 Mold in Ground Spices

**Howard Mold Count**
**Final Action 1974**

(Applicable to garlic powder, paprika, red and cayenne pepper, chili powder, and other ground capsicums)

Weigh 10 g thoroly mixed sample of ground spice and transfer to high-speed blender. Add 200 mL 1% NaOH soln in 3 or 4 successive portions, stirring after each addn, washing down with final portion any material that may stick to walls of blender. Agitate mixt. in blender 1 min at ca 13,000 rpm. With rubber policeman, rub down into mixt. any material sticking to walls and repeat blending 2 min longer. Add 2 or 3 drops 2-octanol to break foam. Mix 100 g of this mixt. with 50 g stabilizer soln, **945.75C(v)**, and count as in **984.29**.

Occasionally blended mixt. contains particles of seed tissue that make it difficult to obtain Newton's rings in prepg slide for mold counting. Clamp devised for holding cover slip in place to obviate this difficulty consists of metal plate with circular opening, 2.5 cm diam., in center of plate; 2 clips attached to edge of plate hold cover slip in position when slide is placed on plate. Seed particles may also be picked off Howard slide with micro-forceps.

Refs.: JAOAC **55,** 78(1972); **61,** 475(1978).

## 984.30 *Geotrichum* Mold Counting

### A. Diagnostic Characteristics

*Geotrichum* mycelial fragments have the same characteristics of mold hyphae as described in **984.29A**. In addition, *Geotrichum* mold filaments are septate, tend to taper toward the tip, and branch at a 45° angle, giving the mold a feathery appearance as shown in Fig. **984.30**.

### B. Determination

Using pipet, **945.75B(p)**, take up 0.5 mL of well mixed sample and apply as streak ca 4 cm long to rot fragment counting slide, **945.75B(q)**. Blow out last drop, if necessary. Prep. addnl slides in same way.

Examine each slide at 30–45×, using transmitted diffused bottom illumination. Count *Geotrichum* mycelial fragments (with 3 or more characteristic hyphae branches, Fig. **984.30**) on 2 *entire* slides.

### C. Calculations

Calc. mycelial fragments/500 g product:

$$N = (S/V\{\text{slides}\}) \times (500/W) \times V\{\text{diln}\}$$

where $S$ = total mycelial fragments counted; $V$\{slides\} = total vol. counted (0.5 mL/slide); $W$ = net wt of sample, g; and $V$\{diln\} = vol. after final diln with stabilizer soln.

## 945.95 Mold in Soft Drinks

***Geotrichum* Mold Count**
**Final Action 1988**

Det. net wt, $W$, of container and transfer contents to 3″ No. 230 sieve, **945.75B(r)**. Transfer residue from sieve to 50 mL graduated centrf. tube. Dil. to 10 mL. Add 1 drop crystal violet staining soln, **945.75C(h)**, and mix thoroly. Add 10 mL stabilizer soln, **945.75C(v)**, and bring total vol., $V$, to 20 mL. Make *Geotrichum* mold count on 2 slides as in **984.30**.

Ref.: JAOAC **57,** 957(1974).

## 980.29 Mold in Citrus Juices

***Geotrichum* Mold Count**
**First Action 1980**
**Final Action 1988**

Use single strength juice as is; dil. conc. to single strength. Pour vol. ≤250 mL (record vol. and wt used) onto 5″ No. 40 sieve, **945.75B(r)**, resting in 2 L beaker. Wash container with $H_2O$ from wash bottle and transfer washings to sieve. Save residue, liq., and washings. Quant. transfer liq. and washings onto 5″ No. 140 sieve resting in second 2 L beaker. Wash first beaker and transfer washings onto No. 140 sieve. Save residue, liq. and washings. Quant. transfer liq. and washings from No. 140 sieve onto 5″ No. 230 sieve. Wash second beaker into sieve. Discard liq. and washings but save residue.

With wash bottle and spatula, quant. transfer residues from Nos. 40 and 140 sieves to No. 230 sieve. Tilt No. 230 sieve at ca 30° and wash residue to lower edge of sieve with $H_2O$. With wash bottle and spatula, transfer residue on No. 230 sieve to 50 mL graduated centrf. tube. If vol. transferred is ≤20 mL, dil. to 20 mL with $H_2O$. Add 5 drops crystal violet soln, **945.75C(h)**, mix well, and dil. to 40 mL with stabilizer soln, **945.75C(v)**. Mix well and proceed as in **984.30**.

If vol. transferred is ≥20 mL, add 5 drops crystal violet soln

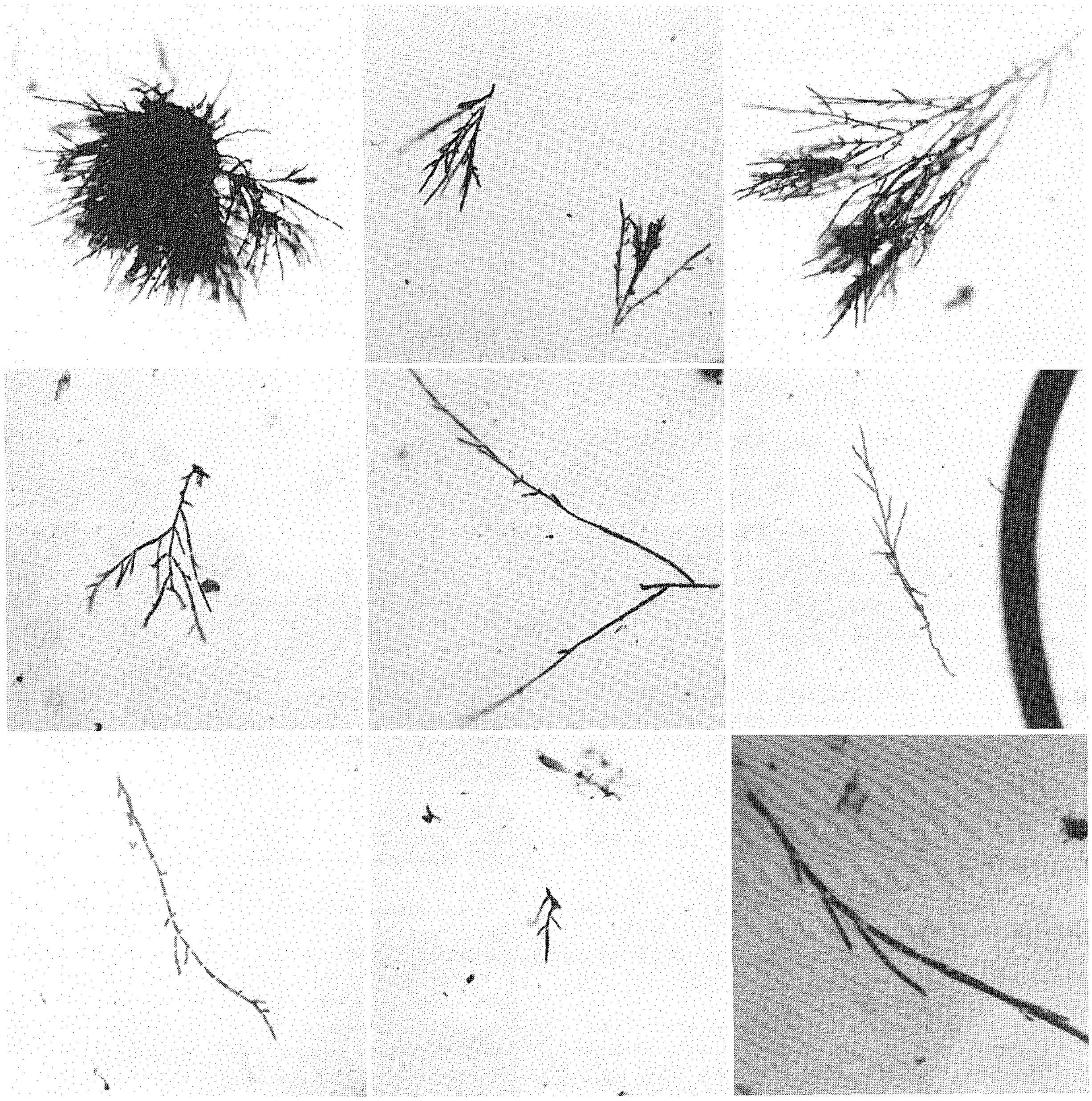


**FIG. 984.30—*Geotrichum* mold fragments**

and centrf. 6 min at 2200 rpm (*see* **970.75**). Dil. pellet to 20 mL with $H_2O$, mix well, and dil. to 40 mL with stabilizer soln. Mix well and proceed as in **984.30**.

Ref.: JAOAC **63,** 483(1980).

## 974.34 Mold in Vegetables, Fruits, and Juices (Canned)

### *Geotrichum* Mold Count

**First Action 1974**
**Final Action 1988**

(Applicable to products where mold is not masked by large amts of tissues)

Det. net wt (g) of can contents. Drain contents 3 min on 8″ No. 8 sieve, **945.75B(r)**, in pan. Remove fruit from sieve with spoon and discard. Wash can and sieve with ca 300 mL $H_2O$ from wash bottle, saving liq. and washings. Quant. transfer combined liq. and washings onto 5″ No. 16 sieve resting in 2 L beaker. Wash residue on sieve with ca 50 mL $H_2O$, and discard residue. Quant. transfer combined liq. and washings onto 5″ No. 230 sieve, tilted at ca 30° angle, and discard liq. and washings. Wash tissue to lower edge of sieve with $H_2O$.

With wash bottle and spatula, transfer residue from sieve to 50 mL graduated thick-walled centrf. tube with min. vol. $H_2O$. For vols $\leq$10 mL, use (**a**); $>$10 mL but $\leq$30 mL, use (**b**); for $>$30 mL, use (**c**).

(**a**) Dil. to 10 mL. Add 1 drop crystal violet staining soln, **945.75C(h)**, and mix thoroly. Add 10 mL stabilizer soln, **945.75C(v)**, to bring total vol. to 20 mL. Proceed as in **984.30**.

(**b**) Dil. to 40 mL. Add 3 drops crystal violet soln. Mix well. Centrf. ca 6 min at ca 2200 rpm, **970.75**. Decant and discard supernate. Bring vol. of sediment in centrf. tube to nearest 5 mL graduation by adding $H_2O$. Note combined vol. of sediment and $H_2O$, add equal vol. stabilizer soln, and mix thoroly but gently. Record mL total vol. of mixt. in centrf. tube, ($V$). Proceed as in **984.30**.

(**c**) Transfer to g-s graduate. Dil. to ≥100 mL ($V$\{prepn\}) and mix well. Quickly pour off two 25 mL aliquots ($V$\{aliq.\} = sum of aliquots taken) into sep. centrf. tubes and proceed as in (**b**). Keep final vols equal after dilg with stabilizer soln; $V$\{diln\} = sum of vol. in both tubes. Proceed as in **984.30**, using pipet to prep. 1 slide from each dild aliquot. Calc. mycelial fragments/500 g product:

$$N = (S/V\{\text{slides}\}) \times (500/W) \times V\{\text{diln}\} \times (V\{\text{prepn}\}/V\{\text{aliq.}\})$$

where $S$ = total mycelial fragments counted; $V$\{slides\} = total vol. counted (0.5 mL/slide); $W$ = net wt of sample, g; $V$\{diln\} = sum of vol. in both centrf. tubes after final diln with stabilizer soln; $V$\{prepn\} = vol. before aliquots removed; and $V$\{aliq.\} = sum of vol. of aliquots taken.

Refs.: JAOAC **57,** 957(1974); **62,** 390(1979); **63,** 287(1980).

## 982.34 Mold in Comminuted Fruits and Vegetables

### *Geotrichum* Mold Count

**First Action 1982**
**Final Action 1988**

### *A. Sample Preparation*

(**a**) *Fruit nectars*.—Add 40 mL nectar and 10 drops of crystal violet stain, **945.75C(h)**, to 40 mL centrf. tube (Corning, Pyrex No. 8340, or equiv.). Mix well and proceed as in **982.34B**.

(**b**) *Purees with no added starch*.—Add 20 mL puree and 10 drops of crystal violet stain, **945.75C(h)**, to 40 mL centrf. tube. Mix well. Bring vol. to 40 mL with $H_2O$ and mix well. Proceed as in **982.34B**.

(**c**) *Purees with starch added*.—Add 50 mL HCl soln (5 + 45) to 50 g fruit puree. Mix well and heat with mag. stirring until starch clears. Neutze soln with 50% KOH or 50% NaOH to pH 7.0 ± 1.0. Transfer 40 mL soln to 40 mL centrf. tube and add 20 drops of crystal violet stain, **945.75C(h)**. Mix well and proceed as in **982.34B**.

(**d**) *Pastes*.—Disperse 1 part paste in 3 parts $H_2O$. If necessary, warm gently to break gel. Transfer 40 mL soln to 40 mL centrf. tube and add 10 drops of crystal violet stain, **945.75C(h)**. Mix well and proceed as in **982.34B**.

### *B. Centrifugation*

Centrf. 10 min at 2200 rpm, **945.75B(f)**. Immediately after centrf. comes to rest, decant aq. layer and read vol. of sediment. Dil. sediment 1 + 3 (v/v) with stabilizer soln, **945.75C(v)**. Proceed as in **984.30**. Express results in mycelial fragments per 100 mL prepn. $N = S \times 100$, where $S$ = total mycelial fragments/mL sample prepn countd (0.5 mL/slide).

Ref.: JAOAC **65,** 1095(1982).

## 980.30 Mold in Cream Style Corn

### *Geotrichum* Mold Count

**First Action 1980**
**Final Action 1988**

Weigh 250 g product onto 5″ No. 16 sieve, **945.75B(r)**, resting in container. Wash can and residue on sieve with ca 1.5 L hot (44–55°) $H_2O$, saving liq. and washings. Discard residue and rewash sieve with ca 300 mL hot $H_2O$. Quant. transfer combined liq. and washings onto 5″ No. 70 sieve. Wash residue on sieve with ca 1 L hot $H_2O$, saving liq. and washings but discarding residue. Quant. transfer combined liq. and washings onto 5″ No. 230 sieve tilted at ca 30°. Wash residue with ca 500 mL hot $H_2O$ and discard liq. and washings. Wash tissue to lower edge of sieve with hot $H_2O$. With wash bottle and spatula, transfer residue from sieve to 100 mL graduate, keeping vol. ≤50 mL. Dil. to 50 mL with $H_2O$. Add 20 drops crystal violet soln, **945.75C(h)**, and mix well. Dil. to 100 mL with stabilizer soln, **945.75C(v)**. Mix well and proceed as in **984.30**, making count on 2 slides.

Ref.: JAOAC **63,** 481(1980).

## 952.23 Rot in Tomato Products (Comminuted)

### Rot Fragment Count

**Final Action 1974**

Weigh sample directly into 400 mL beaker, using elec. top-loading balance with readability of 0.1 g, precision (std dev.) of ±0.05 g, and 1200 g capacity (Sartorius No. 3706, Sartorius Gmbh, Weender Landstr 94/108, D-3400 Gottingen, West Germany, or equiv.). In case of puree or paste, add $H_2O$ to make mixt. of tomato sol. solids content that gives refractive index of 1.3448–13454 at 20° (1.3442–1.3448 at 25°). Use 5.0 g catsup, sauce, or dild puree or paste; and 10.0 g juice.

Add ca 100 mL $H_2O$ to sample in beaker and stir thoroly using glass rod until sample material appears well dispersed. Add 12 drops crystal violet soln, **945.75C(h)**, stir, and let stand 5 min. Add 200 mL $H_2O$ and pour directly from beaker onto a 3 in. diam. No. 60 sieve. Rinse beaker with addnl 200 mL $H_2O$ and pour $H_2O$ directly from beaker over sieve as before. Spread samples evenly over sieve. Tilt sieve to ca 30° angle and carefully wash tissue to lower edge, using stream of $H_2O$ from polyethylene wash bottle (500 mL Nalgene No. 2402-0500 wash bottle with delivery tube molded on side, Nalge Co., 75 Panorama Creek Dr, PO Box 20365, Rochester, NY 14602, or equiv.). Let tissue drain. If necessary, repeat washing and draining steps until tomato tissue is concd at lower edge of sieve. Transfer tissue, portion-wise, with micro-spoon (Scientific Products No. S1571, or equiv.), or small scoop-style spatula to bottom of graduated tube ca 12 × 3 cm. Tissue remaining on sieve should be washed to lower edge as before. Hold sieve at ca 80–90° angle so that some $H_2O$ and tissue is retained at edge of sieve. Immediately take up $H_2O$ and tissue with eyedropper having tip cut off at 2 mm id, and transfer to graduated tube. Repeat process of washing tissue to lower edge of sieve with wash bottle and transferring with dropper until all tissue has been transferred. Bring vol. of $H_2O$ and tissue to 10 mL with $H_2O$. Add stabilizer soln, **945.75C(v)**, to bring vol. to 20 mL and mix well using micro-spoon or spatula. Pipet 4 sep. 0.5 mL portions using pipet, **945.75B(p)**, stirring sample prepn with pipet before drawing up each portion and pipetting from ca center of prepn. Spread 0.5 mL portion evenly over each of 4 counting slides, **945.75B(q)**, letting material flow slowly, and spread uniformly in center of slide to cover area ca 6 × 2 cm. Touch lower end of pipet to slide several times to ensure complete removal of material. Blow out last drop if necessary. Examine each slide at 40–45×, using trans-

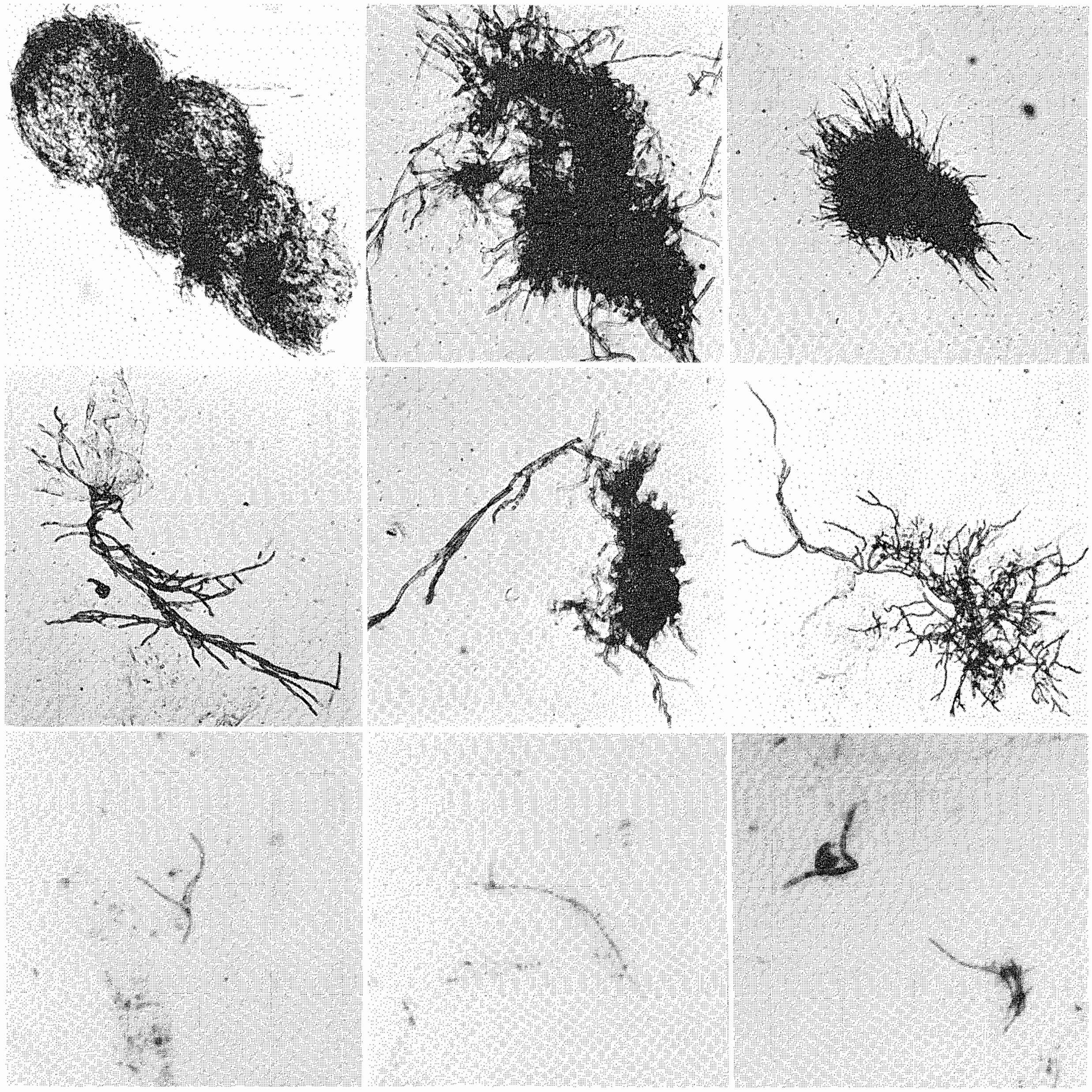


**FIG. 952.23—Rot fragments from tomato puree**

mitted light. Rot fragment is defined as particle of tomato cellular material with one or more mold filaments attached. Some may appear as almost solid masses of mold. See Figs. **984.29B**, **984.30**, **952.23**.

Count number of rot fragments on each of 4 slides. Add results from 4 slides to obtain number of rot fragments/g juice. Add results from 4 slides and multiply by 2 for 5.0 g samples (catsup, sauce, or dild puree or paste). Report number of rot fragments/g product.

Refs.: JAOAC **35,** 337(1952); **53,** 366(1970); **68,** 278, 896(1985). Natl. Canners Assoc. Tomato Products Tables, 2nd rev. (Feb. 1966).

## 945.96★ Yeasts and Spores in Tomato Products

**Final Action**
**Surplus 1970**

(Not applicable to dehydrated products)

*See* **40.086**, 11th ed.

## GENERAL REFERENCES

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(2) Annotated Bibliography of Methods for Examination of Foods. JAOAC **29,** 420(1946); **38,** 1016(1955).
(3) Insect Contaminants of Foods. JAOAC **33,** 898(1950).
(4) Insect Setae. JAOAC **37,** 960(1954).
(5) Radiographic Applications. JAOAC **37,** 148(1954).
(6) "Identification of Stored Products by the Micromorphology of the Exoskeleton." A series published in JAOAC (reprints are no longer available): Elytral patterns, **38,** 776(1955); Adult antennae, **39,** 879(1956); Larval fragments, **39,** 990(1956); Adult legs, **40,** 973(1957); Adult and larval beetle mandibles, **41,** 460(1958); Adult labral characteristics, **41,** 472(1958); Head, thorax, abdomen, **41,** 828(1958); Adult moths, **43,** 444(1960); Larvae of moths, **41,** 704(1958); Cockroach fragments, **41,** 886(1958); Miscellaneous insects, **41,** 206(1958).
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(8) Winton, A. L. and Winton, K. B., "The Structure and Composition of Foods." John Wiley & Sons (4 Vols: 1932–1939).
(9) "Food Microscopy." A series published in Food **25**(1956)–**28**(1959).
(10) "Training Manual for Analytical Entomology in the Food Industry." AOAC, 2200 Wilson Blvd, Arlington, VA 22201 (1977).

# 17. Microbiological Methods

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(When preparing culture media, use distd or deionized $H_2O$ such as Purified Water USP XXII, found to be free from traces of dissolved metals, and bactericidal or inhibitory compds. Use anhyd. salts unless otherwise specified.)

## 970.77 Cross Reference Tables

### *A. Methods for Examination of Foods*

- Beef, ground
  - Virus **975.56**
- Candy and candy coatings
  - *Salmonella* **975.54D**
- Casein
  - *Salmonella* **967.26**
- Cheese powders
  - *Salmonella* **985.42**
- Chocolate, milk
  - *Salmonella*
    - Culture **967.26**
    - Hydrophobic grid membrane filter **985.42**
- Coconut
  - *Salmonella* **975.54D**
- Dairy products
  - Coliforms
    - Dry rehydratable film **989.10**
    - Pectin gel **989.11**
- Egg, powdered
  - *Salmonella* **985.42**
- Eggs and egg products
  - Aerobic plate counts
    - Standard **940.37B**
    - Spiral **977.27**
  - Coliform organisms **940.37C**
  - Direct microscopic count **940.37F**
  - Fungi **940.37E**
  - *Salmonella*
    - Culture **967.26**
    - Fluorescent antibody **975.54D**
  - Staphylococci, hemolytic **940.37D**
  - Streptococci **940.37D**
- Fish meal
  - *Salmonella* **975.54D**
- Food ingredients, raw; and nonprocessed food
  - *Staphylococcus aureus* **980.37**
- Foods
  - Aerobic plate count
    - Hydrophobic grid membrane filter **986.32**
    - Pectin gel **988.18**
  - *Bacillus* **980.31**
  - *Bacillus cereus* **983.26**
  - *Salmonella*
    - Biochemical identification kit **978.24**
    - Colorimetric monoclonal EIA screening **986.35**
    - Colorimetric polyclonal EIA screening **989.14**
    - DNA hybridization screening **987.10**
    - Fluorogenic monoclonal EIA screening **989.15**
  - *Salmonella*, motile
    - Immunodiffusion screening **989.13**
  - *Salmonella* sp., *Escherichia coli,* and other *Enterobacteriaceae* in foods **989.12**
  - Staphylococcal enterotoxin
    - Extraction and separation **980.32**
    - Microslide gel double diffusion **976.31**
  - *Staphylococcus aureus*
    - Most probable number **987.09**
    - Surface plating **975.55**
  - Total coliforms, fecal coliforms, and *Escherichia coli* **983.25**
- Foods, canned
  - *Clostridium botulinum* and its toxins **977.26**
- Foods, canned, low-acid
  - Commercial sterility **972.44**
  - Microleak detection **984.36**
  - Sporeformers **985.41**
- Foods, chilled, frozen, precooked, or prepared, and nutmeats
  - Aerobic plate count **966.23C**
  - Coliform organisms **966.24**
  - *Escherichia coli* **966.24**
- Foods, chilled or frozen
  - *Escherichia coli* **988.19**
- Foods, low-moisture
  - *Salmonella*
    - Colorimetric monoclonal EIA screening **987.11**
- Foods, outbreak
  - *Clostridium perfringens*
    - Alpha toxin estimation **974.38**
    - Plate count for isolation and enumeration **976.30**
- Foods and cosmetics
  - Aerobic plate count **977.27**
- Frog legs
  - *Salmonella* **976.54D**
- Garlic powder
  - *Salmonella* **967.26**
- Mammalian cells
  - *Escherichia coli,* invasiveness **982.36**
- Meat and meat products
  - *Salmonella* **975.54D**
- Mild, fluid
  - Dry rehydratable film
    - Bacterial count **986.33**
    - Coliform count **986.33**
    - Somatic cell count **973.68, 978.25, 978.26, 980.33**
- Milk, nonfat dry
  - *Salmonella*
    - Culture **967.26**
    - Fluorescent antibody **975.54D**
    - Hydrophobic grid membrane filter **985.42**
- Milk products, dried
  - *Salmonella*
    - Culture **967.26**

### *B. Methods for Examination of Organisms*

Virus
Beef, ground **975.56**

## EGGS AND EGG PRODUCTS

### 939.14 Sampling of Eggs and Egg Products
**Microbiological Methods**
**Final Action**

("Compendium of Methods for the Microbiological Examination of Foods," 2nd ed. Prepd by the APHA Intersociety/Agency Committee on Microbiological Methods for Foods. 1984. Marvin L. Speck, Ed., should be used as guide for further study of microorganisms obtained in culturing technics described.)

#### A. Equipment

(**a**) *Liquid eggs*.—Sampling tube or dipper, sterile sample containers with tight closures (pt (500 mL) Mason jars or friction top cans are most practical), alcohol, alcohol lamp or other burner, absorbent cotton, clean cloth or towel, and $H_2O$ pail.

(**b**) *Frozen eggs*.—Elec. (high-speed) or hand drill with 1 × 16" auger, hammer and steel strip (12 × 2 × 0.25"), or other tool for opening cans; tablespoon, hatchet or chisel, precooled sterile containers, etc., as in (**a**).

(**c**) *Dried eggs*.—Grain trier long enough to reach to bottom of containers to be sampled. Clean sample containers with tight closures (pt (500 mL) Mason jars or paperboard cartons), clean cloth or towel, and tablespoon.

#### B. Methods

Take samples from representative number of containers in lot, **925.29**. Sterilize sampling tube or dipper, auger, spoon, and hatchet by wiping with alcohol-soaked cotton and flaming over alcohol lamp or other burner. Between samplings, thoroly wash instruments, dry, and resterilize. Open and sample all containers under as nearly aseptic conditions as possible.

(**a**) *Liquid eggs*.—Thoroly mix contents of container with sterile sample tube or dipper, and transfer ca 400 mL (0.75 pt) to sterile sample container. Keep samples at <5° but avoid freezing. Observe and record odor of each container sampled as normal, abnormal, reject, or musty.

(**b**) *Frozen eggs*.—Remove top layer of egg with sterilized hatchet or chisel. Drill 3 cores from top to bottom of container: first core in center, second core midway between center and periphery, and third core near edge of container. Transfer drillings from container to sample container with sterile spoon. Examine product organoleptically by smelling at opening of fourth drill-hole made after removal of bacteriological sample. (Heat produced by elec. drill intensifies odor of egg material, thus facilitating organoleptic examination.) Record odors as normal, abnormal, reject, or musty. Refrigerate samples with solid $CO_2$ or other suitable refrigerant if analysis is to be delayed or sampling point is at some distance from laboratory.

(**c**) *Dried eggs*.—For small packages, take entire parcel or parcels for sample. For boxes and barrels, remove top layer with sterile spoon or other sterile instrument, and with sterile trier remove ≥3 cores as in (**b**). (Samples should consist of ca 400 mL (0.75 pt).) Aseptically transfer core to sample container with sterile spoon or other suitable instrument. Store samples under refrigeration or in cool place.

Ref.: JAOAC **22,** 625(1939).

### 940.36 Culture Media for Eggs and Egg Products
**Microbiological Methods**
**Final Action**

#### A. Standard Methods Media

(**a**) *Dilution water*.—To prep. stock soln, dissolve 34 g $KH_2PO_4$ in 500 mL $H_2O$, adjust to pH 7.2 with 1*N* NaOH (ca 175 mL), and dil. to 1 L with $H_2O$. To prep. buffered $H_2O$ for dilns, dil. 1.25 mL stock soln to 1 L with boiled and cooled $H_2O$. Autoclave 15 min at 121°.

(**b**) *Buffered glucose broth* (*MR-VP medium*).—For Me red-Voges Proskauer (MR-VP) tests. Dissolve 7.0 g proteose peptone, 5.0 g glucose, and 5.0 g $K_2HPO_4$ in ca 800 mL $H_2O$ with gentle heat and occasional stirring. Filter, cool to 20°, and dil. to 1 L. Dispense 10 mL portions into test tubes and autoclave 12–15 min at 121°. Max exposure to heat should be ≤30 min. Final pH, 6.9 ± 0.2.

(**c**) *Endo medium*.—Suspend 3.5 g $K_2HPO_4$, 10.0 g peptone, 20.0 g agar, and 10 g lactose in 1 L $H_2O$. Boil to dissolve, add $H_2O$ to original vol., and clarify if necessary. Dispense in 100 mL portions and autoclave 15 min at 121°. Final pH, 7.4 ± 0.1. Before use, melt and add 0.25 g $Na_2SO_3$ and 1.0 mL filtered 5% alc. soln basic fuchsin.

(**d**) *Eosin methylene blue agar* (*Levine*).—Dissolve 10.0 g peptone, 2.0 g $K_2HPO_4$, and 15.0 g agar in 1 L $H_2O$. Boil to dissolve and add $H_2O$ to original vol. Dispense in 100 or 200 mL portions and autoclave 15 min at 121°. Final pH, 7.1 ± 0.1. Before use, melt and to each 100 mL add 5 mL sterile 20% lactose soln, 2.0 mL 2% aq. Eosin Y soln, and 1.3 mL 0.5% aq. methylene blue soln.

(**e**) *Koser's citrate broth*.—Dissolve 1.5 g $NaNH_4HPO_4.4H_2O$, 1.0 g $KH_2PO_4$, 0.2 g $MgSO_4.7H_2O$, and 3.0 g Na citrate.$2H_2O$ in 1 L $H_2O$. Dispense in 10 mL portions into test tubes and autoclave 15 min at 121°. Final pH, 6.7 ± 0.1.

(**f**) *Lactose broth*.—Dissolve on $H_2O$ bath, with stirring, 3.0 g beef ext and 5.0 g polypeptone or peptone in 1 L $H_2O$. Add 5.0 g lactose. Dispense into fermentation tubes and autoclave 15 min at 121°. Max. exposure to heat should be ≤30 min. Final pH, 6.7 ± 0.2.

(**g**) *Plate count agar* (*tryptone glucose yeast agar*).—Suspend 5.0 g peptone-tryptone (pancreatic digest of casein), 2.5 g yeast ext, 1.0 g glucose, and 15.0 g agar in 1 L $H_2O$. Heat and boil until all ingredients are dissolved. Autoclave 15 min at 121°. Final pH, 7.0 ± 0.1.

(**h**) *Tryptophane broth*.—Dissolve by heating, with stirring, 10.0 g tryptone or trypticase in 1 L $H_2O$. Dispense in 5 mL portions into test tubes and autoclave 15 min at 121°. Final pH, 6.9 ± 0.2.

#### B. Other Media

(**a**) *Malt agar*.—Dissolve by boiling 30 g malt ext (Difco) and 15.0 g agar in 1 L $H_2O$. Autoclave 15 min at 121°. Just before use, melt malt agar and acidify with 85% lactic acid to pH 3.5. Do not reheat medium after addn of acid.

(**b**) *Milk protein hydrolysate glucose agar*.—BBL dehydrated, or prep. from 9.0 g milk protein hydrolysate, 1 g glucose, 15 g agar, and 1 L $H_2O$; adjust to pH 7.0. Autoclave 15 min at 121°, cool to room temp., and readjust pH to 7.0, if necessary.

(**c**) *Physiological salt soln*.—Dissolve 8.5 g NaCl in 1 L $H_2O$. Autoclave 15 min at 121° and cool to room temp.

(**d**) *Veal infusion agar*.—Mix 500 g ground lean veal and 1 L $H_2O$. Infuse overnight in refrigerator and strain thru cheesecloth without pressure. Dil. to original vol. with $H_2O$

and skim off any fat. Steam in Arnold sterilizer 30 min and filter thru paper. Add 10.0 g peptone (Difco), 5.0 g NaCl, and 15.0 g agar.

Steam in Arnold sterilizer to dissolve ingredients. Adjust to pH 7.6 and steam in Arnold sterilizer 15 min. Filter thru buchner with paper pulp mat, with suction. (Use egg albumen for clarification when necessary. Add fresh white of 1 egg previously beaten with 50 mL medium or its equiv. in desiccated egg white (1.5 g) to each L of medium before adjusting pH and after cooling to 50°. Shake thoroly to ensure soln of egg white. Let stand 20 min. Heat in Arnold sterilizer 15 min to coagulate egg white. Shake vigorously and reheat. Filter, adjust to pH 7.6, steam in Arnold sterilizer 15 min, and filter.)

Place 10 mL portions in test tubes or 80 mL portions into bottles. Autoclave 20 min at 121°; final pH, 7.4.

For hemolytic tests, cool melted agar to 45° and add 5% defibrinated horse, sheep, or rabbit blood prior to pouring plates (0.5 mL blood/10 mL medium).

## 940.37 Technics for Eggs and Egg Products

### Microbiological Methods

### Final Action

### *A. Preparation of Sample*

(**a**) *Liquid eggs*.—Thoroly mix sample with sterile spoon or sterile mech. stirrer and prep. 1:10 diln by aseptically weighing 11 g egg material into sterile wide-mouth g-s or screw-cap bottle; add 99 g sterile diln $H_2O$, **940.36A(a)**, or sterile physiological salt soln, **940.36B(c)**, and 1 tablespoonful sterile glass shot. Thoroly agitate 1:10 diln to ensure complete soln or distribution of egg material in diluent by shaking each container rapidly 25 times, each shake being up-and-down movement of ca 30 cm, time interval not exceeding 7 sec. Let bubbles escape. Transfer representative portion from 1:10 diln for higher serial dilns as needed. Proceed as in **940.37B–F(a)**. Pour all plates and inoculate other media within 15 min after prepn of first diln to avoid growth or death of microorganisms.

(**b**) *Frozen eggs*.—Thaw frozen egg material as rapidly as possible to avoid increase in number of microorganisms present and at temp. low enough to prevent destruction of the microorganisms (≤45° for ≤15 min). (Frequent rotary shaking of sample container aids in thawing frozen material. Thawing temp. may be maintained by use of $H_2O$ bath or bacteriological incubator.) Proceed as in (**a**).

(**c**) *Dried eggs*.—Thoroly mix sample with sterile spoon or spatula. Prep. 1:10 diln as in (**a**). If material is relatively insol. (stored samples), use 0.1*N* LiOH as diluent. Prep. serial dilns as in (**a**) and proceed as in **940.37B–F(b)**.

### *B. Plate Counts*

Inoculate one set of petri plates with 1 mL portion of each suitable diln. Pour plates with tryptone glucose yeast agar or milk protein hydrolysate glucose agar previously cooled to 42–45°. Incubate inoculated plates 3 days at 32°. Count plates with aid of Quebec colony counter, if available. Express final results as number of viable microorganisms/g egg material.

### *C. Incidence of Coliform Group*

(**a**) Inoculate 1.0 mL portions from suitable dilns of egg material into fermentation tubes of lactose broth. Incubate 24–48 hr at 35°. Streak eosin methylene blue or Endo medium plates from all lactose broth cultures showing gas production. Incubate plates 24–48 hr at 35°. Examine plates of differential media for colonies of microorganisms of coliform group. Record number of coliform bacteria/g egg material as reciprocal of highest diln showing pos. confirmation on differential media.

(**b**) *Biochemical reaction* (*optional*).—Inoculate from colonies of coliform types of bacteria appearing on differential agar plates to agar slants, **940.36A(g)** or **940.36B(b)**. Incubate 24 hr at 35°. Purify cultures for further study. Obtain IMViC biochem. reactions of purified cultures by following tests:

*Kovacs test* (indole production), **966.24(a)**;

*Acid production in Me red indicator*, **966.24(b)**;

*Acetylmethylcarbinol production*, **966.24(b)**;

*Koser sodium citrate test* (utilization of Na citrate as sole source of C), **966.24(c)**.

*Note:* Follow methods for biochem. reactions recommended in "Standard Methods for Examination of Water and Waste Water," 16th ed., 1985, American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1015 15th St NW, Washington, DC 20005.

### *D. Incidence of Hemolytic Staphylococci and Streptococci —Procedure*

Inoculate petri plates with 1 mL portions of suitable dilns of sample. Pour plates with veal infusion agar contg 5% defibrinated horse, sheep, or rabbit blood (0.5 mL blood/10 mL medium). Cool agar to 45° and add blood just before pouring plates. Incubate plates 24 hr at 35°. Confirm presence of coccus types of microorganisms by microscopic examination of smears taken from representative colonies and stained by Gram method. Express final results as number/g.

### *E. Tests for Fungi—Procedure*

Inoculate petri plates with 1 mL portions of suitable dilns of sample. Pour inoculated plates with malt agar, **940.36B(a)**, previously cooled to 42–45°. Incubate plates 5 days at 20° or at room temp., if 20° incubator is not available. Express final results as number of fungi/g egg material. Confirm yeast colonies by microscopic examination of smears stained by Gram method.

### *F. Direct Microscopic Counts*

*North aniline oil-methylene blue stain*.—Mix 3.0 mL aniline oil with 10.0 mL alcohol, and slowly add 1.5 mL HCl with const agitation. Add 30.0 mL satd alc. methylene blue soln, dil. to 100.0 mL with $H_2O$, and filter.

(**a**) *Liquid and frozen eggs*.—Place 0.01 mL undild egg material on clean, dry microscopic slide and spread over area of 2 sq cm (circular area with diam. of 1.6 cm suggested). Let film prepn dry on level surface at 35–40°. Immerse in xylene ≤1 min; then immerse in alcohol ≤1 min. Stain ≥45 sec in North aniline oil-methylene blue stain (10–20 min preferred; exposure up to 2 hr does not overstain). Wash slide by repeated immersions in $H_2O$ and dry thoroly before examination. Observe subsequent operations and precaution as in "Standard Methods for Examination of Dairy Products," 15th ed., 1985, American Public Health Association. Express final result as number of bacteria/g egg material (double microscopic factor, since 2 sq cm area is used).

(**b**) *Dried eggs*.—Place 0.01 mL of 1:10 or 1:100 diln of dried egg material on clean, dry microscopic slide and spread over 2 sq cm.

*Note:* 0.1*N* LiOH may be used as diluent and is preferred for samples that are relatively insol. Circular area with diam. of 1.6 cm is preferable. Addn of drop of $H_2O$ to each film facilitates uniform spreading.

Proceed as in (**a**). Double microscopic factor, since area of 2 sq cm is used, and multiply count by 10 or 100, depending on whether film was prepd from 1:10 or 1:100 diln.

Ref.: JAOAC **36,** 91, 316(1953).

# CHILLED, FROZEN, PRECOOKED, OR PREPARED FOODS, AND NUTMEATS

## 966.23 Microbiological Methods

**First Action 1966**
**Final Action 1989**

(For the detn of aerobic plate count, most probable number of coliform bacteria and *Escherichia coli,* and *Staphylococcus* in products such as frozen cooked meat, poultry, and vegetable products; cooked and/or breaded seafood; bakery products; salads; tree nut meats; and ingredients of food samples collected during sanitation inspections of food producing establishments, unless specific directions are given for that product.)

### *A. Media and Reagents*

Ingredients and reagents used to prep. following media may be product of any manufacturer if comparative tests show that satisfactory results are obtained. Use pure carbohydrates suitable for biological use; ACS reagent grade inorg. chemicals; and dyes certified by "Biological Stain Commission" for use in media.

For convenience, dehydrated media of any brand equiv. to formulation may be used. Test each lot of medium for sterility and growth-promoting qualities of suitable organisms (e.g., inoculate media contg lactose with coliform bacteria, *Staphylococcus* media with *Staphylococcus,* etc.).

Det. pH before autoclaving with pH meter stdzd against std buffers, **964.24**. Adjust pH, when necessary, by adding 1*N* NaOH or 1*N* HCl so that stated final pH results after autoclaving.

Use sterile glass or plastic, 100 × 15 mm, petri dishes.

(**a**) *Plate count agar.—See* **940.36A(g)**.

(**b**) *Lauryl sulfate tryptose broth.*—Dissolve 20.0 g trypticase or tryptose (pancreatic digest of casein), 5.0 g NaCl, 5.0 g lactose, 2.75 g $K_2HPO_4$, 2.75 g $KH_2PO_4$, and 0.1 g Na lauryl sulfate in 1 L $H_2O$ with gentle heat, if necessary. Dispense 10 mL portions into 20 × 150 mm test tubes contg inverted fermentation tubes 10 × 75 mm. Autoclave 15 min at 121°. Final pH, 6.8 ± 0.1.

(**c**) *Brilliant green lactose bile (BGLB) broth.*—Dissolve 10.0 g peptone and 10.0 g lactose in ca 500 mL $H_2O$. Add soln (pH 7.0–7.5) of 20 g dehydrated oxgall or oxbile in 200 mL $H_2O$. Dil. to 975 mL and adjust pH to 7.4. Add 13.3 mL 0.1% soln of brilliant green, and dil. to 1 L with $H_2O$. Filter thru cotton and dispense 10 mL portions into 20 × 150 mm test tubes contg inverted 10 × 75 mm fermentation tubes. Autoclave 15 min at 121°. Final pH, 7.2 ± 0.1.

(**d**) *Eosin methylene blue agar (Levine).—See* **940.36A(d)**.

(**e**) *Baird-Parker medium (egg tellurite glycine pyruvate agar, ETGPA).*—(*1*) *Basal medium.*—Suspend 10.0 g tryptone, 5.0 g beef ext, 1.0 g yeast ext, 10 g Na pyruvate, 12.0 g glycine, 5.0 g $LiCl.6H_2O$, and 20.0 g agar in 950 mL $H_2O$. Heat to bp with frequent agitation to dissolve ingredients completely. Dispense 95 mL portions into screw-capped bottles. Autoclave 15 min at 121°. Final pH, 7.0 ± 0.2 at 25°. Store ≤1 month at 4 ± 1°.

(*2*) *Enrichment.*—Bacto EY tellurite enrichment (Difco Laboratories) or prep. as follows: Soak fresh eggs ca 1 min in diln of satd $HgCl_2$ soln (1 + 1000). Aseptically crack eggs and sep. yolks from whites. Blend yolk and physiological saline soln, **940.36B(c)**, (3 + 7, v/v) in high-speed blender ca 5 sec. To 50 mL egg yolk emulsion add 10 mL filter sterilized 1% K tellurite soln. Mix and store at 4 ± 1°.

(*3*) *Complete medium.*—Add 5 mL warmed enrichment to 95 mL molten basal medium cooled to 45–50°. Mix well, avoiding bubbles, and pour 15–18 mL into sterile 100 × 15 mm petri dishes. Store plates at room temp. (≤25°) for ≤5 days before use. Medium should be densely opaque; do not use nonopaque plates. Dry plates before use by 1 of following methods: (*a*) in convection oven or incubator 30 min at 50° with lids removed and agar surface downward; (*b*) in forced-draft oven or incubator 2 hr at 50° with lids on and agar surface upward; (*c*) in incubator 4 hr at 35° with lids on and agar surface upward; or (*d*) on laboratory bench 16–18 hr at room temp. with lids on and agar surface upward.

(*4*) *Interpretation.*—Colonies of *S. aureus* are typically circular, smooth, convex, moist, 2–3 mm in diam. on uncrowded plates, gray-black to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone (ppt) and frequently with outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasional non-lipolytic strains may be encountered which have same appearance, except that surrounding opaque and clear zones are absent. Colonies isolated from frozen or desiccated foods which have been stored for extended periods are frequently less black than typical colonies and may have rough appearance and dry texture.

(**f**) *Trypticase (tryptic) soy broth with 10% sodium chloride.*—Add 95 g NaCl to 1 L of soln of 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g $K_2HPO_4$, and 2.5 g glucose. Heat gently if necessary. Dispense into 16–20 mm diam. tubes to depth of 5–8 cm. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2.

(**g**) *EC broth.*—Dissolve 20.0 g trypticase or tryptose (pancreatic digest of casein), 1.5 g Bacto bile salt No. 3 or bile salt mixt., 5.0 g lactose, 4.0 g $K_2HPO_4$, 1.5 g $KH_2PO_4$, and 5.0 g NaCl in 1 L $H_2O$. Dispense 8 mL into 16 × 150 mm test tubes contg inverted 10 × 75 mm fermentation tube. Autoclave 15 min at 121°. Final pH, 6.9 ± 0.1.

(**h**) *Brain-heart infusion.—See* **967.25A(r)**. Dispense into bottles or tubes for storage and autoclave 15 min at 121°.

(**i**) *Desiccated coagulase plasma (rabbit) with EDTA.*—Reconstitute according to manufacturer's directions. If not available, reconstitute *desiccated coagulase plasma (rabbit)* and add $Na_2H_2EDTA$ to final concn of 0.1% in reconstituted plasma.

(**j**) *Tryptophane broth.—See* **940.36A(h)** but dispense in 10 mL portions.

(**k**) *Buffered glucose broth (MR-VP medium).—See* **940.36A(b)**. BBL Microbiological Systems, No. 11383, or equiv.

(**l**) *Koser's citrate broth.—See* **940.36A(e)**.

(**m**) *Butterfield's buffered phosphate diluent.*—(*1*) *Stock soln.*—Dissolve 34.0 g $KH_2PO_4$ in 500 mL $H_2O$, adjust to pH 7.2 with ca 175 mL 1*N* NaOH, and dil. to 1 L. Store in refrigerator. (*2*) *Diluent.*—Dil. 1.25 mL stock soln to 1 L with $H_2O$. Prep. diln blanks with this soln, dispensing enough to allow for losses during autoclaving. Autoclave 15 min at 121°.

### *B. Preparation of Sample*

(Prep. all decimal dilns with 90 mL sterile diluent plus 10 mL previous diln unless otherwise specified. Shake all dilns 25 times in 30 cm arc. Pipets must accurately deliver required vol. Do not use to deliver <10% of their total vol. For example, to deliver 1 mL, do not use pipet >10 mL; to deliver 0.1 mL, do not use pipet >1 mL.)

(**a**) *Frozen and/or prepared foods.*—Use balance with capacity of ≥2 kg and sensitivity of 0.1 g to aseptically weigh 50 g unthawed (if frozen) sample into sterile high-speed blender jar. Add 450 mL diluent, (**m**)(*2*), and blend 2 min. (If necessary to temper frozen sample to remove 50 g portion, hold

≤18 hr at 2–5°.) Not >15 min should elapse from time sample is blended until all dilns are in appropriate media.

If entire sample consists of <50 g, weigh portion equiv. to ½ sample and add vol. of sterile diluent required to make 1:10 diln. Total vol. in blender jar must completely cover blades.

(**b**) *Tree nut meat halves and larger pieces*.—Aseptically weigh 50 g sample into sterile jar. Add 50 mL diluent, (**m**)(*2*), and shake vigorously (50 times thru 30 cm arc) to obtain $10^0$ diln. Let stand 3–5 min and shake just before making serial dilns and inoculations.

(**c**) *Nut meal*.—Aseptically weigh 10 g sample into sterile jar. Add 90 mL diluent, (**m**)(*2*), and shake vigorously (50 times thru 30 cm arc) to obtain $10^{-1}$ diln. Let stand 3–5 min and shake to resuspend just before making serial dilns and inoculations.

### *C. Aerobic Plate Count*

Seed duplicate petri dishes in dilns of 1:10, 1:100, 1:1000, etc., using plate count agar, (**a**). Ordinarily 1:100 thru 1:10,000 are satisfactory. Place 1 mL appropriate diln in each plate, and add molten agar (cooled to 42–45°) within 15 min from time of original diln. Incubate 48 ± 2 hr at 35° and count duplicate plates in suitable range (30–300 colonies). If plates do not contain 30–300 colonies, record diln counted and note number of colonies found. Average counts obtained and report as aerobic plate count/g.

## 966.24 Coliform Group and *Escherichia coli* in Tree Nut Meats

**Microbiological Method**

**Final Action 1971**

Seed 3-tube most probable number (MPN) series into lauryl sulfate tryptose broth, (**b**), using 1 mL inocula of 1:10, 1:100, and 1:1000 dilns, with triplicate tubes at each diln. (For nut meats (halves and larger pieces), begin MPN detn with $10^0$ diln; for nut meal, begin with $10^{-1}$ diln.) Incubate 48 ± 2 hr at 35° for gas formation as evidenced by displacement of liq. in insert tube or by vigorous effervescence when tubes are shaken *gently*. Examine tubes for gas formation at 24 and 48 hr intervals. Transfer, using 3 mm loop, from gassing tubes to BGLB, (**c**) (omit this transfer for tree nuts), and EC broth, (**g**), at time gas formation is noted.

Incubate BGLB broth 48 ± 2 hr at 35°. Using MPN Table **966.24**, compute MPN on basis of number of tubes of BGLB broth producing gas by end of incubation period. Report as MPN of coliform bacteria/g.

Incubate EC broth 48 ± 2 hr at 45.5 ± 0.05° in covered $H_2O$ bath. Submerge broth tubes in bath so that $H_2O$ level is above highest level of medium. Examine tubes for gas formation at 24 and 48 hr intervals. Streak gas-pos. tubes on Levine's eosin methylene blue agar plates, (**d**), and incubate plates 24 ± 2 hr at 35°.

Pick 2 or more well isolated typical colonies from Levine's eosin methylene blue agar plates and transfer to agar slants prepd from agar medium, (**a**). Incubate 18–24 hr at 35°. If typical colonies are not present, pick 2 or more colonies most likely to be *E. coli*. Pick ≥2 from every plate.

Transfer growth from plate count agar slants into following broths for identification by biochem. tests:

(**a**) *Tryptophane broth*.—Incubate broth, (**j**), 24 ± 2 hr at 35° and test for indole by adding 0.2–0.3 mL Kovacs reagent, **967.25B(a)**, to 24 hr culture. Test is pos. if upper layer turns red.

(**b**) *MR-VP medium*.—Incubate medium, (**k**), 48 ± 2 hr at 35°. Aseptically transfer 1 mL culture to 13 × 100 mm test tube to test for acetylmethylcarbinol. Add 0.6 mL 5% alc. α-naphthol soln, 0.2 mL KOH soln (4 + 10), and few crystals of creatine. Shake and let stand 2 hr. Test is pos. if eosin pink develops. Alternatively, *see* **967.27D(c)**(*1*).

Incubate remainder of MR-VP medium for addnl 48 hr and test for Me red reaction by adding 5 drops Me red soln to culture. Test is pos. if culture turns red; neg., if yellow. (Prep. Me red soln by dissolving 0.1 g Me red in 300 mL 90% alcohol and dilg to 500 mL with $H_2O$.)

(**c**) *Koser citrate broth*, (*l*).—Incubate 96 hr at 35° and record growth as + or −.

(**d**) *Lauryl sulfate tryptose broth*, (*b*).—Incubate 48 ± 2 hr at 35°. Examine tubes for gas formation.

(**e**) *Gram stain*.—Perform Gram stain on 18 hr agar slant ("Standard Methods for the Examination of Water and Waste Water," 16th ed., 1985). Coliform organisms will stain red (neg.); Gram-pos. organisms will stain blue-black.

(**f**) *Classification*.—Classify biochem. types as follows:

| *Indole* | *MR* | *VP* | *Citrate* | *Type* |
|---|---|---|---|---|
| + | + | − | − | Typical *E. coli* |
| − | + | − | − | Atypical *E. coli* |
| + | + | − | + | Typical Intermediate |
| − | + | − | + | Atypical Intermediate |
| − | − | + | + | Typical *Enterobacter aerogenes* |
| + | − | + | + | Atypical *Enterobacter aerogenes* |

**Table 966.24 Most Probable Numbers (MPN) per 1 g of Sample, Using 3 Tubes with Each of 0.1, 0.01, and 0.001 g Portions**

| Positive Tubes | | | | Positive Tubes | | | | Positive Tubes | | | | Positive Tubes | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 0.1 | 0.01 | 0.001 | MPN | 0.1 | 0.01 | 0.001 | MPN | 0.1 | 0.01 | 0.001 | MPN | 0.1 | 0.01 | 0.001 | MPN |
| 0 | 0 | 0 | <3 | 1 | 0 | 0 | 3.6 | 2 | 0 | 0 | 9.1 | 3 | 0 | 0 | 23 |
| 0 | 0 | 1 | 3 | 1 | 0 | 1 | 7.2 | 2 | 0 | 1 | 14 | 3 | 0 | 1 | 39 |
| 0 | 0 | 2 | 6 | 1 | 0 | 2 | 11 | 2 | 0 | 2 | 20 | 3 | 0 | 2 | 64 |
| 0 | 0 | 3 | 9 | 1 | 0 | 3 | 15 | 2 | 0 | 3 | 26 | 3 | 0 | 3 | 95 |
| 0 | 1 | 0 | 3 | 1 | 1 | 0 | 7.3 | 2 | 1 | 0 | 15 | 3 | 1 | 0 | 43 |
| 0 | 1 | 1 | 6.1 | 1 | 1 | 1 | 11 | 2 | 1 | 1 | 20 | 3 | 1 | 1 | 75 |
| 0 | 1 | 2 | 9.2 | 1 | 1 | 2 | 15 | 2 | 1 | 2 | 27 | 3 | 1 | 2 | 120 |
| 0 | 1 | 3 | 12. | 1 | 1 | 3 | 19 | 2 | 1 | 3 | 34 | 3 | 1 | 3 | 160 |
| 0 | 2 | 0 | 6.2 | 1 | 2 | 0 | 11 | 2 | 2 | 0 | 21 | 3 | 2 | 0 | 93 |
| 0 | 2 | 1 | 9.3 | 1 | 2 | 1 | 15 | 2 | 2 | 1 | 28 | 3 | 2 | 1 | 150 |
| 0 | 2 | 2 | 12 | 1 | 2 | 2 | 20 | 2 | 2 | 2 | 35 | 3 | 2 | 2 | 210 |
| 0 | 2 | 3 | 16 | 1 | 2 | 3 | 24 | 2 | 2 | 3 | 42 | 3 | 2 | 3 | 290 |
| 0 | 3 | 0 | 9.4 | 1 | 3 | 0 | 16 | 2 | 3 | 0 | 29 | 3 | 3 | 0 | 240 |
| 0 | 3 | 1 | 13 | 1 | 3 | 1 | 20 | 2 | 3 | 1 | 36 | 3 | 3 | 1 | 460 |
| 0 | 3 | 2 | 16 | 1 | 3 | 2 | 24 | 2 | 3 | 2 | 44 | 3 | 3 | 2 | 1100 |
| 0 | 3 | 3 | 19 | 1 | 3 | 3 | 29 | 2 | 3 | 3 | 53 | 3 | 3 | 3 | >1100 |

Other groupings may appear; in such cases cultures are usually mixed. Restreak to det. their purity.

Compute MPN of *E. coli*/g, considering Gram neg., nonspore-forming rods producing gas in lactose and producing ++−− or −+−− IMViC patterns as *E. coli*.

Refs.: JAOAC **49,** 270, 276(1966); **51,** 865, 867(1968); **58,** 1154(1975).

## 977.27 Bacteria in Foods and Cosmetics

### Spiral Plate Method

**First Action 1977**
**Final Action 1981**

#### A. Principle

Bacterial suspension from prepd sample of food or cosmetic is deposited continuously on surface of rotating agar plate. Resultant track on surface is in form of Archimedes spiral. Vol. is decreased while dispensing stylus moves from center to edge so that exponential relationship exists between vol. deposited and radius of agar. On incubation, colonies develop along lines where liq. was deposited. Counting grid is calibrated for sample vol. associated with different areas of agar. No. colonies per known area is counted and calcd to bacterial concn.

#### B. Apparatus

*Spiral plating machine.*—For use with 150 × 15 mm (100 × 15 mm may be used) petri dishes and adjusted to deliver total vol. of 0.035 mL/plate. Platform carrying plate is rotated at ca 50 rpm and is connected mech. to lead screw driving hollow syringe dispenser. Backflow syringe, 2-way valve, and vac. trap control loading and dispensing of sample, disposal of residual sample, and rinsing of system. Liq. is dispensed from backflow syringe thru thin wall Teflon tubing thru stylus to surface of agar plate. (Available com. from Spiral Systems Marketing, 4853 Cordell Ave, Suite A10, Bethesda, MD 20014.)

#### C. Plates

Pour 40–45 mL portions plate count agar, **940.36A(g)**, into 150 × 15 mm (100 × 15 mm may be used) petri dishes; let harden and dry to smooth, even surface.

#### D. Calibration of Spiral Counters

To det. vol. associated with different parts of counting grid, prep. 11 bacterial suspensions by dilg 1:1 from $10^6$ to $10^3$ cells/mL (use nonspreaders). Plate all dilns in duplicate by both **966.23D** and spiral plater, using same medium and incubator. Count spiral plates as in **977.27G** and divide by av. count/mL by **966.23C** to calc. vol. of counted grid area.

$$\text{mL in counted area} = \frac{\text{No. spiral colonies on area}}{\text{count/mL by } \mathbf{966.23C}}$$

#### E. Preparation of Samples

Weigh 50 g sample into sterile blender jar, add 450 mL diln $H_2O$, **940.36A(a)**, and blend 2 min. If necessary, let settle few min before removing portion of supernate for spiral plating. (Presence of particles may clog tubing.)

Liqs may be used directly or after dilg 1 + 9 with diln $H_2O$.

#### F. Operation

Check stylus tip angle by letting vac. hold microscope cover slip against face of stylus tip at 1 mm above platform. Cover slip should be parallel to rotating platform in all directions. Adjust angle if necessary. Check stylus at start position.

Clean stylus tip before use and between plating each sample by rinsing 1 sec with com. 5.25% NaOCl soln and then 1 sec with sterile $H_2O$. Identify 3 disposable polyethylene sample cups and fill with com. 5.25% NaOCl soln, sterile $H_2O$, and sample. Turn vac. filling valve to "on" and move sample holder into position under stylus tip. Lower stylus into NaOCl soln and lift out twice. Repeat with $H_2O$. Lower stylus into sample soln. Draw soln thru stylus until continuous column of liq. is present in tube above vac. filling valve. With tip of stylus still below surface of sample, close vac. valve. Raise stylus and move sample holder out of way.

Identify lid of agar plate and remove lid. Place dish on turntable, and lower stylus until tip rests freely on agar surface. Start app. and let rotate until stylus is lifted and app. stops automatically. Remove dish and replace cover. Incubate 48 ± 3 hr at 35 ± 1°.

After all samples have been plated, flush app. with NaOCl soln and $H_2O$. When not in use, leave filled with $H_2O$.

#### G. Counting Spiral Plates

Transparent viewing grid consists of 13.2 cm circle divided into 5 areas by 4 concentric circles equidistant along diam. (marked 1 (furthest) and 4 (nearest) to center) and into eight 45° wedges or octants, marked A thru H. Thus, each octant is subdivided by 4 arcs linearly equidistant from each other. Outer ring of 2 opposite octants (e.g., A and E) is further subdivided in half by arc in middle (marked ½), and outer ring thus formed is divided in half by line toward center. Addnl lines are provided for use with 10 cm plates.

After incubation, center plate over grid. Choose any octant sector and count colonies from outer edge toward center until 20 colonies have been counted. Continue counting remaining colonies contained in segment in which 20th colony was observed. Record this count together with No. segment that included 20th colony (i.e., ½, 1, 2, 3, or 4). Count opposite similar segment and add together. If 20 colonies are not contained in an octant, count all colonies on plate and designate as T (total). If total No. colonies counted exceeds 75 in completing count in segment contg 20th colony, count will generally be low because of coincidence error associated with crowding of colony. In this case, count circumferentially adjacent annular segments starting with sector 1 until ≥50 colonies are counted, and complete count of remaining colonies in segment in which 50th colony was observed.

Divide No. colonies counted (or sum of 2 sector counts) by corresponding vol. sectors counted in mL to obtain bacterial count/mL. Use as vol. that calcd for that sector(s) from calibration, **977.27D**, based on std plate count.

Refs.: JAOAC **60,** 807(1977); **64,** 408(1981).

## 986.32 Aerobic Plate Count in Foods

### Hydrophobic Grid Membrane Filter Method

**First Action 1986**
**Final Action 1987**

#### A. Principle

Hydrophobic grid membrane filter (HGMF) uses membrane filter imprinted with hydrophobic material in grid pattern. Hydrophobic lines act as barriers to spread of colonies, thereby dividing membrane filter surface into sep. compartments of equal and known size. Number of squares occupied by colonies is enumerated and converted to most probable number value of organisms by using formula given below.

#### B. Apparatus, Culture Media, and Reagents

(**a**) *Hydrophobic grid membrane filter (HGMF).*—Membrane filter has pore size of 0.45 μm and is imprinted with nontoxic hydrophobic material in grid pattern. ISO-GRID (available from QA Laboratories, Ltd, 135 The West Mall, Toronto, Ontario, Canada M9C 1C2) or equiv. meets these specifications.

(**b**) *Filtration units for HGMF*.—Equipped with 5 μm mesh prefilter to remove food particles during filtration. One unit is required for each sample. ISO-GRID (available from QA Laboratories, Ltd) or equiv. meets these specifications.

(**c**) *Pipets*.—1.0 mL serological with 0.1 mL graduations, 1.1 or 2.2 mL milk pipets are satisfactory. 5.0 mL serological with 0.1 mL graduations.

(**d**) *Blender*.—Waring Blendor, or equiv. multispeed model, with low-speed operation at 10 000–12 000 rpm, and 250 mL glass or metal blender jars with covers. One jar is required for each sample.

(**e**) *Vacuum pump*.—$H_2O$ aspirator vac. source is satisfactory.

(**f**) *Manifold or vacuum flask*.

(**g**) *Peptone/Tween 80 (PT) diluent*.—Dissolve 1.0 g peptone (Difco 0118) and 10.0 g Tween 80 in 1 L $H_2O$. Dispense enough vol. into diln bottles to give 90 ± 1 mL or 99 ± 1 mL after autoclaving 15 min at 121°.

(**h**) *Tryptic soy-fast green agar (TSFA)*.—15.0 g tryptone, 5.0 g phytone (or soytone), 5.0 g NaCl, 0.25 g fast green FCF (CI No. 42053), and 15.0 g agar dild to 1 L with $H_2O$. Heat to boiling. Autoclave 15 min at 121°. Temper to 50–55°. Aseptically adjust pH to 7.3 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(**i**) *Tris buffer*.—1.0M. Dissolve 121.1 g tris(hydroxymethyl)amino methane in ca 500 mL $H_2O$. Adjust soln to desired pH with concd HCl and dil. to 1 L with $H_2O$. Store at either room temp. or 4–6°.

(**j**) *Acetate buffer*.—1.0M. Dissolve 60 mL glacial acetic acid in ca 500 mL $H_2O$. Adjust soln to desired pH with 5M NaOH and dil. to 1 L with $H_2O$. Store at 4–6°.

(**k**) *Amylase stock soln*.—Dil. 10 g α-amylase (Sigma Chemical Co., No. A1278 or equiv.) to 100 mL with tris buffer, pH 7.0. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**l**) *Cellulase stock soln*.—Dil. 10 g cellulase (Sigma No. C0901 or equiv.) to 100 mL with acetate buffer, pH 5.0. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**m**) *Diastase stock soln*.—Dil. 10 g diastase (Sigma No. A6880 or equiv.) to 100 mL with tris buffer, pH 7.0. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**n**) *Hemicellulase stock soln*.—Dil. 10 g hemicellulase (Sigma No. H2125 or equiv.) to 100 mL with acetate buffer, pH 5.5. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**o**) *Trypsin stock soln*.—Dil. 10 g trypsin (Difco No. 0153 or equiv.) to 100 mL with tris buffer, pH 7.6. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material; then filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**p**) *Lecithinase (phospholipase $A_2$) stock soln*.—Dil. com. enzyme soln (Sigma No. P9139 or equiv.) to 25 units/mL with tris buffer, pH 8.0. Filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**q**) *Pectinase stock soln*.—Use com. enzyme soln of pectinase from *Aspergillus niger*, contg 3–6 units/mg protein, dissolved in 40% glycerol (Sigma No. P5146 or equiv.). Filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

(**r**) *Protease stock soln*.—Use com. enzyme soln of protease from *Bacillus subtilis*, containing 7–15 units/mg protein (Biuret) in aq. soln (Sigma No. P8775 or equiv.) Filter-sterilize using 0.45 μm membrane filter. Store up to 1 week at 4–6° or up to 3 months at −18°.

### *C. Sample Preparation*

(**a**) *Liquid egg*.—Thoroly mix sample with sterile spoon or spatula and prep. 1:10 diln by aseptically weighing 11 g egg material into sterile wide-mouth g-s or screw-cap bottle; add 99 mL PT diluent, (**g**), and 1 tablespoon of sterile glass shot. Thoroly agitate 1:10 diln to ensure complete soln or distribution of egg material in diluent by shaking each container rapidly 25 times, each shake being up-and-down movement of ca 30 cm, time interval not exceeding 7 s. Let bubbles escape. Transfer representative portion from 1:10 diln for higher serial dilns as needed. If enzyme treatment is needed (see Table **986.32**), combine 5 mL of 1:10 diln with 1 mL enzyme stock soln. Incubate 20–30 min at 35–37° in $H_2O$ bath. Correct for addnl diln factor by filtering 1.2 mL of enzyme-treated sample.

(**b**) *Other liquid samples*.—Mix contents of sample container thoroly. To prep. 1:10 diln, aseptically transfer 10 mL sample into 90 mL PT diluent, (**g**). Mix by shaking bottle 25 times thru 30 cm arc in 7 s. Transfer representative portions

**Table 986.32 Enzyme Treatments for Foods**[a]

| Food | Enzyme |
|---|---|
| Skim milk | none |
| Raw milk | none |
| Fluid dairy products other than skim milk | trypsin |
| Ice cream: without stabilizers | trypsin |
| contg gums | hemicellulase |
| contg cellulose derivatives | cellulase |
| Spray-dried milks | trypsin |
| Cheeses | trypsin |
| Spray-dried cheese powders | cellulase or protease[b] |
| Sour cream | diastase |
| Yoghurt | trypsin |
| Butter | none |
| Margarine | none |
| Egg: liq. or powder | trypsin |
| Raw beef, pork, poultry | trypsin |
| Cooked meat or poultry | trypsin |
| Flour | none |
| Rice | none |
| Chocolate | amylase |
| Breakfast cereals | cellulase |
| Cake mixes | amylase |
| Fruit puree (e.g., fig paste) | pectinase |
| Raw vegetables | none |
| Lecithin | lecithinase |
| Food colorings | none |
| Gums | hemicellulase |
| Citrus juices | pectinase |
| Infant formula | trypsin |
| Sodium caseinate | protease |
| Nut meats | none |
| Shrimp | none |
| Oysters | trypsin |

[a] Based on analysis of 1 mL of 1:10 diln. Foods tested at dilns of 1:100 or higher do not usually need enzyme treatment.
[b] Varies, depending on individual product.

from 1:10 diln for higher serial dilns as needed. If enzyme treatment is needed (see Table **986.32**), combine 5 mL of 1:10 diln with 1 mL enzyme stock soln. Incubate 20–30 min at 35–37° in $H_2O$ bath. Correct for addnl diln factor by filtering 1.2 mL of enzyme-treated sample.

(**c**) *Whole egg powder*.—Thoroly mix sample with sterile spoon or spatula and prep. 1:10 diln by aseptically weighing 11 g egg material into sterile wide-mouth g-s or screw-cap bottle; add 99 mL PT diluent, (**g**), and 1 tablespoon of sterile glass shot. Thoroly agitate 1:10 diln to ensure complete soln or distribution of egg material in diluent by shaking each container rapidly 25 times, each shake being up-and-down movement of ca 30 cm, time interval not exceeding 7 s. Let bubbles escape. Transfer representative portion from 1:10 diln for higher serial dilns as needed. If testing 1:10 diln is necessary, prep. 1:100 diln and combine 10 mL of 1:100 diln with 1 mL trypsin stock soln, (**o**). Incubate 20–30 min at 35–37° in $H_2O$ bath. Filter entire 11 mL vol. to test 1:10 diln.

(**d**) *Other foods*.—To prep. 1:10 diln, aseptically weigh 10 g sample into sterile blender jar. Add 90 mL PT diluent, (**g**), and blend 2 min at low speed (10 000–12 000 rpm). Transfer representative portion from 1:10 diln for higher serial dilns as needed. If enzyme treatment is needed (see Table **986.32**), combine 5 mL of 1:10 diln with 1 mL enzyme stock soln. Incubate 20–30 min at 35–37° in $H_2O$ bath. Correct for addnl diln factor by filtering 1.2 mL of enzyme-treated sample.

### *D. Analysis*

Select appropriate diln for analysis, depending on desired counting range. Ordinarily, 1:100 diln is satisfactory, producing counting range of 100/g or mL to 500 000/g or mL. Use 1:10 diln if very low counts are expected.

(See Figs **986.32A** and **986.32B**). Turn on vac. source. Place sterile filtration unit on manifold or vac. flask. Open clamp A. Rotate back funnel portion C. Aseptically place sterile HGMF on surface of base D. Rotate funnel forward. Clamp shut by sliding jaws L of stainless steel clamp over entire length of flanges B extending from both sides of funnel C and base D, and rotating moving arm K into horizontal (locked) position.

Aseptically add ca 15–20 mL sterile $H_2O$ to funnel. Pipet required vol. of appropriate diln into funnel. Apply free end of vac. tubing E to suction hole F to draw liq. thru prefilter mesh G. Aseptically add addnl 10–15 mL sterile $H_2O$ to funnel and draw thru mesh as before. Close clamp A to direct vac. to base of filtration unit and draw liq. thru HGMF.

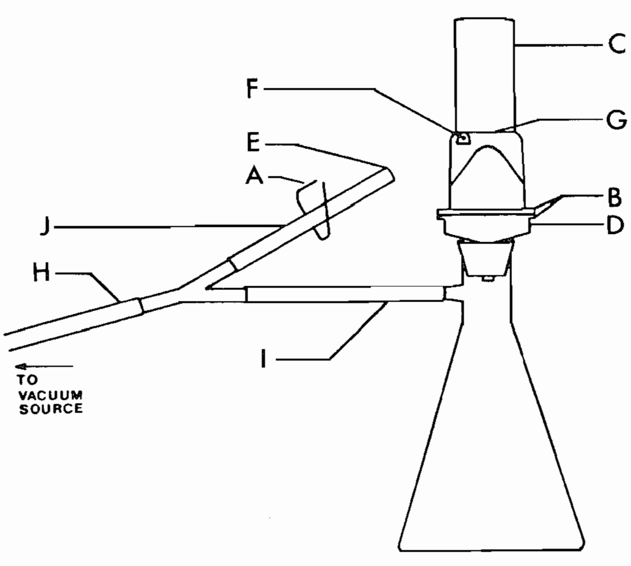



**FIG. 986.32A—Filtration unit**

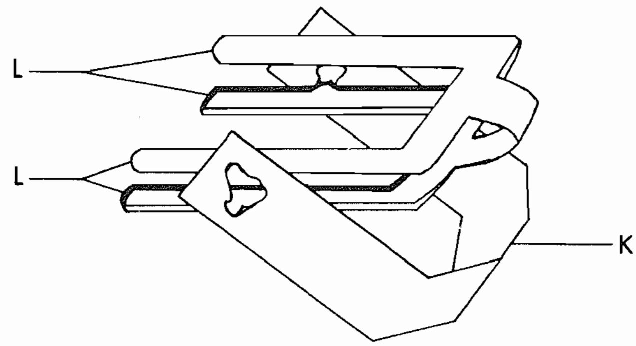



**FIG. 986.32B—Filtration unit clamp**

Open clamp A. Rotate moving arm K of stainless steel clamp into unlocked (ca 45° angle) position and slide jaws L off of flanges B. Rotate back funnel C. Aseptically remove HGMF and place on surface of pre-dried TSFA (**h**) plate. Avoid trapping air bubbles between filter and agar.

(**a**) *Raw milk, pasteurized milks and creams, and egg powders*.—Incubate 48 ± 3 h at 32°. Colonies will be various shades of green. Count all squares contg one or more colonies (pos. squares) except that if a single colony has clearly spread to adjacent squares, count it as one pos. square. Convert pos. square count to MPN with the formula, MPN = $[N \log_e (N/(N - x))]$, where $N$ = total number of squares and $x$ = number of pos. squares. Multiply by reciprocal of diln factor and report as MPN of total bacteria/g or mL.

(**b**) *Liquid egg*.—Incubate 3 days (72 ± 3 h) at 32°. Proceed as in (**a**).

(**c**) *All other foods*.—Incubate 48 ± 3 h at 35°. Proceed as in (**a**).

Ref.: JAOAC **69**, 671(1986).

## 988.18 Aerobic Plate Count
### Pectin Gel Method
### First Action 1988

### *A. Principle*

Method uses pretreated petri plates contg thin "hardener" layer, and liq. medium contg nutrients with pectin as only gelling agent. Liq. medium, 12–15 mL, is poured into pretreated petri plate and undild or dild sample is added. Plate is rotated and rocked to mix sample and medium. Plates are then allowed to stand on level surface 30–40 min until medium solidifies. Total process is done at ambient temp. Plates are then incubated and counted.

### *B. Materials*

*Note:* Before pectin base medium formulated from individual ingredients is used, comparability to commercially available medium must be demonstrated.

*Pectin gel tubes and plates*.—Pectin gel is available as sterile liq. in individual tubes contg sufficient gel to pour 1 plate. Use tubes of Redigel and pretreated petri plates (RCR Scientific, Inc., 206 W Lincoln Ave, Goshen, IN 46526), or equiv. that meets specifications.

To prep. plate count pectin gel from individual ingredients, suspend 5.0 g pancreatic digest of casein, 2.5 g yeast ext, and 1.0 g glucose, in 500 mL $H_2O$. Suspend 15 g low methoxyl pectin in 500 mL $H_2O$. Heat individual mixts until all ingredients are dissolved. Autoclave solns 15 min at 121°. Combine nutrient and pectin solns and adjust pH to 7.0 ± 0.1. To prep. pretreated petri plates, prep. hardener layer mixt. of 1% agar with 0.02 $CaCl_2$ concn. Sterilize mixt. by autoclaving 15 min at 121°. Aseptically dispense 5 mL portions of mixt. into sterile petri plates.

### C. Preparation of Samples

Prep. all decimal dilns with 90 mL sterile diluent (Butterfield's phosphate buffer) plus 10 mL of previous diln unless otherwise specified. Shake all dilns 25 times in 30 cm arc. Pipets must accurately deliver required vol. Do not use to deliver <10% of their total vol. For example, to deliver 1 mL, do not use pipet >10 mL; to deliver 0.1 mL, do not use pipet >1 mL.

(**a**) *Dairy products.*—Measure (or weigh) 11 mL (or g) sample and dil. in 99 mL Butterfield's diluent. For solid samples, blend 2 min at 10 000 to 12 000 rpm. Prep. addnl dilns so that total colonies/plate is in 25–250 range. Incubate plates 48 ± 3 h at 32 ± 1°.

(**b**) *Nondairy products.*—Weigh 50 g sample into 450 mL Butterfield's diluent and blend 2 min at 10 000 to 12 000 rpm. Prep. further dilns by dispensing 10 mL sample into 90 mL diluent so that total colonies/plate is in 30–300 range. Incubate plates 48 ± 2 h at 35 ± 1°.

### D. Determination

(*1*) Lift lid of pretreated petri plate and pour liq. pectin gel from 1 tube (12–15 mL) into plate. Replace lid and swirl plate to cover bottom with pectin gel. Prep. number of plates needed for samples being run (duplicate plates for each diln). Plates *must* be used within 5 min after liq. pectin gel is poured.

(*2*) Add 1 mL inoculum (sample) to liq. pectin gel in petri plate. Touch pipet tip once to dry spot on inside wall of plate (above level of liq. pectin gel) after dispensing sample to rest point in pipet tip. *Immediately* rotate and rock plate to mix sample thoroly with pectin gel. Do not spill pectin gel over sides of plate. (*Note:* This step is primary difference in procedure between pectin gel and agar-based media. *Do not* add inoculum (sample) to pretreated petri plate and pour pectin gel over it. This would lock sample in one small area of plate without sepn of individual colonies.)

(*3*) Let inoculated plates stand on level surface until pectin gel is solid (ca 30–40 min), and then incubate 48 ± 2 h at 35 ± 1° for nondairy products and 48 ± 3 h at 32 ± 1° for dairy products.

(*4*) Count duplicate plates in suitable range (30–300 colonies for nondairy products, 25–250 colonies for dairy products). If plates do not contain proper range of colonies, record diln counted and note number of colonies found. Average counts obtained and report as aerobic plate count/g or mL.

Ref.: JAOAC **71,** 343(1988).

## COLIFORMS

## 989.11 Coliforms in Dairy Products

**Pectin Gel Method**

**First Action 1989**

### A. Principle

Method uses pretreated plates contg thin "hardener" layer and liq. medium contg nutrients with pectin as sole gelling agent. Liq. medium (10–12 mL) is poured into pretreated plate and undild or dild sample is added. Plate is rotated and rocked to mix sample and medium. Plates are then allowed to rest on level surface until medium solidifies. Then, 3–4 mL liq. medium is poured as overlay and allowed to solidify. Total process is done at ambient temp. Plates are then incubated and counted as for agar-based prepns.

### B. Materials

*Note:* Pectin base medium may be formulated from individual ingredients; suitability for analysis must be demonstrated.

*Pectin gel and plates.*—Violet red bile (VRB) pectin gel is available as sterile liq. in individual units contg sufficient gel to pour 1 plate or in units to pour 8 plates. VRB Redigel and pretreated plates (RCR Scientific, Inc., 206 W Lincoln Ave, Goshen, IN 46526), or equiv., meet specifications of method.

To prep. VRB pectin gel from individual ingredients, suspend 7.0 g pancreatic digest of gelatin, 3.0 g yeast ext, 10.0 g lactose, 1.5 g bile salts No. 3, 5.0 g NaCl, 0.03 g neutral red, and 0.002 g crystal violet in 500 mL $H_2O$. Suspend 15 g low methoxyl pectin in 500 mL $H_2O$. Heat individual mixts until all ingredients are dissolved. Autoclave solns 15 min at 121°. Combine nutrient and pectin solns and adjust pH to 7.4 ± 0.2. To prep. pretreated petri plates, prep. hardener layer mixt. of 1% agar with 0.02 $CaCl_2$ concn. Sterilize mixt. by autoclaving 15 min at 121°. Aseptically dispense 5 mL portions of mixt. into sterile petri plates.

### C. Preparation of Samples

To prep. dilns, measure (or weigh) 11 mL (or g) sample and dil. in 99 mL Butterfield's or 2% Na citrate diluent. For solid samples, blend 2 min at 10 000 to 12 000 rpm. Prep. addnl dilns so that total colonies/plate is in 25–250 range. Incubate plates 48 ± 3 h at 32 ± 1°. Shake all dilns 25 times in 30 cm arc. Pipets must accurately deliver required vol. Do not use to deliver <10% of their total vol. For example, to deliver 1 mL, do not use pipet > 10 mL; to deliver 0.1 mL, do not use pipet >1 mL.

### D. Determination

(*1*) Lift lid of pretreated petri plate and pour ca 75% (10-12 mL) of liq. medium from tube into plate. (*Note:* Remove cap from each tube of liq. pectin gel medium as it is needed to pour plate.) Prep. number of plates, in duplicate, needed for samples being run. Replace lid and swirl plate to cover bottom with liq. medium. Plates must be used within 5 min.

(*2*) Add inoculum (sample) to liq. pectin gel in petri plate. Touch pipet tip once to dry spot on inside wall of plate (above level of liq. medium) after dispensing sample to rest point in pipet tip. *Immediately* rotate and rock plate to mix sample thoroly with pectin gel. Do not spill mixt. over sides of plate. (*Note:* This step is primary difference in procedure between pectin gel and agar-based media. *Do not* add inoculum (sample) to pretreated petri plate and pour pectin gel over it. This would lock sample in one small area of plate without sepn of individual colonies.)

(*3*) Let inoculated plates stand on level surface until pectin gel is solid, and then pour remaining medium (3–4 mL) from tube as overlay and let gel solidify. Incubate in same manner as for agar-based plates (24 ± 2 h at 32 ± 1°).

(*4*) After 24 h incubation, count all red or pink colonies. Report as coliforms/mL or g.

(*5*) Pick 5 colonies of each type present on each plate and transfer to brilliant green lactose bile broth fermentation tubes, **966.23A(c)**. Incubate 48 ± 3 h at 32 ± 1° and check for gas production, which is considered pos. for coliforms.

(*6*) If any picks from step 5 are neg. for gas production, adjust counts (step *4*) accordingly.

Ref.: JAOAC **72,** 298(1989).

## 986.33 Bacterial and Coliform Counts in Milk

### Dry Rehydratable Film Methods

**First Action 1986**
**Final Action 1988**

***A. Principle***

Method uses bacterial culture plates of dry medium and cold $H_2O$-sol. gel. Undild or dild samples are added directly to plates at rate of 1.0 mL per plate. Pressure, when applied to plastic spreader placed on overlay film, spreads sample over ca 20 sq. cm growth area. Gelling agent is allowed to solidify and plates are incubated and then counted. Either pipet or plate loop continuous pipetting syringe can be used for sample addn for bacterial count analyses.

***B. Apparatus***

(**a**) *Std method plates*.—Plates contain std methods media nutrients, **940.36A(g)**, cold $H_2O$-sol. gelling agent coated onto film base, overlay film coated with gelling agent, and 2,3,5-triphenyltetrazolium chloride indicator. Circular growth area of single plate contains ca twenty 1 cm squares outlined on film base. Petrifilm Aerobic Count Plates® (available from Medical-Surgical Division/3M, 225-5S 3M Center, St. Paul, MN 55144) or equiv. meets these specifications.

(**b**) *Violet red bile plates*.—Plates contain violet red bile nutrients conforming to APHA standards as given in Compendium of Methods for the Microbiological Examination of Foods, 2nd ed., 1984 (American Public Health Association, 1015 15th St, NW, Washington, DC 20005), cold $H_2O$ sol. gelling agent, and 2,3,5-triphenyltetrazolium chloride. Petrifilm VRB Plates® (available from Medical-Surgical Division/3M), or equiv. meets these specifications.

(**c**) *Plastic spreader*.—Provided with Petrifilm plates, consists of concave side and smooth flat side, designed to spread milk sample evenly over plate growth area.

(**d**) *Pipets*.—Calibrated for bacteriological use of plate loop continuous pipetting syringe to deliver 1.0 mL.

(**e**) *Colony counter*.—Std app., Quebec model preferred, or one providing equiv. magnification and visibility.

***C. Analysis***

(**a**) *Bacterial colony count*.—Use Petrifilm Aerobic Count Plates or equiv. plates. Place plate on flat surface. Lift top film and inoculate 1 mL sample onto center of film base. Carefully roll top film down onto inoculum. Distribute sample over prescribed growth area with downward pressure on center of plastic spreader device (recessed side down). Leave plate undisturbed 1 min to permit gel to solidify. Incubate plates 48 ± 3 h at 32° ± 1°.

In incubator, place plates in horizontal position, clear side up, in stacks not exceeding 10 units. Count plates promptly after incubation period. If impossible to count at once, store plates after required incubation at 0–4.4° for not >24 h. This should be avoided as a routine practice.

Use std colony counter for counting purposes. Magnifier-illuminator may also be used to facilitate counting. Colonies stain in various shades of red. Count all colonies in countable range (30–300 colonies).

To compute bacterial count, multiply total number of colonies per plate (or av. number of colonies per plate if counting duplicate plates of same diln) by reciprocal of diln used. When counting colonies on duplicate plates of consecutive dilns, compute mean number of colonies for each diln before detg av. bacterial count. Estd counts can be made on plates with >300 colonies and should be reported as estd counts. In making such counts, circular growth area can be considered to contain ca twenty 1 cm squares. To isolate colonies for further identification, lift top film and pick colony from gel.

(**b**) *Coliform count*.—Use Petrifilm Coliform Count Plates or equiv. plates. Proceed as in (**a**), but distribute sample over plate by using plastic spreader, flat side down. Incubate plates 24 ± 2 h at 32° ± 1°. Count as in (**a**), but count only red colonies that have one or more gas bubbles associated (within 1 colony diam.) with them. Count all colonies in countable range (15–150 colonies). Red colonies without gas bubbles are not counted as coliform organisms.

Ref.: JAOAC **69**, 527(1986).

## 989.10 Bacterial and Coliform Counts in Dairy Products

### Dry Rehydratable Film Methods

**First Action 1989**

Method Performance:
AEROBIC COUNT
Chocolate milk:
$s_r$ = 0.102; $s_R$ = 0.177; $RSD_r$ = 4.3%; $RSD_R$ = 7.5%
Cheese:
$s_r$ = 0.113; $s_R$ = 0.117; $RSD_r$ = 3.6%; $RSD_R$ = 3.7%
Nonfat dry milk:
$s_r$ = 0.151; $s_R$ = 0.230; $RSD_r$ = 4.5%; $RSD_R$ = 6.9%
Evaporated milk:
$s_r$ = 0.193; $s_R$ = 0.198; $RSD_r$ = 8.3%; $RSD_R$ = 8.5%
Ice cream:
$s_r$ = 0.180; $s_R$ = 0.222; $RSD_r$ = 6.9%; $RSD_R$ = 8.5%

COLIFORM COUNT
Chocolate milk:
$s_r$ = 0.164; $s_R$ = 0.257; $RSD_r$ = 9.2%; $RSD_R$ = 14.4%
Cheese:
$s_r$ = 0.221; $s_R$ = 0.225; $RSD_r$ = 10.4%; $RSD_R$ = 10.6%
Nonfat dry milk:
$s_r$ = 0.197; $s_R$ = 0.151; $RSD_r$ = 8.5%; $RSD_R$ = 4.5%
Evaporated milk:
$s_r$ = 0.200; $s_R$ = 0.225; $RSD_r$ = 13.0%; $RSD_R$ = 13.0%
Ice cream:
$s_r$ = 0.081; $s_R$ = 0.131; $RSD_r$ = 4.1%; $RSD_R$ = 6.6%

***A. Principle***

Method uses bacterial culture plates of dry medium and cold $H_2O$-sol. gel. Undild or dild samples are added to plates at rate of 1.0 mL per plate. Pressure, when applied to plastic spreader placed on overlay film, spreads sample over ca 20 sq. cm growth area. Gelling agent is allowed to solidify and plates are incubated and then counted. Pipet, plate loop continuous pipetting syringe, or automatic pipet can be used for sample addn for bacterial count analyses.

***B. Apparatus and Reagent***

(**a**) *Aerobic count plates*.—Plates contain std methods media nutrients, **940.36A(g)**, cold $H_2O$-sol. gelling agent coated onto film base, overlay film coated with gelling agent, and 2,3,5-triphenyltetrazolium chloride indicator. Circular growth area of single plate contains ca twenty 1 cm squares outlined on film base. Petrifilm Aerobic Count Plates™ (available from Medical-Surgical Division/3M, 225-5S 3M Center, St. Paul, MN 55144) meet these specifications.

(**b**) *Coliform count plates*.—Plates contain violet red bile nutrients conforming to APHA standards as given in Compendium of Methods for the Microbiological Examination of Foods, 2nd ed., 1984 (American Public Health Association, 1015 18th St, NW, Washington, DC 20005), cold $H_2O$-sol. gelling agent, and 2,3,5-triphenyltetrazolium chloride indicator. Petrifilm Coliform Count Plates™ (available from Medical-Surgical Division/3M) meet these specifications.

(**c**) *Plastic spreader*.—Provided with Petrifilm plates, consists of recessed side and smooth flat side, designed to spread sample evenly over plate growth area.

(**d**) *Pipets*.—Calibrated for bacteriological use, or plate loop continuous pipetting syringe to deliver 1.0 mL. Automatic pipet to deliver 1.0 mL may be used.

(**e**) *Colony counter*.—Std app., Quebec model preferred, or one providing equiv. magnification and visibility.

(**f**) *Dilution water*.—*See* **940.36A(a)**.

### C. Sample Preparation

(**a**) For total plate counts: Aseptically prep. 1:10 diln (11 g/99 mL diln $H_2O$). Mix well and plate. Prep. addnl dilns as required. Ordinarily, 1:10 and 1:100 dilns are sufficient.

(**b**) For coliform counts:

*(1) Cream, half-and-half, condensed mild, egg nog, cottage cheese, butter, margarine, and related products*.—Make 1:5 diln (24.75 g/99 mL diln $H_2O$). Mix well and plate 1 mL on each of 2 plates. Multiply total of counts on 2 plates by 2.5 to obtain count/g.

*(2) Sour cream, dips, and yogurt*.—Proceed as in *(1)* except after diln, adjust pH to 6.6–7.2 with 1.0N NaOH (ca 0.1 mL/g sample).

*(3) Buttermilk*.—Make 1:10 diln (11 g/99 mL diln $H_2O$). Adjust pH to 6.6–7.2 with 1.0N NaOH (ca 0.1 mL/g samples). Mix well and plate 1 mL on each of 2 plates. Multiply total of counts on 2 plates by 5 to obtain count/g.

*(4) Ice cream, sherbet, and mixes*.—Hydrate dry-film plates with 1 mL sterile diln $H_2O$ and allow at least 1 h for gel to solidify. Then, lift top film of prehydrated dry-film coliform count plate (gel will adhere to top film) and dispense 0.5 mL of 2:3 homogenate (10 g/5 mL diln $H_2O$) onto bottom film of each of 3 plates. Replace top film gently over sample. Add counts on the 3 plates to obtain count/g. Alternatively, plate 1 plate and multiply result by 3 to obtain count/g.

*(5) Cheese*.—Proceed as in *(1)*. Do not use citrate buffer to homogenize sample.

*(6) Chocolate milk*.—Proceed as in *(1)*.

### D. Analysis

(**a**) *Bacterial colony count*.—Use dry-film aerobic count plates. Place plate on flat surface. Lift top film and inoculate 1 mL sample onto center of film base. Carefully roll top film down onto inoculum. Distribute sample over prescribed growth area with downward pressure on center of plastic spreader device (recessed side down). Leave plate undisturbed 1 min to permit gel to solidify. Incubate plates 48 ± 3 h at 32 ± 1°.

In incubator, place plates in horizontal position, clear side up, in stacks not exceeding 20 units. Count plates promptly after incubation period. If impossible to count at once after required incubation, store plates at 0–4.4° for not >24 h. This should be avoided as a routine practice.

Use std colony counter for counting purposes. Magnifier-illuminator may also be used to facilitate counting. Colonies stain in various shades of red. Count all colonies in countable range (25–250 colonies).

To compute bacterial count, multiply total number of colonies per plate (or av. number of colonies per plate if counting duplicate plates of same diln) by reciprocal of diln used. When counting colonies on duplicate plates of consecutive dilns, compute mean number of colonies for each diln before detg av. bacterial count. Estd counts can be made on plates with >250 colonies and should be reported as estd counts. In making such counts, circular growth area can be considered to contain ca twenty 1 cm squares. To isolate colonies for further identification, lift top film and pick colony from gel.

(**b**) *Coliform count*.—Use dry-film coliform count plates. Proceed as in (**a**), but distribute prepd sample over plate by using plastic spreader, flat side down. Incubate plates 24 ± 2 h at 32 ± 1°. Count as in (**a**), but count only red colonies that have one or more gas bubbles associated (within 1 colony diam.) with them. Count all colonies in countable range (15–150 colonies). Red colonies without gas bubbles are not counted as coliform organisms.

Ref.: JAOAC **72,** 312(1989).

## 978.23 Fecal Coliforms in Shellfish Growing Waters

**Medium A-1 Method**

**First Action 1978**
**Final Action 1979**

(Applicable to enumeration of fecal coliforms and also as presumptive test for *Escherichia coli* in shellfish growing waters)

### A. Apparatus

(**a**) *Pipets*.—1.0 mL serological with 0.1 mL graduations and 10.0 mL with 0.1 mL graduations. Pipets conforming to APHA stds as given in "Standard Methods for the Examination of Dairy Products," 15th ed., 1985, American Public Health Association, 1015 15th St, NW, Washington, DC 20005, may also be used.

(**b**) *Incubator*.—Air, 35 ± 0.5°.

(**c**) *Water bath*.—Covered, circulating, 44.5 ± 0.2°.

(**d**) *Dilution bottles or tubes*.—Borosilicate glass, with glass or rubber stoppers or polyethylene screw caps equipped with Teflon liners.

### B. Media

*Note:* Because geographical differences may affect performance of Medium A-1 method, det. comparability with LST-EC tube method prior to using Medium A-1. Moreover, this medium must be made from individual ingredients. Preformulated Medium A-1 is unacceptable.

(**a**) *Butterfield's buffered phosphate diluent*.—*See* **966.23A(m)**.

(**b**) *Medium A-1 broth*.—Dissolve 5 g lactose, 20 g tryptone, 5 g NaCl, and 0.5 g salicin in 1 L $H_2O$. Heat to dissolve ingredients, pipet in 1 mL Triton X-100 (Rohm & Haas Co.), and adjust pH to 6.9 ± 0.1. For 10 mL sample aliquots, prep. and use double strength medium. To achieve approx. same level of medium and inoculum in all tubes, dispense 10 mL portions of single strength broth into 150 × 18 mm tubes contg inverted fermentation vials; use 175 × 22 mm tubes contg inverted fermentation vials for double strength broth. Autoclave 10 min at 121°. Formation of flocculent ppt, particularly in double strength medium, is common and does not impair performance. Store in dark at room temp. and use within 7 days. Store dehydrated ingredients and/or medium under conditions that will prevent absorption of moisture.

### C. Determination

Shake sample and each successive diln bottle vigorously using 25 complete up and down movements of ca 30 cm in 7 sec. Inoculate $H_2O$ sample directly into tubes contg A-1 Medium in suitable decimal dilns using 3 or 5 tubes/diln with

Butterfield's buffered phosphate diluent. Place inoculated tubes into air incubator and incubate 3 hr at 35 ± 0.5°. Transfer tubes to $H_2O$ bath and incubate 21 ± 2 hr at 44.5 ± 0.2°. Maintain $H_2O$ level in bath above level of liq. in inoculated tubes. Presence of gas in inverted vial or of dissolved gas which can be removed by slight agitation of tube constitutes pos. test. Use std Most Probable Number (MPN) tables, Table **966.24** or Table **978.23**, to det. MPN values. Report results as fecal coliform MPN/100 mL sample.

Ref.: JAOAC **61,** 1317(1978).

## 983.25 Total Coliforms, Fecal Coliforms, and *Escherichia coli* in Foods

### Hydrophobic Grid Membrane Filter Method

**First Action 1983**
**Final Action 1985**

### *A. Principle*

Hydrophobic grid membrane filter (HGMF) uses membrane filter imprinted with hydrophobic material in grid pattern. Hydrophobic lines act as barriers to spread of colonies, thereby dividing membrane filter surface into sep. compartments of equal and known size. Number of squares occupied by colonies is enumerated and converted to most probable number value of organisms by using formula given below.

### *B. Apparatus, Culture Media and Reagents*

(**a**) *Hydrophobic grid membrane filter (HGMF)*.—Membrane filter has pore size of 0.45 μm and is imprinted with nontoxic hydrophobic material in grid pattern. ISO-GRID (available from QA Laboratories Ltd, 135 The West Mall, Toronto, Ontario, Canada M9C 1C2) or equiv. meets these specifications.

(**b**) *Filtration units for HGMF*.—Equipped with 5 μm mesh prefilter to remove food particles during filtration. One unit is required for each sample. ISO-GRID (available from QA Laboratories Ltd.) or equiv. meets these specifications.

(**c**) *Pipets*.—1.0 mL serological with 0.1 mL graduations; 1.1 mL or 2.2 mL milk pipets are satisfactory. 5.0 mL serological with 0.1 mL graduations.

(**d**) *Blender*.—Waring Blendor, or equiv., multispeed model, with low-speed operation at 10 000–12 000 rpm, and 250 mL glass or metal blender jars with covers. One jar is required for each sample.

(**e**) *Vac. pump*.—Water aspirator vac. source is satisfactory.

(**f**) *Manifold or vac. flask*.

(**g**) *Filter paper*.—Whatman No. 1 or No. 4, or equiv.

(**h**) *Peptone/Tween 80 diluent*.—Dissolve 1.0 g peptone (Difco 0118) and 10.0 g Tween 80 in 1 L $H_2O$. Dispense enough vol. into diln bottles to give 90 ± 1 mL after autoclaving 15 min at 121°.

**Table 978.23 Most Probable Numbers per 100 mL of Sample, Planting 5 Portions in Each of 3 Dilutions in Geometric Series**

| Number of Positive Tubes | | | | Number of Positive Tubes | | | | Number of Positive Tubes | | | | Number of Positive Tubes | | | | Number of Positive Tubes | | | | Number of Positive Tubes | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 mL | 1 mL | 0.1 mL | MPN | 10 mL | 1 mL | 0.1 mL | MPN | 10 mL | 1 mL | 0.1 mL | MPN | 10 mL | 1 mL | 0.1 mL | MPN | 10 mL | 1 mL | 0.1 mL | MPN | 10 mL | 1 mL | 0.1 mL | MPN |
| 0 | 0 | 0 | | 1 | 0 | 0 | 2.0 | 2 | 0 | 0 | 4.5 | 3 | 0 | 0 | 7.8 | 4 | 0 | 0 | 13 | 5 | 0 | 0 | 23 |
| 0 | 0 | 1 | 1.8 | 1 | 0 | 1 | 4.0 | 2 | 0 | 1 | 6.8 | 3 | 0 | 1 | 11 | 4 | 0 | 1 | 17 | 5 | 0 | 1 | 31 |
| 0 | 0 | 2 | 3.6 | 1 | 0 | 2 | 6.0 | 2 | 0 | 2 | 9.1 | 3 | 0 | 2 | 13 | 4 | 0 | 2 | 21 | 5 | 0 | 2 | 43 |
| 0 | 0 | 3 | 5.4 | 1 | 0 | 3 | 8.0 | 2 | 0 | 3 | 12 | 3 | 0 | 3 | 16 | 4 | 0 | 3 | 25 | 5 | 0 | 3 | 58 |
| 0 | 0 | 4 | 7.2 | 1 | 0 | 4 | 10 | 2 | 0 | 4 | 14 | 3 | 0 | 4 | 20 | 4 | 0 | 4 | 30 | 5 | 0 | 4 | 76 |
| 0 | 0 | 5 | 9.0 | 1 | 0 | 5 | 12 | 2 | 0 | 5 | 16 | 3 | 0 | 5 | 23 | 4 | 0 | 5 | 36 | 5 | 0 | 5 | 95 |
| 0 | 1 | 0 | 1.8 | 1 | 1 | 0 | 4.0 | 2 | 1 | 0 | 6.8 | 3 | 1 | 0 | 11 | 4 | 1 | 0 | 17 | 5 | 1 | 0 | 33 |
| 0 | 1 | 1 | 3.6 | 1 | 1 | 1 | 6.1 | 2 | 1 | 1 | 9.2 | 3 | 1 | 1 | 14 | 4 | 1 | 1 | 21 | 5 | 1 | 1 | 46 |
| 0 | 1 | 2 | 5.5 | 1 | 1 | 2 | 8.1 | 2 | 1 | 2 | 12 | 3 | 1 | 2 | 17 | 4 | 1 | 2 | 26 | 5 | 1 | 2 | 64 |
| 0 | 1 | 3 | 7.3 | 1 | 1 | 3 | 10 | 2 | 1 | 3 | 14 | 3 | 1 | 3 | 20 | 4 | 1 | 3 | 31 | 5 | 1 | 3 | 84 |
| 0 | 1 | 4 | 9.1 | 1 | 1 | 4 | 12 | 2 | 1 | 4 | 17 | 3 | 1 | 4 | 23 | 4 | 1 | 4 | 36 | 5 | 1 | 4 | 110 |
| 0 | 1 | 5 | 11 | 1 | 1 | 5 | 14 | 2 | 1 | 5 | 19 | 3 | 1 | 5 | 27 | 4 | 1 | 5 | 42 | 5 | 1 | 5 | 130 |
| 0 | 2 | 0 | 3.7 | 1 | 2 | 0 | 6.1 | 2 | 2 | 0 | 9.3 | 3 | 2 | 0 | 14 | 4 | 2 | 0 | 22 | 5 | 2 | 0 | 49 |
| 0 | 2 | 1 | 5.5 | 1 | 2 | 1 | 8.2 | 2 | 2 | 1 | 12 | 3 | 2 | 1 | 17 | 4 | 2 | 1 | 26 | 5 | 2 | 1 | 70 |
| 0 | 2 | 2 | 7.4 | 1 | 2 | 2 | 10 | 2 | 2 | 2 | 14 | 3 | 2 | 2 | 20 | 4 | 2 | 2 | 32 | 5 | 2 | 2 | 95 |
| 0 | 2 | 3 | 9.2 | 1 | 2 | 3 | 12 | 2 | 2 | 3 | 17 | 3 | 2 | 3 | 24 | 4 | 2 | 3 | 38 | 5 | 2 | 3 | 120 |
| 0 | 2 | 4 | 11 | 1 | 2 | 4 | 15 | 2 | 2 | 4 | 19 | 3 | 2 | 4 | 27 | 4 | 2 | 4 | 44 | 5 | 2 | 4 | 150 |
| 0 | 2 | 5 | 13 | 1 | 2 | 5 | 17 | 2 | 2 | 5 | 22 | 3 | 2 | 5 | 31 | 4 | 2 | 5 | 50 | 5 | 2 | 5 | 180 |
| 0 | 3 | 0 | 5.6 | 1 | 3 | 0 | 8.3 | 2 | 3 | 0 | 12 | 3 | 3 | 0 | 17 | 4 | 3 | 0 | 27 | 5 | 3 | 0 | 79 |
| 0 | 3 | 1 | 7.4 | 1 | 3 | 1 | 10 | 2 | 3 | 1 | 14 | 3 | 3 | 1 | 21 | 4 | 3 | 1 | 33 | 5 | 3 | 1 | 110 |
| 0 | 3 | 2 | 9.3 | 1 | 3 | 2 | 13 | 2 | 3 | 2 | 17 | 3 | 3 | 2 | 24 | 4 | 3 | 2 | 39 | 5 | 3 | 2 | 140 |
| 0 | 3 | 3 | 11 | 1 | 3 | 3 | 15 | 2 | 3 | 3 | 20 | 3 | 3 | 3 | 28 | 4 | 3 | 3 | 45 | 5 | 3 | 3 | 180 |
| 0 | 3 | 4 | 13 | 1 | 3 | 4 | 17 | 2 | 3 | 4 | 22 | 3 | 3 | 4 | 31 | 4 | 3 | 4 | 52 | 5 | 3 | 4 | 210 |
| 0 | 3 | 5 | 15 | 1 | 3 | 5 | 19 | 2 | 3 | 5 | 25 | 3 | 3 | 5 | 35 | 4 | 3 | 5 | 59 | 5 | 3 | 5 | 250 |
| 0 | 4 | 0 | 7.5 | 1 | 4 | 0 | 11 | 2 | 4 | 0 | 15 | 3 | 4 | 0 | 21 | 4 | 4 | 0 | 34 | 5 | 4 | 0 | 130 |
| 0 | 4 | 1 | 9.4 | 1 | 4 | 1 | 13 | 2 | 4 | 1 | 17 | 3 | 4 | 1 | 24 | 4 | 4 | 1 | 40 | 5 | 4 | 1 | 170 |
| 0 | 4 | 2 | 11 | 1 | 4 | 2 | 15 | 2 | 4 | 2 | 20 | 3 | 4 | 2 | 28 | 4 | 4 | 2 | 47 | 5 | 4 | 2 | 220 |
| 0 | 4 | 3 | 13 | 1 | 4 | 3 | 17 | 2 | 4 | 3 | 23 | 3 | 4 | 3 | 32 | 4 | 4 | 3 | 54 | 5 | 4 | 3 | 280 |
| 0 | 4 | 4 | 15 | 1 | 4 | 4 | 19 | 2 | 4 | 4 | 25 | 3 | 4 | 4 | 36 | 4 | 4 | 4 | 62 | 5 | 4 | 4 | 350 |
| 0 | 4 | 5 | 17 | 1 | 4 | 5 | 22 | 2 | 4 | 5 | 28 | 3 | 4 | 5 | 40 | 4 | 4 | 5 | 69 | 5 | 4 | 5 | 430 |
| 0 | 5 | 0 | 9.4 | 1 | 5 | 0 | 13 | 2 | 5 | 0 | 17 | 3 | 5 | 0 | 25 | 4 | 5 | 0 | 41 | 5 | 5 | 0 | 240 |
| 0 | 5 | 1 | 11 | 1 | 5 | 1 | 15 | 2 | 5 | 1 | 20 | 3 | 5 | 1 | 29 | 4 | 5 | 1 | 48 | 5 | 5 | 1 | 350 |
| 0 | 5 | 2 | 13 | 1 | 5 | 2 | 17 | 2 | 5 | 2 | 23 | 3 | 5 | 2 | 32 | 4 | 5 | 2 | 56 | 5 | 5 | 2 | 540 |
| 0 | 5 | 3 | 15 | 1 | 5 | 3 | 19 | 2 | 5 | 3 | 26 | 3 | 5 | 3 | 37 | 4 | 5 | 3 | 64 | 5 | 5 | 3 | 920 |
| 0 | 5 | 4 | 17 | 1 | 5 | 4 | 22 | 2 | 5 | 4 | 29 | 3 | 5 | 4 | 41 | 4 | 5 | 4 | 72 | 5 | 5 | 4 | 1,600 |
| 0 | 5 | 5 | 19 | 1 | 5 | 5 | 24 | 2 | 5 | 5 | 32 | 3 | 5 | 5 | 45 | 4 | 5 | 5 | 81 | | | | |

(**i**) *M-FC agar*.—10.0 g tryptose, 5.0 g proteose peptone No. 3, 3.0 g yeast ext, 5.0 g NaCl, 12.5 g lactose, 1.5 g bile salts No. 3, 0.1 g aniline blue, and 15.0 g agar dild to 1 L with $H_2O$ (mFC Agar, Difco 0677, is satisfactory). Heat to boiling. Temper to 50–55°. Adjust pH to 7.4 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(**j**) *Tryptone bile agar* (*TBA*).—20.0 g tryptone, 1.5 g bile salts No. 3, and 15.0 g agar dild to 1 L with $H_2O$ (Tryptone bile agar (Oxoid CM 595) is satisfactory). Heat to boiling. Autoclave 15 min at 121°. Temper to 50–55°. Adjust pH to 7.2 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(**k**) *Tryptic soy-magnesium sulfate agar* (*TSAM*).—15.0 g tryptone, 5.0 g phytone (or soytone), 5.0 g NaCl, 1.5 g $MgSO_4.7H_2O$, and 15.0 g agar dild to 1 L with $H_2O$. Heat to boiling. Autoclave 15 min at 121°. Temper to 50–55°. Adjust pH to 7.3 ± 0.1. Dispense ca 18 mL portions into 100 × 15 mm petri dishes. Surface-dry plated medium before use.

(**l**) *Indole reagent*.—(*1*) *Soln A:* Dissolve 2.5 g *p*-dimethylamino benzaldehyde and 10 mL HCl in 90 mL alcohol. (*2*) *Soln B:* Dissolve 2.0 g potassium persulfate in 200 mL $H_2O$. Mix equal vols of Soln A and Soln B just before use.

(**m**) *Tris buffer*.—1.0M, pH 7.6. Dissolve 121.1 g tris(hydroxymethylamino)methane and dil. to 1 L with $H_2O$. Adjust pH to 7.6 with 1N HCl.

(**n**) *Trypsin stock soln*.—Dil. 10 g trypsin to 100 mL with tris buffer. Warm to 35° if necessary to aid soln. Filter thru Whatman No. 1 paper (or equiv.) to remove insoluble material, then filter-sterilize using 0.45 μm membrane filter.

#### C. Sample Preparation

(**a**) *Nut meat pieces*.—Aseptically weigh 50 g sample into sterile jar. Add 50 mL peptone/Tween 80 diluent (**h**) and shake vigorously (50 times thru 30 cm arc). Let stand 3–5 min and shake just before doing filtrations.

(**b**) *Cheese*.—Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (**h**) and blend 2 min at low speed (10 000–12 000 rpm). Aseptically combine, in 16 × 150 mm tube, 3.5 mL of this 1:10 homogenate and 3.5 mL trypsin soln (**n**). Incubate 20–30 min at 35 ± 0.5° in $H_2O$ bath. Vortex to remix suspension just before doing filtrations.

(**c**) *Other foods needing digestion*.—Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (**h**) and blend 2 min at low speed (10 000–12 000 rpm). Aseptically combine in 16 × 150 mm tube 5.0 mL of this 1:10 homogenate and 1.0 mL trypsin soln (**n**). Incubate 20–30 min at 35 ± 0.5° in $H_2O$ bath. Vortex to remix suspension just before doing filtrations.

(**d**) *Other foods*.—Aseptically weigh 10 g sample into sterile blender jar. Add 90 mL diluent (**h**) and blend 2 min at low speed (10 000–12 000 rpm).

#### D. Analysis

(*See* Figs. **986.32A** and **986.32B**). Turn on vac. source. Place sterile filtration unit on manifold or vac. flask. Open clamp A. Rotate back funnel portion C. Aseptically place sterile HGMF on surface of base D. Rotate funnel forward. Clamp shut by sliding jaws L of stainless steel clamp over entire length of flanges B extending from both sides of funnel C and base D, and rotating moving arm K into horizontal (locked) position.

Aseptically add ca 15–20 mL sterile $H_2O$ to funnel. Pipet required volume (see below) of sample suspension into funnel. Apply free end of vac. tubing E to suction hole F to draw liq. thru prefilter mesh G. Aseptically add addnl 10–15 mL $H_2O$ to funnel and draw thru mesh as before. Close clamp A to direct vac. to base of filtration unit and draw liq. thru HGMF.

| Food | Filtering Diln | Filtering Vol., mL | Multiplication Factor |
|---|---|---|---|
| Nut meat pieces | $10^0$ | 0.5 | 2 |
| Cheese | $10^{-1}$ | 2.0[a] | 10 |
| Other foods needing digestion | $10^{-1}$ | 1.2[a] | 10 |
| Other foods | $10^{-1}$ | 1.0 | 10 |

[a] Filtering vol. of enzyme-digested suspension.

Open clamp A. Rotate moving arm K of stainless steel clamp into unlocked (ca 45° angle) position and slide jaws L off of flanges B. Rotate back funnel C. Aseptically remove HGMF and place on surface of pre-dried agar plate (see below). Avoid trapping air bubbles between filter and agar.

(**a**) *Total coliform count*.—Place HGMF on surface of pre-dried M-FC agar (**i**). Incubate 24 ± 2 h at 35°. Count all squares contg one or more blue colonies. Include any shade of blue. Score each square as either pos. (blue) or neg. Convert pos. square count to MPN with the formula

$$\text{MPN} = [N \log_e (N/(N - x))]$$

where $N$ = total number of squares and $x$ = number of pos. squares. Multiply by reciprocal of diln factor and report as MPN of total coliform bacteria/g.

(**b**) *Fecal coliform count*.—Place HGMF on surface of pre-dried TSAM (**k**). Incubate 4–5 h at 25° for dry foods and 4–5 h at 35° for all other foods. Transfer HGMF to surface of pre-dried M-FC agar (**i**) and incubate 24 ± 2 h at 44.5 ± 0.5° in closed container. Proceed as in (**a**), and report as MPN of fecal coliform bacteria/g.

(**c**) *E. coli count*.—Place HGMF on surface of pre-dried TSAM (**k**). Incubate 4–5 h at 25° for dry foods and 4–5 h at 35° for all other foods. Transfer HGMF to surface of pre-dried TBA (**j**) and incubate 24 ± 2 h at 44.5° ± 0.5° in closed container. Prepare indole reagent (**l**) by combining equal vol. of Soln A and Soln B. Place 9 cm filter paper disk in petri dish lid and flood with 1–2 mL indole reagent (**l**). Transfer HGMF to filter paper, ensuring that no air bubbles are trapped between HGMF and paper. Let stand 10–15 min, then transfer HGMF back to surface of TBA. Count all squares contg one or more pink (indole pos.) colonies. Score each square as either pos. (pink) or neg. Convert pos. square count to MPN with formula above. Multiply by reciprocal of diln factor and report as MPN of *E. coli* (biotype I)/g.

Refs.: JAOAC **66,** 897(1983); **67,** 812(1984).

## ESCHERICHIA COLI

### 988.19 *Escherichia coli* in Chilled or Frozen Foods

**Fluorogenic Assay for Glucuronidase**

**First Action 1988**

(Applicable only to chilled or frozen foods, except chilled or frozen shellfish)

#### A. Principle

Lauryl sulfate tryptose broth with added 4-methyl-umbelliferyl-β-D-glucuronide (MUG) is used as medium in 3-tube MPN method. Tubes are incubated 24 ± 2 h at 35°. Fluorescent-pos. tubes are streaked onto eosin methylene blue agar (Levine) plates, which are incubated 24 ± 2 h at 35°. Typical colonies are picked and confirmed as *E. coli*.

#### B. Media and Reagents

See introductory par. to **966.23A**.

(**a**) *Plate count agar*.—See **940.36A(g)**.

(**b**) *Eosin methylene blue agar* (*Levine*).—See **940.36A(d)**.

(**c**) *Tryptophane broth.*—Dissolve by heating, with stirring, 10.0 g tryptone or trypticase in 1 L $H_2O$. Dispense in 10 mL portions into test tubes and autoclave 15 min at 121°. Final pH, 6.9 ± 0.2.

(**d**) *Buffered glucose broth (MR-VP medium).*—For Me red-Voges Proskauer (MR-VP) tests. Dissolve 7.0 g proteose peptone, 5.0 g glucose, and 5.0 g $K_2HPO_4$ in ca 800 mL $H_2O$ with gentle heat and occasional stirring. Filter, cool to 20°, and dil. to 1 L. Dispense 10 mL portions into test tubes and autoclave 12–15 min at 121°. Max. exposure to heat should be ≤30 min. Final pH, 6.9 ± 0.2. BBL, Division of Bioquest, or Difco dehydrated medium may be used.

(**e**) *Koser's citrate broth.*—See **940.36A(e)**.

(**f**) *Butterfield's buffered phosphate diluent.*—See **966.23A(m)**.

(**g**) *Lauryl sulfate tryptose broth with MUG.*—Prep. lauryl sulfate tryptose broth, **966.23A(b)**, and add 50 mg 4-methyl-umbelliferyl-β-D-glucuronide (MUG). Dissolve with gentle heat, if necessary. Dispense 10 mL portions into 20 × 150 mm test tubes. Autoclave 15 min at 121°. Final pH, 6.8 ± 0.1. Difco dehydrated medium, or equiv., may be used.

(**h**) *Peptone dilution water.*—Dissolve 1.0 g peptone in 1 L $H_2O$. Prep. diln blanks with this soln, dispensing enough to allow for losses during autoclaving. Adjust pH to 7.0 ± 0.1. Autoclave 15 min at 121°.

(**i**) *Lactose broth.*—Dissolve on $H_2O$ bath, with stirring, 3.0 g beef ext and 5.0 g polypeptone or peptone in 1 L $H_2O$. Add 5.0 g lactose. Dispense 450 mL portions into 750 mL flasks and autoclave 15 min at 121°. Max. exposure to heat should be ≤30 min. Final pH, 6.7 ± 0.2.

### C. Preparation of Sample

Prep. all decimal dilns with 90 mL sterile diluent, peptone diln $H_2O$ (**h**) or **966.23A(m)**(*2*), plus 10 mL previous diln unless otherwise specified. Shake all dilns 25 times in 30 cm arc. Pipets must accurately deliver required vol. Do not use to deliver <10% of their total vol. For example, to deliver 1 mL, do not use pipet >10 mL; to deliver 0.1 mL, do not use pipet >1 mL.

*Frozen or chilled foods.*—Use balance with capacity of ≥2 kg and sensitivity of 0.1 g to aseptically weigh 50 g unthawed (if frozen) sample into sterile high-speed blender jar. Add 450 mL diluent, (**i**) or **966.23A(m)**(*2*), and blend 2 min at 10 000–12 000 rpm. (If necessary to temper frozen sample to remove 50 g portion, hold ≤18 h at 2–5°.) Not >15 min should elapse from time sample is blended until all dilns are made in appropriate media.

If entire sample consists of <50 g, weigh portion equiv. to $^1/_2$ sample and add vol. of sterile diluent required to make 1:10 diln. Total vol. in blender jar must completely cover blades.

### D. Determination

*Notes: (1)* Test tubes used in MPN method should be checked under UV light to be sure glass does not fluoresce. (*2*) To avoid false-pos. fluoresence, longwave UV light used in method should not exceed 6 watts (Blak-Ray, Model UVL-56 (available from UVP, Inc.), or equiv.).

Seed 3-tube most probable number (MPN) series into lauryl sulfate tryptose broth contg MUG (**g**), using 1 mL inocula of 1:10, 1:100, and 1:1000 dilns, with triplicate tubes at each diln. Incubate 24 ± 2 h at 35° and examine for fluorescence of medium when tube is held under longwave UV light (366 nm).

Streak fluorescent-pos. tubes on eosin methylene blue agar plates (**b**), and incubate plates 24 ± 2 h at 35°.

Pick 2 or more well isolated typical colonies from eosin methylene blue agar plates and transfer to agar slants prepd from agar medium (**a**). Incubate 18–24 h at 35°. If typical colonies are not present, pick 2 or more colonies most likely to be *E. coli*. Pick ≥2 from every plate.

Confirm *E. coli* as specified in **966.24**.

Ref.: JAOAC **71**, 589(1988).

## 982.36 Invasiveness by *Escherichia coli* of Mammalian Cells

**Microbiological Method**

**First Action 1982**

**Final Action 1987**

### A. Principle

Invasiveness is detected by intracellular growth on monolayer of HeLa cells on slides. To minimize extracellular bacterial multiplication, host-pathogen interaction is resolved into 2 phases, infective and intracellular, using appropriate substrates and the following protocols: growth of monolayer in chamber slides, using controlled inoculum and period of incubation; detn of optimal pre-infection growth conditions for pathogen; washing pathogen to remove toxic end products; infection of host cell under controlled conditions of number and multiplicity of infection, and medium and length of incubation; subsequent removal of unattached bacteria; use of post-infection medium to permit only intracellular bacterial growth for limited period.

### B. Culture Media

(**a**) *Minimal essential medium (MEM).*—Eagle-type with Earle's salts. Dissolve 126.4 mg L-arginine.HCl, 24 mg L-cystine, 292 mg L-glutamine, 41.9 mg L-histidine.HCl.$H_2O$, 52.5 mg L-isoleucine, 52.4 mg L-leucine, 73.1 mg L-lysine.HCl, 14.9 mg L-methionine, 33.0 mg L-phenylalanine, 47.6 mg L-threonine, 10.2 mg L-tryptophan, 36.2 mg L-tryosine, 46.8 mg L-valine, 1 mg D-calcium pantothenate, 1 mg choline chloride, 1 mg folic acid, 2 mg isoinositol, 1 mg pyridoxal HCl, 1 mg nicotinamide, 0.1 mg riboflavin, 1 mg thiamine.HCl, 1 g glucose, 265 mg $CaCl_2.2H_2O$, 400 mg KCl, 200 mg $MgSO_4.7H_2O$, 6.8 g NaCl, 2.2 g $NaHCO_3$, 140 mg $NaH_2PO_4.H_2O$, and 10 mg phenol red in 1 L $H_2O$. Sterilize by filtration. Final pH should be 7.2 ± 0.2. Check sterility of all culture fluids before use. Store at 4–8°.

(**b**) *Fetal bovine serum (FBS).*—Sterile, virus-screened, myco-plasma-free, obtained aseptically during slaughter (Flow Laboratories, Inc., 7655 Old Springhouse Rd, McLean, VA 22102). Store at 4–8°.

(**c**) *Antibiotic concentrate (AC).*—Dissolve 500 000 international units (IU) penicillin G and 500 mg streptomycin (Flow Laboratories, Inc.) in 100 mL $H_2O$ and sterilize by filtration. Store at −10°.

(**d**) *MEM-FBS-AC medium.*—Routine medium for cultivation of HeLa mammalian cells. Mix 90 mL MEM (**a**), 10 mL FBS (**b**), and 1 mL AC (**c**). Store at 4–8°.

(**e**) *MEM-FBS medium.*—Medium for cultivation of HeLa cells before infection. Mix 90 mL MEM (**a**) and 10 mL FBS (**b**). Store at 4–8°.

(**f**) *Earle's salts.*—Prep. without phenol red as follows: Dissolve 6.8 g NaCl, 400 mg KCl, 265 mg $CaCl_2$, 200 mg $MgSO_4.7H_2O$, 140 mg $NaH_2PO_4.H_2O$, 1.0 g glucose, and 2.2 g $NaHCO_3$ in 1 L $H_2O$. Sterilize by filtration. Final pH should be 7.2 ± 0.2.

(**g**) *Veal infusion broth.*—Dissolve 500 g veal (infusion) and 10 g proteose peptone in 1 L $H_2O$ with gentle heating. Dispense 5 mL portions into 13 × 100 mm screw-cap tubes. Autoclave 15 min at 121°. Final pH should be 7.3 ± 0.2.

(**h**) *Brain-heart infusion (BHI).*—Dissolve 12.5 g BHI (powder) in 1 L Earle's salts (**f**). Sterilize by filtration. Final pH should be 7.2 ± 0.2.

(**i**) *Bile salts No. 3*.—Dissolve 5 g bile salts No. 3 formulation in 1 L Earle's salts (**f**). Sterilize by filtration.

(**j**) *Heat-inactivated HFBS*.—Heat FBS (**b**) 2 h at 55 ± 1°. Store at 4–8°.

(**k**) *HFBS-BHI-BS medium*.—Mix 20 mL heat-inactivated FBS (**j**), 10 mL BHI (**h**), 10 mL bile salts No. 3 (**i**), and 60 mL Earle's salts (**f**). Store at 4–8°.

(**l**) *Veal infusion agar slant*.—For maintenance of cultures, dissolve 500 g veal (infusion), 10 g proteose peptone No. 3, 5 g NaCl, and 15 g agar in 1 L $H_2O$ with gentle heating. Dispense 7 mL aliquots to 16 × 150 mm screw-cap tubes. Autoclave 15 min at 121°. Final pH should be 7.3 ± 0.2.

(**m**) *Dulbecco's phosphate-buffered saline (PBS)*.—Dissolve 8.0 g NaCl, 200 mg KCl, 1.15 g $Na_2HPO_4$, 200 mg $KH_2PO_4$, 100 mg $CaCl_2$, and 100 mg $MgCl_2.6H_2O$ in 1 L $H_2O$. Sterilize by filtration. Final pH 7.2 ± 0.2.

(**n**) *Calcium- and magnesium-free Dulbecco's PBS*.—Dissolve 8.0 g NaCl, 200 mg KCl, 1.15 g $Na_2HPO_4$, and 200 mg $KH_2PO_4$ in 1 L $H_2O$. Sterilize by filtration. Final pH 7.2 ± 0.2.

(**o**) *Calcium magnesium, phenol red-free Hanks' PBS*.—Dissolve 8.0 g NaCl, 400 mg KCl, 90 mg $Na_2HPO_4.7H_2O$, 60 mg $KH_2PO_4$, 1.0 g glucose, and 350 mg $NaHCO_3$ in 1 L $H_2O$. Sterilize by filtration. Final pH 7.2 ± 0.2.

(**p**) *Trypsin stock soln*.—2.5%. Suspend 2.5 g 1:250 trypsin (Difco Laboratories) in 100 mL Ca- and Mg-free Hanks' PBS (**o**) and let particles settle. Sterilize by filtration. Dil. 10 mL stock soln with 90 mL sterile Ca- and Mg-free Dulbecco's PBS (**n**) to prep. 0.25% trypsin. Store at −10°.

(**q**) *Gentamicin stock soln*.—Dissolve 50 mg gentamicin (Schering Corp., 2000 Galloping Hill Rd, Kenilworth, NJ 07033) in 100 mL Dulbecco's PBS (**m**) to give soln contg 500 μg/mL. Dil 1 + 9 with Dulbecco's PBS to soln contg 50 μg/mL. Store at 4–8°.

(**r**) *Lysozyme soln*.—Weigh 0.3 g lysozyme, 3× crystalline, salt-free, ca 12 000 Shugar units/mg (Calbiochem Corp.), into 100 mL Dulbecco's PBS and stir to dissolve. Store at 4–8° not >2 weeks.

(**s**) *Intracellular growth phase medium*.—Mix 80 mL MEM-FBS medium (**e**), 10 mL gentamicin soln (50 μg/mL) (**q**), and 10 mL lysozyme soln (**r**). Prep. immediately before use.

### C. Diagnostic Reagents

(**a**) *May-Grunwald stain*.—Weigh 2.5 g stain (EM Science) into 50 mL absolute MeOH, dissolve by grinding, and dil. to 1 L with MeOH. Stir 16 h at 37°. Hold stain 1 month at 22° (room temp.). Filter for use.

(**b**) *Giemsa stain*.—Dissolve 1 g stain (EM Science, No. GX0080) in 66 mL glycerol by heating 1.5–2.0 h at 55–60°. Add 66 mL absolute MeOH. Store stain 2 weeks in tightly stoppered bottle at 22°. Dil. stock soln (1 + 9) before use.

(**c**) *Decolorizing and dehydrating reagents*.—Acetone; acetone-xylene (50 + 50) and (33 + 67); xylene.

(**d**) *Mounting medium*.—Dil. mounting medium with xylene to give easily dispensed colloidal suspension; 20 mL Permount® (Fisher Scientific Co.) dild with 5 mL xylene is satisfactory.

(**e**) *Human cervical epithelial cell culture*.—ATCC HeLa culture. Other cultures, including Henle 407 human intestine and human laryngeal carcinoma gave comparable data; however, HeLa cell culture was more suitable with regard to culture characteristics.

### D. Apparatus

(**a**) *Water baths*.—Maintained at 35 ± 1° and 55 ± 1°.

(**b**) *Microscopes*.—Standard 900 × magnification; inverted stage, 100× magnification (Preiser Scientific, 94 Oliver St, St Albans, WV 25177), or equiv.; microscope illuminator.

(**c**) *Carbon dioxide incubator*.—95% air-5% $CO_2$-moisture-satd atmosphere, maintained at 36 ± 1° (Lab-Line Instruments, Inc. Melrose Park, IL 60160, or equiv.).

(**d**) *Tissue culture chamber slides*.—Clean microscope slides mounted with partitions on plastic gasket to facilitate multiple testing. Lab-Tek units contg 4 chambers are satisfactory (Nunc, Inc., 200 N. Aurora Rd, Naperville, IL 60566), or equiv.

(**e**) *Culture containers*.—Sterile 3 fluid oz (85 mL) glass prescription bottles or plastic tissue culture flasks (Costar, 205 Broadway, Cambridge, MA 02139, or equiv.).

(**f**) *Glass cover slips*.—1 × 2 in. (2.5 × 5.1 cm).

(**g**) *Cell-counting chamber*.—Spencer Bright Line, Fuchs-Rosenthal (Preiser Scientific), or equiv.

(**h**) *Refrigerated centrifuge with adapter*.—To accommodate 13 × 100 mm tubes and covered centrf. cups to prevent aerosolization of pathogens.

(**i**) *Membrane filters*.—0.45 μm pore diam. (Millipore Corp., or equiv.).

### E. Preparation of HeLa Cell Culture

Using std cell culture technics, grow HeLa strain on inner surface of 3 oz glass or plastic container, using 5 mL MEM-FBS-AC medium, (**d**), for 7 days at 36° in $CO_2$ incubator. Replace with fresh culture medium on fourth day to prevent accumulation of toxic metabolites. In prepg cells in monolayer for transfer to chamber slides, wash once with 5 mL Dulbecco's PBS (**m**) prewarmed at 36°. Add 5 mL prewarmed (36°) 0.25% trypsin and hold at room temp. 2 min. Aseptically remove ca 4.5 mL trypsin. Incubate flask at 36° with occasional agitation. After monolayer has detached and cells are fairly uniformly distributed in residual trypsin, add 25 mL prewarmed (36°) MEM-FBS medium, (**e**). Est. cell density, using counting chamber. Add MEM-FBS medium, if necessary, to dil. suspension to density of 1 × $10^5$ cells/mL. With occasional agitation, rapidly transfer 1 mL aliquots to chambers of slide. Incubate 20–24 h at 36° in $CO_2$ incubator. Aseptically remove spent medium before infection. Wash each monolayer once with 1 mL prewarmed (36°) Earle's salts, (**f**), and 1 mL prewarmed (36°) uninoculated infection medium, (**k**) (see below).

### F. Preparation of Bacteria

Inoculate, with needle, 5 mL veal infusion broth, (**g**), using growth from veal infusion agar slant (**l**) incubated at 22°. Incubate presumptive *E. coli* broth cultures 18–24 h at 36°. Centrf. suspension 20 min at 1200 × g at 18°. Resuspend cells in equal vol. of Earle's salts, (**f**). Recentrifuge 20 min at 1200 × g. Resuspend cells in 5 mL Earle's salts. Dil. latter suspension with prewarmed (36°) HFBS-BHI-BS medium, (**k**), to final density of 5 × $10^7$ cells/mL. Add 0.2 mL of each suspension to prepd chamber (above). Use 0.2 mL HFBS-BHI-BS for uninoculated neg. control.

### G. Infection Stage

Incubate chambers 2.5 h at 36° in $CO_2$ incubator. Time factor is critical; shorter period results in min. number of infected host cells and longer period may result in cytotoxic effect arising from medium and possibly bacterial metabolites.

### H. Intracellular Growth Stage

Remove infection medium from chamber with Pasteur pipet. To prevent contamination, use sep. pipet for each chamber. Wash each chamber twice with 1 mL aliquots of prewarmed (36°) Earle's salts. Subsequently wash with 1 mL aliquot of prewarmed intracellular growth phase medium (**s**) prepd immediately before use. Add 0.8 mL prewarmed intracellular growth phase medium to each chamber. Incubate 5 h at 36° in

$CO_2$ incubator. Control of extracellular growth is critical at this stage; sensitivity of culture to gentamicin and other antibiotics should be examined by std procedures before pathogenicity testing. Problem is critical in meats and dairy products where antibiotics may have been used in therapy or in feeds.

### *I. Staining*

Remove fluid contents of chambers. Wash monolayer 3 times with 1 mL Dulbecco's PBS (**n**). Add 1 mL absolute MeOH fixative per chamber. Hold at room temp. 5 min. Remove MeOH and side walls of chamber slide. Insert single-edge razor blade between gasket and slide, and gently pry gasket from slide. If necessary, cautiously remove remnants of gasket from slide with razor blade. Do *not* let specimen dry while slide is prepd for staining. Immerse slides in May-Grunwald stain (**a**) 10 min. Withdraw slides, remove excess stain, and immerse in Giemsa stain (**b**) 20 min. Withdraw slides, remove excess stain, and immerse in $H_2O$ 10–20 s. Briefly rinse twice in acetone. Briefly immerse slides in following sequence of solvs; acetone-xylene (50 + 50), acetone-xylene (33 + 67), and xylene. Evenly distribute 4 drops of mounting medium, (**d**) to slide. Place large cover slip on prepn. Remove excess mounting medium and xylene by gently blotting. Gently apply pressure to remove air bubbles from prepn.

### *J. Detection and Criteria of Invasiveness*

Examine specimens with 900× magnification. Criterion for intracellular location of bacteria is parfocality of cytoplasmic ground substance and bacteria. If invasive, *E. coli* occurs within cytoplasm. Frequently, they may be located along nuclear membrane. In addition, they may be elongated. Finally, bacteria may occur within a membrane (phagolysosome) individually or in groups, indicative of intracellular growth. Examine, at random, 10 fields contg 15–25 HeLa cells. Count bacteria in each cell. Criterion for infection is ≥5 bacteria per cell. Criterion for invasiveness of bacterial culture is ≥1.0% infected HeLa cells.

HeLa cell results with *E. coli* strains must be confirmed by Sereny keratoconjunctivitis test.

Refs.: *Acta Microbiol. Acad. Sci. Hung.* **2**, 292(1955); **4**, 367(1957). *J. Hyg. Epidemiol. Microbiol. Immunol.* **3**, 292(1959). JAOAC **65**, 602(1982).

## 984.34 Detection of *Escherichia coli* Producing Heat-Labile Enterotoxin

**DNA Colony Hybridization Method**

**First Action 1984**
**Final Action 1987**

(*Caution:* This procedure uses radioactive and mutagenic compounds. Personnel must receive adequate training and monitoring and have proper facilities available for handling these substances.)

### *A. Method Performance*

| Results | Percent | 95% Confidence Range (approx.) |
|---|---|---|
| Correct | 96.9 | 95–99 |
| False positive | 2.1 | 1–5 |
| False negative | 4.6 | 1–11 |

Of 13 laboratories, 8 (62%) correctly identified all unknown samples (25/25); 11 laboratories (85%) identified ≥96% of the samples.

### *B. Principle*

Isolated and purified genes (DNA) that code for determinants of bacterial virulence can be used to detect pathogenic strains. Specific fragments of DNA are isolated by cleaving plasmid DNA with appropriate restriction endonucleases and sepg resulting pieces by gel electrophoresis. Purified fragments are radioactively labeled in vitro. Bacterial cultures to be tested are spotted on nitrocellulose filters on agar medium and incubated until colonies are visible. Cells are lysed in situ, DNA is fixed to filter, and radioactive virulence gene DNA fragments are added. Colonies which contain same gene as radioactive DNA will bind this DNA and become radioactive. These colonies are detected by autoradiography.

### *C. Reagents*

(Prep. all media according to manufacturer's instructions.)

(**a**) *10X M9 Salts.*—Dissolve 10 g $NH_4Cl$, 60 g $Na_2HPO_4$, 30 g $KH_2PO_4$, and 5 g NaCl in final vol. of 1 L $H_2O$. Dispense into 100 mL aliquots and autoclave 15 min at 121°.

(**b**) *Amplification medium.*—Sterilize all components sep. Aseptically combine 100 mL 10X M9 salts (**a**), 835 mL $H_2O$, 10 mL 0.1M $MgSO_4$, 10 mL 0.01M $CaCl_2$, 25 mL 20% (w/v) casamino acids, 20 mL 20% (w/v) glucose, and 0.2 mL thiamine (10 mg/mL).

(**c**) *TE Buffer.*—Combine 10 mL 1.0M Tris-HCl (tris-hydroxymethyl aminomethane HCl) and 2 mL 0.5M $Na_2EDTA$. Adjust to pH 8.0 with 10N NaOH. Add $H_2O$ to final vol. of 1 L.

(**d**) *TES Buffer.*—Combine 30 mL 1.0M Tris, 10 mL 0.5M $Na_2EDTA$, and 10 mL 5.0M NaCl. Add $H_2O$ to final vol. of 1 L.

(**e**) *CsCl saturated isopropanol.*—Add ca 50 mL TE buffer (**c**) to ca 350 mL isopropanol. Add solid CsCl (reagent or optical grade) until bottom layer is satd.

(**f**) *Triton lytic mix.*—Add 0.1 mL Triton X-100, 5 mL 1.0M Tris, pH 8.0, and 12.5 mL 0.5M $Na_2EDTA$, pH 8.0, to $H_2O$ (final vol. 100 mL).

(**g**) *10X TBE electrophoresis buffer.*—Dissolve 108 g Tris, 9.3 g $Na_2EDTA$, and 55 g boric acid in ca 800 mL $H_2O$. Adjust pH to 8.2 with concd HCl and bring final vol. to 1 L with $H_2O$.

(**h**) *10X HindIII reaction buffer.*—Combine 50 mL 1.0M Tris, pH 8.0, 10 mL 1.0M $MgCl_2$, 10 mL 5.0M NaCl, and 10 mL 100 mM dithiothreitol in final vol. of 1 L $H_2O$.

(**i**) *Stop soln.*—Combine 1.0 mL 10% (w/v) sodium dodecyl sulfate, 10 mg bromophenol blue, 2 mL 0.5M $Na_2EDTA$, pH 8.0,5 g glycerol in a final vol. of 10 mL of $H_2O$.

(**j**) *10X nick translation buffer.*—Combine 500 μL 1.0M Tris, pH 7.8, 50 μL 1.0M $MgCl_2$, 7 μL 2-mercaptoethanol, and 500 μL nuclease-free bovine serum albumin (1 mg/mL). (Commercially available nick translation kits contain similar reagents. Follow supplier's instructions.)

(**k**) *Hybridization mixture.*—Combine 22 mL distd formamide, 12.5 mL 20X SSC (**l**), 0.5 mL 10% (w/v) sodium dodecyl sulfate, 5.0 mL 10X Denhardt's soln (**m**), 0.1 mL 0.5M $Na_2EDTA$, pH 8.0, and 9.9 mL $H_2O$.

(**l**) *20X Standard saline citrate soln (SSC).*—Add 175.4 g NaCl and 88.2 g Na citrate to final vol. of 1 L $H_2O$. 5X and 2X SSC may be prepd by dilg 20X SSC with $H_2O$.

(**m**) *10X Denhardt's soln.*—Combine 2.0 g Ficoll (400,000 mol. wt), 2.0 g polyvinyl pyrrolidone (360,000 mol. wt), and 2.0 g nuclease-free bovine serum albumin in 1 L $H_2O$. Store 5 mL aliquots at −20°.

(**n**) *Calf thymus DNA.*—Dissolve 1 g purified calf thymus DNA in 100 mL $H_2O$ by stirring for several hours. Sonicate until av. mol. wt is 300,000–500,000 which may be detd by electrophoresis with appropriate stds. Store in 1 mL portions at −20°.

(**o**) *Brain heart infusion broth.*—Prep. and sterilize according to supplier's instructions.

(**p**) *DE 52 column chromatography medium.*—Prep. according to manufacturer's instructions in loading buffer, (**q**).

(**q**) *DE 52 loading buffer.*—Combine 30 mL 5.0M NaCl, 0.2 mL 0.5M $Na_2EDTA$, and 10 mL 1.0M Tris (pH 8.0) in final vol. of 1 L $H_2O$.

(**r**) *DE 52 eluting buffer.*—Combine 200 mL 5.0M NaCl, 0.2 mL 0.5M $Na_2EDTA$, and 10 mL 1.0M Tris (pH 8.0) in final vol. of 1 L $H_2O$.

(**s**) *Sephadex G-50 column chromatography medium.*—Prep. according to manufacturer's instructions in TE buffer, (**c**).

### D. Apparatus and Materials

(**a**) *Preparative ultracentrifuge and fixed angle rotor.*—100,000 × g and 13 mL tubes.

(**b**) *Shaker.*—In 37 ± 1° $H_2O$ bath with clips for holding 1 L erlenmeyers.

(**c**) *Longwave ultraviolet lamp.*—302 nm transilluminator preferred. Camera for photographing gels is useful.

(**d**) *Refrigerated superspeed centrifuge and fixed angle rotor.*—37,000 × g and −20°, capable of holding 50 mL tubes and adapters for 15 or 30 mL tubes.

(**e**) *Siliconized glass tubes.*—15 or 30 mL capable of withstanding 10,000 × g.

(**f**) *Spectrophotometer or colorimeter and sample holder.*—Measure bacterial cell growth at 550 or 600 nm.

(**g**) *Escherichia coli strain C600(pEWD299)(ATCC 37218).*—Contains cloned heat-labile enterotoxin gene. Pos. and neg. strains such as *E. coli* H10407 (ATCC 35401) and plasmid pBR313 (ATCC 37018) are needed as controls during hybridization.

(**h**) *Alpha-$^{32}$P deoxycytosine triphosphate.*—dCTP, 2000–3000 Ci/mmole, aq. stabilized (ICN Biomedicals, Inc., ICN Plaza, 3300 Hyland Ave, Costa Mesa, CA 92626; New England Nuclear, 549 Albany St, Boston, MA 02118; Amersham Corp., Div. of Amersham International, 2636 S. Clearbrook Dr, Arlington Heights, IL 60005-4692).

(**i**) *Ultralow temperature freezer.*—Capable of −70° is preferred; however, −20° (not frost-free) may be substituted.

(**j**) *Vacuum desiccator.*—Large enough to contain 15 or 30 mL tubes.

(**k**) *Polycarbonate tubes.*—50 mL.

(**l**) *Variable volume micropipettors and tips.*—To cover range of 1–1000 μL.

(**m**) *Electrophoresis apparatus.*—Horizontal and vertical units with bed dimensions ca 12 × 12 cm and appropriate power supplies (to 125 mA; 200 V).

(**n**) *Incubator.*—$H_2O$ bath or dry heating block capable of maintaining 37 ± 1°.

(**o**) *Plastic conical centrifuge tubes.*—500 and 1500 μL sizes able to withstand 15,000 × g with appropriate racks.

(**p**) *Centrifuge.*—For spinning tubes (**o**) at greater than 10,000 × g.

(**q**) *Dialysis tubing.*—¼ in. diam., 10–12,000 molecular weight cut-off. Boil 3 min before use.

(**r**) *Glass wool.*—Boiled or siliconized.

(**s**) *Disposable plastic syringes.*—1 mL.

(**t**) *Vacuum side arm flask.*—250 mL for degassing.

(**u**) *Cooling block or refrigerated $H_2O$ bath.*—15 ± 1°.

(**v**) *Plastic column.*—Disposable, ca 4 × 0.9 cm.

(**w**) *Scintillation counter.*—Or Geiger-Mueller counter if calibrated in cpm.

(**x**) *Nitrocellulose filters.*—0.45 μm pore size, 82 mm diam.

(**y**) *Absorbent paper filters.*—82 mm diam.; similar in characteristics to Whatman No. 1.

(**z**) *Petri dishes.*—100 × 15 or 20 mm, plastic.

(**aa**) *Vacuum oven.*—Maintain 80 ± 3°.

(**bb**) *X-ray film.*—8 × 10 in. is convenient size.

(**cc**) *X-ray film holder cassette.*—With intensifying screens (Kodak regular or Dupont Cronex lightening plus).

### E. Isolation of Plasmid DNA

Inoculate 25 mL brain heart infusion broth (**o**) contg 10 μg ampicillin (filter-sterilized)/mL with frozen stock of strain C600 (pEWD299). Incubate overnight at 37° with shaking. Read *A* at 550 nm, using 25-fold diln. Inoculate 1.5 L amplification medium (**b**) to $A_{550}$ = 0.02. [*Note:* This procedure can be scaled up to 10 L.] Shake or aerate well at 37°. When $A_{550}$ = 0.4, add solid chloramphenicol to 100 μg/mL. Reduce shaking to 75 rpm or aeration to 2 Lpm. Incubate overnight. Harvest cells by centrifugation at 4°, resuspend pellets in TES buffer (**d**), and centrf. again. Resuspend cells in 8 mL 25% sucrose (w/v, nuclease-free) in TE buffer (**c**) in 50 mL polycarbonate centrf. tube. Add 1 mL 1% lysozyme (egg white, grade 1), mix gently, and let sit on ice 5 min. Add 13 mL Triton lytic mix (**f**), stir briefly to mix, and incubate on ice 30 min. Centrf. 30 min at 27,000 × g. Decant supernate thru gauze. If pellet is very soft, centrf. again at 37,000 × g for 30 min and combine this supernate with first one. Measure vol. of supernate (to 0.1 mL) and add 0.97 g solid CsCl for each mL. Add soln to ultracentrf. tubes and layer on surface 0.1 mL ethidium bromide, 10 mg/mL, for each mL supernate (before addn of CsCl) on liq. surface. [*Caution:* Ethidium bromide is mutagenic. Handle with care.] Fill tubes with light mineral oil, balance to 50 mg, and cap or seal. Centrf. 40 h in fixed angle rotor at 100,000 × g or 18 h in vertical rotor at 180,000 × g at room temp. (23°).

Observe ultracentrifuge tube in subdued room light, without fluorescent lights. Locate lower, orange band with longwave UV light and remove band with needle and syringe. Place band into polystyrene or siliconized glass tube. Ext and discard ethidium bromide with isopropanol satd with TE buffer and CsCl (**e**). Repeat until pink color is gone and then ext twice more. Measure remaining sample vol. and add 3 vols $H_2O$ and 25 μg yeast transfer RNA (2.5 mg/mL, stored at −20°). After addition of water, add one-ninth total vol. of 3.0M Na acetate-10mM $MgCl_2$. Add 2.5 vols −20° alcohol and hold at −20° 1 h. Centrf. 10 min. at 9,000 × g at 0°. Discard supernate and let pellets drain until alcohol odor is gone. Tubes may be dried 15 min in vac. desiccator but do not over-dry. Gently resuspend pellet in 1 mL TE buffer (**c**). Est. DNA concn by electrophoresis against known stds. If $A_{258}$ is measured, DNA concn will be over-estd because of presence of RNA. [For pure DNA, $A_{258}$ = 1.0 corresponds to 50 μg/mL and ratio $A_{258}/A_{280} \approx 1.8$]. Store DNA in plastic or siliconized glass tubes at 4°.

## Enterotoxin Gene DNA Isolation

### F. Enzyme Titration

Titr. restriction endonuclease against plasmid (pEWD299), using estd DNA concn to det. correct amt of enzyme. Usually, one unit of enzyme will digest about 1 μg DNA. However, this can vary by several fold, depending on plasmid, enzyme, or impurities. Generally, it is best to follow methods suggested by supplier.

If *Hind*III is used to cleave pEWD299, an 850 base-pair fragment will be generated which contains nucleotide sequence for entire B subunit and about one-third of the A subunit of the heat-labile enterotoxin. Dispense ca 1 μg DNA into four 500 μL conical plastic tubes. Add 2.5 μL 10X – *Hind*III reaction buffer (**h**). Add 0, 2, 5, or 25 units of enzyme to each tube. Add 2.5 μL bovine serum albumin (1 mg/mL, nuclease-free). Add $H_2O$ to bring vol. to 25 μL. Incubate 1 h at 37°. Add 5 μL stop soln (**i**) and electrophorese 25 μL of each mixture for 3 h at 100 V in 0.7% agarose in 1X TBE dild from (**g**). As control, run 30–50 ng linear bacteriophage lambda DNA. Stain gel with ethidium bromide (2 μg/mL) until lambda DNA band is visible under longwave UV light. If record is desired, rinse gel briefly with $H_2O$, and photograph with 302 nm transilluminator and camera with Wratten No. 23A or 9 filter.

### G. Preparative Digest

Scale up titrn digest using lowest amt of enzyme that achieves complete digestion. After 1 h of incubation at 37°, add one-tenth vol. of stop soln (**i**). Prep. 10% polyacrylamide gel. For 50 mL gel, combine 32.5 mL $H_2O$, 5.0 mL 10X TBE (**g**), 12.5 mL 40% acrylamide (w/v; caution: acrylamide monomer is a neurotoxin). Degas 15–30 min in sidearm flask under vac. Add 0.5 mL freshly prepd 10% (w/v) ammonium persulfate soln. Add 50 μL TEMED (*N,N,N',N'*-tetramethylethylenediamine) but mix gently so as not to aerate degassed soln. Pour vertical gel which should harden in 10–20 min. Layer digest on gel and electrophorese for 3 h at 100 V in 1X TBE (diluted from **g**). Stain with ethidium bromide (2 μg/mL) until bands are visible with longwave UV. Slice 850 base-pair band (nearest bottom) from gel and place into dialysis tubing with 1–2 mL 1X TBE. Place bag in horizontal electrophoresis unit and cover with 1X TBE. Electroelute band from gel at 50 V for 16 h. Reverse polarity of electrodes and turn on power for 15 s at 150 V. Remove buffer contg DNA from dialysis bag with plastic pipet. Add one-tenth vol. of stop soln (**i**). Repeat electrophoresis and electroelution as described above.

### H. DE52 Chromatography

Prep. DE52 according to manufacturer's instructions, using loading buffer (0.15M NaCl, 1mM $Na_2EDTA$, pH 8.0, 0.01M Tris, pH 8.0). Construct 0.3 to 0.4 mL DE52 column in 1 mL plastic syringe plugged with boiled or siliconized glass wool. Wash column with 2 mL loading buffer. Apply 1–2 mL DNA to top of column bed. Wash column with 3–4 mL loading buffer. Elute DNA with 10 column vols of eluting buffer (1.0M NaCl, 1mM $Na_2EDTA$, pH 8.0, 10mM Tris, pH 8.0) in 0.3 mL aliquots. Collect 0.3 mL fractions in 500 μL plastic tubes. Most of DNA should be in first 2 or 3 fractions. Spot 2 μL of each fraction onto 1% agarose with 2 μg/mL ethidium bromide and illuminate with UV light. Fractions contg DNA will fluoresce; pool these fractions and alcohol-ppt by measuring total vol. and adding 10 μL transfer-RNA (2.5 mg/mL) and one-ninth soln vol. of 3.0M NaOAC-10mM $MgCl_2$. Add 2.5 vols of –20° alcohol and hold at –20° ≥1 h. Centrf. at 10,000 × g for 10 min. Discard supernate and gently rinse pellet (which may not be visible) with 0.5 mL –20° alcohol. Drain well until alcohol odor is gone but do not dry completely. Gently resuspend DNA pellet in 200–300 μL TE buffer (**c**).

### I. In Vitro DNA Labeling

Kits are available commercially for nick translation reaction. Following procedure uses 50 ng DNA rather than 1 μg often suggested by suppliers. To 500 μL conical plastic centrf. tube, add 50 ng DNA (as prepd above in max. vol. of 5 μL). Add 3 μL 10X reaction buffer. Add 1.5 μL of soln 333 μM in each of deoxyadenosine triphosphate, deoxyguanosine triphosphate, and thymidine triphosphate. Add 16 μL alpha-$^{32}$P deoxycytosine triphosphate (dCTP) (**h**). Add $H_2O$ to final vol. of 26 μL. Add 2 μL DNase I (100 ng/mL, dild immediately before use). Incubate 10 min at 15°. Add 2 μL DNA polymerase I (1 IU/μL). Incubate 1 h at 15°. Add 2 μL 0.5M $Na_2EDTA$, pH 8.0. Prep. 2 mL column of Sephadex G-50 (prepd according to manufacturer's instructions) using TE buffer (**c**). Load reaction mix onto column and elute by adding 100 μL portions of TE buffer (**c**). Collect twenty 2-drop fractions into 500 μL tubes. Spot 2 μL of each fraction onto 2 × 2 cm paper squares (e.g., Whatman 3MM), dry, add scintillation fluid (e.g., 5 g 2,5-diphenyloxazole/L toluene), and count. Geiger-counter may suffice to assay fractions. Labeled DNA is eluted from column usually between fractions 6 and 12. Unincorporated dCTP elutes as larger peak, starting between fractions 12 and 15. Pool fractions from earliest peak and count 2 μL as previously described. Using 3000 Ci/mmol of dCT$^{32}$P, specific activities of 2–8 × $10^8$ cpm/μg usually result.

### J. Colony Hybridization Filter Preparation

When received, transfer sample cultures to 5 mL rich broth and incubate at 37° for 18–24 h. Aseptically add 2 mL culture to 0.5 mL sterile 50% (v/v) glycerol. Store at –70° if possible. [*Note:* Frost-free freezers will decrease culture viability. If cultures must be stored at –20°, use non-frost-free unit. This caveat holds for all frozen material in this procedure.]

Boil nitrocellulose filters (0.45 μm, 82 mm diam.) for 2–3 min in ca 2 L $H_2O$. While still wet, flatten filters (to minimize wrinkles) between paper filters (such as Whatman No. 1 or Schleicher and Schuell No. 597), using forceps to avoid touching filters. Loosely wrap filters in Al foil and sterilize at 121°, 15 lb, for 10–20 min on liq. (slow exhaust) cycle.

Store at room temp. Aseptically inoculate ca 5 mL rich broth with portion of frozen bacterial culture. [It is not necessary to completely thaw culture and it may be re-used several times.] Incubate cultures 18–24 h at 37°. Aseptically, place sterile nitrocellulose filter on dry MacConkey agar plate. Ensure that no bubbles are trapped under filter and that it wets completely. Discard filters that do not lie flat. Label filters, using soft lead pencil or by perforating filter in distinctive pattern with needle. This may be more easily done after baking at 80° (see below). Filters marked with 5 mm square grid are useful for arranging cultures in orderly array. Inoculate filters with sterile microbiological needle, using 1:100-fold diln, in sterile normal saline, of overnight culture. Always inoculate each filter with known pos. and neg. control cultures. Record location of each culture; 30–50 cultures should fit on each filter. It is vital that filters and the resulting autoradiogram can be oriented unambiguously. Make duplicate filters, since procedure may have to be repeated. Incubate filters 18–24 h at 37°. Mark cultures which have failed to grow, or a false-neg. result may be reported.

Lyse colonies by transferring filters for 10 min onto paper filters (in 100 × 15 mm plastic petri plates) wetted with 1.5 mL 0.5M NaOH. Ensure that no bubbles are trapped under filters. Transfer nitrocellulose filters for ≥1 min each, to series of 3 paper filters each wetted with 1.5 mL 1.0M ammonium acetate-0.02N NaOH. Shift nitrocellulose filter to fourth ammonium acetate-NaOH filter for 10 min. Keep filters horizontal during transfers so that lysed colonies will not run together. Air dry nitrocellulose filter on absorbent paper ≥30 min. Bake in vac. oven 2 h at 80°. Cool filters to room temp. and label with $H_2O$-proof ink or pencil. Store between paper filters under vac.

### K. Colony Hybridization

Freshly prep. 50 mL hybridization mixt. (**k**). Boil 0.5 mL sonicated calf thymus DNA (**n**) 10 min and add to hybridization mixt. (**k**). Pre-incubate each nitrocellulose filter 3 h at 37° in 100 × 15 mm plastic petri dish contg 5 mL hybridization mixt. with boiled calf thymus DNA. After 3 h, alkali-denature radioactive toxin gene DNA. Det. vol. of DNA required to contain 1 × $10^6$ cpm. Correct for 14.2-day half-life of $^{32}P$. Add 1 × $10^6$ cpm DNA to 500 μL plastic conical tube and bring total vol. to 300 μL with $H_2O$. Add 6 μL 10N NaOH and mix briefly with pipet tip. After 10 min, neutze with 6 μL 10N HOAc. Boil 0.5 mL sonicated calf thymus DNA for 10 min and add 50 mL hybridization mixt. (**k**). Place nitrocellulose filter into 5.0 mL fresh hybridization mixt. and 1 × $10^6$ cpm alkali denatured and neutzd probe DNA. Incubate 18–24 h at 37°.

Rinse filters 5–10 s in 10–15 mL 5X SSC (dild from soln l)-0.1% (w/v) sodium dodecyl sulfate (SDS). Place filter into clean petri dish and cover with 10–15 mL 5X SSC-0.1% SDS and incubate 1 h at 70°. Place filter in fresh 5X SSC-0.1% SDS and incubate addnl 1 h. Rinse filter 5–10 s in 2X SSC (dild from soln l). Air-dry 15–30 min. Mount filter with small pieces of tape onto paper and cover with plastic sheet (such as document holder).

### *L. Autoradiography*

In dark room, place film on plastic-covered filters in cassette film holder with intensifying screens. Enclose film holder in plastic bag and expose film preferably at −70° but at least −20°. Exposure length is dictated by amt of radioactive DNA bound to filter. If increase of 2–3 cps is observed when Geiger-Mueller counter is held over filter, it is likely that pos. reaction will be visible after 1 d exposure. After exposure, let cassette reach room temp. Develop following manufacturer's instructions. If spots are too faint for analysis, expose new film for longer period.

### *M. Interpretation of Results*

DNA of cells having gene for heat-labile enterotoxin of *Escherichia coli* bind radioactive toxin gene DNA. Film will be exposed and dark spots will appear after development. Since colony size and hybridization efficiency can vary, this test is best used qual. and not quant. If there are dark areas on film where no colonies should be, unhybridized radioactive DNA has probably not been completely washed away. Rewash filter twice in 5X SSC-0.1% SDS at 70° for 1 h. Let dry and expose film. After film development, make pos. or neg. detn of each unknown culture by comparing intensity of spot with pos. and neg. control cultures. Neg. control should show no darkening of film or, at most, very faint darkening. Pos. control should show distinct darkening of film clearly discernible above background.

### *N. Troubleshooting*

If autoradiograms are unsatisfactory, a number of factors might be responsible. False-neg. results could be due to spontaneous loss of virulence determinants, insufficient growth of colonies on filters, failure to bake filters to fix DNA, or insufficient radioactivity during hybridization. False-pos. results may result from insufficient filter washing after hybridization, failure to add Denhardt's soln or sonicated calf thymus DNA, or use of probe DNA fragment which was not purified adequately. Possible remedies include use of new bacterial cultures, prepg new filters with lysed colonies, reviewing procedures and reagent composition, rewashing filters, or checking darkroom methods.

Ref.: JAOAC **67**, 801(1984).

## 986.34 Enterotoxigenic *Escherichia coli*

**DNA Colony Hybridization Method Using Synthetic Oligodeoxyribonucleotides and Paper Filters**

**First Action 1986**
**Final Action 1987**

(*Caution:* This procedure uses radioactive compd. Personnel must receive adequate training and monitoring and have proper facilities available for handling this substance.)

### *A. Principle*

Chemically synthesized pieces of DNA (oligodeoxyribonucleotides) that code for regions of genes detg bacterial virulence can be used to identify pathogenic strains of bacteria. These oligomers are radioactively labeled in vitro and hybridized with colonies of bacterial cells that have been lysed and fixed to paper filters. Colonies contg same region of a gene will bind labeled DNA and become radioactive. Such colonies can be detected by autoradiography.

### *B. Reagents*

(Prep. all media according to manufacturer's instructions and use analytical grade materials whenever possible. *Note:* DNA often adheres to unsiliconized glass. When working with solns contg DNA, use siliconized glassware or disposable plasticware unless otherwise specified.)

(**a**) *Lysis mixture A.*—Combine 50 mL 10N NaOH, (**s**), 300 mL 5.0M NaCl, (**u**), and 650 mL $H_2O$.

(**b**) *Lysis mixture B.*—Combine 50 mL 2.0M Tris, pH 7.0, (**v**), 400 mL 5.0M NaCl, (**u**), and 550 mL $H_2O$.

(**c**) *Hybridization mixture.*—Combine in plastic tube or beaker: 28.9 mL $H_2O$, 15.0 mL 20X SSC, (**d**), 5.0 mL 50X Denhardt's soln, (**e**), and 0.1 mL 0.5M EDTA soln, pH 8.0, (**f**). Final vol. is 49 mL. Use immediately.

(**d**) *20X std saline citrate soln (SSC).*—Dissolve 175.4 g NaCl and 88.2 g Na citrate in final vol. of 1 L $H_2O$.

(**e**) *50X Denhardt's soln.*—Dissolve 2.0 g Ficoll (av. molecular wt 400 000), 2.0 g polyvinyl pyrrolidone (av. molecular wt 360 000), and 2.0 g bovine serum albumin in 200 mL $H_2O$. Store at −20° in 5.0 mL aliquots.

(**f**) *0.5M Disodium ethylenediamine tetraacetate soln, pH 8.0.*—Dissolve 186.12 g $Na_2EDTA$ in 800–900 mL $H_2O$. Adjust to pH 8.0 with 10N NaOH, (**s**). Dil. to 1 L with $H_2O$.

(**g**) *Sonicated calf thymus DNA.*—Dissolve 1 g purified calf thymus DNA in 100 mL $H_2O$ by stirring 3–4 h. Sonicate until av. molecular wt is 300 000–500 000, which may be detd by electrophoresis with appropriate stds such as 123-base ladder (Bethesda Research Laboratories (BRL), Div. Life Technologies, Inc., 8717 Grovemont Circle, Gaithersburg, MD 20877). Store in 1 mL portions in 13 × 100 mm screw-cap tubes. Glass may be used in this instance only.

(**h**) *6X SSC soln.*—Combine 300 mL 20X SSC, (**d**), with 700 mL $H_2O$.

(**i**) *2X SSC soln.*—Combine 100 mL 20X SSC, (**d**), with 900 mL $H_2O$.

(**j**) *Synthetic DNA stock soln.*—Approx. 150–350 μg/mL. ($A_{260}$ = 5–10 units.) Soln of 22-base, single stranded DNA molecules [STH (human) and STP (porcine) oligodeoxyribonucleotide probes for enterotoxin genes] will have concn ca 20–50μM. Store at −20°.

(**k**) *Synthetic DNA working soln.*—Dil. stock soln, (**j**), in $H_2O$ to 10μM. Store at −20°.

(**l**) *2.0M Tris soln, pH 7.6.*—Dissolve 242.28 g Tris in ca 800 mL $H_2O$. Adjust to pH 7.6 with concd HCl. Dil. to 1 L with $H_2O$.

**(m)** *1.0M $MgCl_2$ soln.*—Dissolve 9.52 g $MgCl_2$ in final vol. of 100 mL $H_2O$.

**(n)** *0.5M Dithiothreitol soln.*—Weigh 0.77 g dithiothreitol and combine with $H_2O$ to final vol. of 10.0 mL. Store at 4°.

**(o)** *10mM Spermidine soln.*—Dissolve 14.5 mg spermidine in final vol. of 10.0 mL $H_2O$. Store at −20°.

**(p)** *10X Kinase buffer.*—Combine 2.5 mL 2.0M Tris, pH 7.6, **(l)**, 1.0 mL 1.0M $MgCl_2$, **(m)**, 1.0 mL 0.5M dithiothreitol, **(n)**, 1.0 mL 10mM spermidine, **(o)**, 20 μL 0.5M EDTA, **(f)**, and 4.5 mL $H_2O$. Store at 4°.

**(q)** *($\gamma$-$^{32}P$) ATP.*—Aq. soln of adenosine triphosphate, specific activity 3000–7000 Ci/mmole. ("Crude" prepn from ICN Biomedicals, Inc., ICN Plaza, 3300 Hyland Ave, Costa Mesa, CA 92626, or equiv.). Store at −70° if possible.

**(r)** *Bacteriophage T4 polynucleotide kinase.*—20 units/μL (BRL or equiv.).

**(s)** *10N NaOH soln.*—Dissolve 400 g NaOH in final vol. of 1 L $H_2O$.

**(t)** *2.0M Tris soln, pH 8.0.*—Follow instructions for **(l)** but adjust pH to 8.0.

**(u)** *5.0M NaCl soln.*—Dissolve 292.2 g NaCl in final vol. of 1 L $H_2O$.

**(v)** *2.0M Tris soln, pH 7.0.*—Follow instructions for **(l)** but adjust pH to 7.0.

**(w)** *Glycerol freezing soln.*—Combine 50.0 mL glycerol and 50.0 mL $H_2O$. Dispense 0.5 mL aliquots into 1 dram vials. Sterilize by autoclaving 15 min at 121°.

**(x)** *NACS PREPAC column loading buffer.*—Dissolve 19.3 g ammonium acetate in final vol. of 1 L $H_2O$.

**(y)** *NACS PREPAC column eluting buffer.*—Dissolve 308.4 g ammonium acetate in final vol. of 1 L $H_2O$.

**(z)** *Brain heart infusion or trypticase soy broth and agar.*—For microbial growth.

**(aa)** *Scintillation fluid.*—Dissolve 5.0 g 2,5-diphenyloxazole in 1 L toluene.

**(bb)** *ST probe soln.*—Combine equal vols of STH and STP working soln, **(k)**.

**(cc)** *Phosphoramidite soln.*—0.5 g (Applied Biosystems, Inc., 850 Lincoln Centre Dr, Foster City, CA 94404; American Bionetics, Inc., 21377 Cabot Blvd, Hayward, CA 94545, or equiv.), reagent grade (≥95%), made up to 0.1M using anhyd. $CH_3CN$, **(nn)**, and glass syringe transfer procedures with protection from atm. $H_2O$. Vortex mix until dissolved.

**(dd)** *Thiophenol soln.*—Mix 80 mL *p*-dioxane (≤0.01% $H_2O$), 80 mL triethylamine ($99^+$%), and 40 mL thiophenol ($99^+$%) ("Gold Label," Aldrich Chemical Co., or equiv.).

**(ee)** *1H-Tetrazole soln.*—Add 300 mL anhyd. $CH_3CN$, **(nn)**, to 10 g resublimed tetrazole, **(oo)**, with protection from atm. $H_2O$, and sonicate until dissolved. Warm (30–40°), if necessary.

**(ff)** *Ammonium hydroxide soln.*—28–30% $NH_3$, as supplied.

**(gg)** *Acetic anhydride soln.*—Combine 160 mL tetrahydrofuran (≤0.01% $H_2O$), 20 mL 2,6-lutidine ("Spectro Grade," Eastman Kodak Co., or equiv), and 20 mL acetic anhydride ($99^+$%).

**(hh)** *4-Dimethylaminopyridine soln.*—Dissolve 13 g recrystd 4-dimethylaminopyridine, **(pp)**, in 200 mL tetrahydrofuran (≤0.01% $H_2O$).

**(ii)** *Trichloroacetic acid soln.*—Weigh 125 g trichloroacetic acid (Aldrich Chemical Co., Inc., No. 25,139, or equiv., $99^+$%) in beaker with min. exposure to atm. moisture and transfer to storage container using 4 L $CH_2Cl_2$ (≤0.006% $H_2O$).

**(jj)** *Iodine soln.*—Combine 320 mL tetrahydrofuran, 80 mL 2,6-lutidine, and 10.2 g I crystals. Sonicate until dissolved. Add 8.0 mL $H_2O$, dropwise, with stirring.

**(kk)** *Dimethoxytrityl (DMT) assay soln.*—Dissolve 19 g *p*-toluenesulfonic acid monohydrate in 1 L LC grade $CH_3CN$ (0.1M).

**(ll)** *Triethylammonium acetate (TEAA) buffer.*—With const stirring, add 28 mL triethylamine, **(qq)**, to 1.8 L $H_2O$ followed by 10 mL glacial acetic acid. Titr. slowly with more acid to pH 7.0 and then vac. filter thru type HA 0.45 μm filter (Millipore Corp. or equiv.).

**(mm)** *Detritylation soln.*—Add 3 mL glacial acetic acid to 97 mL $H_2O$.

**(nn)** *Anhydrous acetonitrile.*—Store 1 L LC grade $CH_3CN$ (≤0.007% $H_2O$, Burdick & Jackson Laboratories, Inc., or equiv.) over type 4A molecular sieves ≥24 h.

**(oo)** *Resublimed 1H-tetrazole.*—Sublime 20 g 1*H*-tetrazole ($99^+$%, Aldrich "Gold Label" or equiv.) in std sublimation app. at ≤0.25 torr and 130–140°. (Yields ca 15 g sublimate.)

**(pp)** *Recrystallized 4-dimethylaminopyridine.*—Dissolve 200 g 4-dimethylaminopyridine in ca 1 L hot (50–60°) tetrahydrofuran contg 20 g decolorizing charcoal. Filter while still hot thru glass fiber paper (Grade 934-AH, "Reeve Angel," Whatman, Inc., or equiv).

**(qq)** *Triethylamine.*—$99^+$% (Aldrich "Gold Label" or equiv. LC grade).

### *C. Apparatus and Materials*

**(a)** *Labware.*—100 × 15 mm glass petri plates; plastic beakers and tubes to contain up to 100 mL; 100 × 15 or 20 mm plastic petri plates; plastic conical tubes to contain up to 500 μL; plastic pipets to cover range 1–10 mL; variable vol. micropipettors and tips to cover range 1–1000 μL.

**(b)** *Incubators.*—*(1)* Capable of maintaining 37 ± 1°; *(2)* capable of maintaining 40 ± 1°; *(3)* capable of maintaining 50 ± 1°; *(4)* $H_2O$ bath or dry block capable of maintaining 37 ± 1°.

**(c)** *UV spectrophotometer.*—To measure DNA concn at 260 nm. (1 $A_{260}$ unit is 50 μg/mL for double stranded DNA and 33 μg/mL for single stranded DNA.)

**(d)** *Ultralow temperature freezer.*—Capable of maintaining −70° is preferred, but freezer (not frost-free) at −20° may be substituted.

**(e)** *Freezer.*—Capable of maintaining −20° (not frost-free).

**(f)** *Cellulose filters.*—No. 541 (Whatman), 82–85 mm diam.

**(g)** *Absorbent filters.*—Whatman No. 1 or similar, ca 85 mm diam.

**(h)** *NACS PREPAC column.*—DNA binding resin (BRL or equiv.).

**(i)** *Scintillation counter.*—Or Geiger-Mueller counter if calibrated in cpm.

**(j)** *X-ray film and developing chemicals.*—8 × 10 in. is convenient size. Kodak XAR X-ray film or equiv.

**(k)** *Darkroom.*—Facilities for X-ray film development with appropriate safelight.

**(l)** *X-ray film holder cassette.*—With intensifying screens (Kodak regular, Eastman Kodak Co.; Dupont Cronex Lightening Plus, E.I. Dupont de Nemours & Co.; or equiv.).

**(m)** *Centrifuge.*—Capable of spinning 500 μL conical plastic tubes (Eppendorf Model 5412, Brinkmann Instruments, Inc., or equiv.).

**(n)** *Vacuum desiccator.*—Needed only if prepd colony hybridization filters must be stored 1 week.

**(o)** *DNA synthesizer.*—Manual or automated synthesis system (i.e., Applied Biosystems synthesizer Model 380A; other synthesis systems providing equiv. results are also acceptable).

**(p)** *Synthesis ("reaction") columns.*—1 μmol long chain alkylamine-functionalized controlled pore glass, either prepacked or handpacked (Applied Biosystems or equiv.).

(**q**) *Fraction collector*.—To collect fractions from automated synthesis system. Should have auxilliary signal input.

(**r**) *Liquid chromatographic system*.—App. with gradient elution capability, UV detection at 254 or 260 nm, and μBondapak® $C_{18}$, 7.8 mm × 30 cm column (Waters Associates, Inc., or equiv.).

(**s**) *Rotary vacuum centrifuge*.—To conc. LC-purified oligodeoxyribonucleotides (SpeedVac concentrator/dryer, Savant Instruments, Inc., 110-103 Bi-County Blvd, Farmingdale, NY 11735, or equiv.).

(**t**) *Glass syringes*.—Capacity up to 10 mL for transfer of anhyd. $CH_3CN$ with protection from atm. moisture.

(**u**) *Type HV, 0.45 μm filters*.—To remove LC column particulates (Millipore or equiv.).

### D. Colony Hybridization Filter Preparation

Transfer candidate cultures to 5 mL brain heart infusion or trypticase soy broth and incubate 18–24 h at 37°. If culture must be stored before analysis can be performed, aseptically add 2.0 mL culture to 0.5 mL freezing soln, (**w**). Store at −70° if possible. (Note: Frost-free freezers will decrease culture viability and may result in loss of virulence determinants. If cultures must be stored at −20°, use non-frost-free unit. This precaution holds for all frozen material in this procedure.)

Aseptically inoculate 5 mL rich broth with portion of frozen bacterial culture. Sterile cotton swabs are well suited for this purpose. Always include known pos. and neg. control cultures on every fiber (see below). (If culture is not thawed, it may be reused innumerable times.) Incubate culture 18–24 h at 37°. At same time, aseptically prepare 100 × 15 mm petri plates contg either brain heart infusion or trypticase soy agar and dry inverted 18–24 h at 37°. After inoculating cultures in orderly array and ensuring that resulting colonies will not ultimately merge while growing, inoculate agar plates with test cultures, using sterile microbiological needle, toothpick, cotton swab, or replicator; 9–10 mm is convenient distance between cultures. Record location of each culture; it is vital that culture patterns and resulting autoradiogram(s) can be oriented unambiguously. Prep. multiple plates and concomitant filters because hybridization procedure may have to be repeated and number of steps to be repeated is thereby lessened. Incubate plates inverted 18–24 h at 37°. Mark cultures failing to grow; otherwise, false-neg. results may be reported.

Label Whatman No. 541 cellulose filters, (**f**), 82–85 mm diam., using soft lead pencil, and also mark filter so it can be oriented unambiguously after replication. (*Note:* Other manufacturers make filters with physical properties equiv. to Whatman No. 541. However, DNA binding abilities of such filters are not always suitable for use in DNA hybridization.) Apply filter so that side with pencil markings faces colony array on agar surface of plate contg colonies. Wetting initial edge of filter paper and rolling to opposite edge usually eliminates formation of air pockets. If air bubbles are entrapped between filter and agar plate, remove by applying gentle pressure with glass spreader. This maneuver also ensures more efficient attachment of cultures to filter paper, but care must be taken to avoid spreading colonies because of excessive pressure. Filters may be peeled from plate immediately, but more definitive reactions are usually obtained if filter remains situated 1–2 h. (*Note:* Colony array on filter is now mirror image of array originally applied to agar plate.)

Lyse colonies replicated onto filters by transferring filters with colony side up onto absorbent cellulose filters, ca 85 mm diam. (such as Whatman No. 1 or Schleicher & Schuell No. 597) contained in glass 100 × 15 mm petri plates and previously wetted with 1.5–2.0 mL lysis mixt. A, (**a**). Be sure that no air is entrapped between filters. Heat filters in glass plate for 3–5 min in steam. Transfer steamed filters to glass petri plates contg absorbent cellulose filters previously wetted with 1.5–2.0 mL lysis mixt. B, (**b**). Again, be sure that no air pockets result. Maintain filters in horizontal position when transferring so that lysed colonies (DNA) will not become confluent. Let filters become completely neutralized by remaining situated 5–10 min.

If filters are not to be used immediately, air-dry on absorbent paper at room temp. and store under vac. between filter papers. Such filters have been kept ca 1 year without noticeable change in results.

### E. Oligodeoxyribonucleotide Synthesis

(*Note* 1: A number of companies will custom-synthesize oligodeoxyribonucleotides. Also, several oligodeoxyribonucleotide synthesis systems are com. available, both automated and manual. Results are generally satisfactory if manufacturer's instructions are followed. This method uses one of com. available, automated synthesizers and procedure described below is meant to serve only as example.)

(*Note* 2: All solns for prepn and isolation of synthetic oligodeoxyribonucleotides should be prepd in deionized $H_2O$ passed thru 0.2 μm filter ("Versacap Filter Unit," Gelman Sciences, Inc., or equiv.).)

According to manufacturer's instructions, use Applied Biosystems, "fast" cycle but with following modifications of step times: trichloroacetic acid to column detritylation step, 75 s (retained in fraction collector); $CH_3CN$ to column post-detritylation step, 50 s (also retained and pooled with above in fraction collector); $CH_3CN$ to column, pre-coupling step, 120 s; coupling step, 180 s; capping step, 120 s. Synthesis is ended with dimethoxytrityl (DMT) group retained at 5′ terminus. Automated cleavage from support is achieved with concd $NH_4OH$ at room temp. for 1 h. Dil. delivered $NH_4OH$ soln with 1 mL concd $NH_4OH$, heat 10 h at 60° in 3.7 mL vial with Teflon-lined screw cap (Supelco, or equiv.). Let cool to room temp. Add 50 μL triethylamine, (**qq**). Evap. $NH_3$ with N stream to ca 2 mL.

### F. Quantitation of Coupling Yield

To det. isolated product yield (see below) and ensure satisfactory coupling at each addn, theoretical yields of product must be calcd. Dil. each collected fraction (from detritylation and post-detritylation steps above) to 5 mL with DMT-assay soln (**kk**). Mix each fraction well and read *A* at 530 nm. Use assay soln (**kk**) as reference std. Compare *A* with that of previous fraction to det. coupling efficiency of each step (generally 97–99%). To det. overall theoretical yield, multiply all individual step-yields.

### G. Oligodeoxyribonucleotide Purification and Isolation

To det. chromatgc properties of prepn, perform anal. run. Set detector for 0.1 AUFS. Inject 10 μL soln evapd to 2.0 mL. In ambient temp. column, start 20–30% gradient (at 1%/min) of $CH_3CN$ in triethylammonium acetate buffer, (**ll**). Continue at 30% $CH_3CN$ after 10 min. Generally, major DMT-product elutes at 10 ± 3 min. After elution time is detd, repeat chromatgy on preparative scale (inject 100 μL crude soln, 1.0 AUFS). Collect center position of major peak.

### H. Oligodeoxyribonucleotide Processing

Before synthetic oligonucleotide can be used as substrate for polynucleotide kinase, LC solvs and DMT group must be removed. Conc. collected LC fraction using N ca 10–20 min to remove most $CH_3CN$. Conc. sample to dryness using concentrator/dryer, (**s**). Add 1 mL 3% (v/v) acetic acid to remove DMT protecting group. Vortex-mix to dissolve. After 5–10 min at room temp., freeze in crushed dry-ice and conc. using concentrator/dryer, (**s**). Dissolve residue in 1 mL $H_2O$. Add 1 mL anal. grade ethyl acetate to ext org. impurities and vortex-mix thoroly. Let org. layer sep. from aq. layer contg DNA and possible LC column particulates (centrf. if necessary). Remove org. layer with Pasteur pipet and discard. If insoluble LC column particulates are present, syringe-filter DNA soln thru type HV, 0.45 μm filter (**u**). Let DNA soln gravity-filter and collect residual soln by rapidly depressing syringe plunger. Remove 50 μL aliquot from 1 mL filtered DNA soln for $A$ measurement. Conc. both remaining sample and $A$ aliquot to dryness. Dissolve aliquot in 1 mL $H_2O$ and measure $A$ at 260 nm. Since $^1/_{20}$ of sample has been removed, multiply reading by 19 to obtain $A$ units in total purified sample. Discard $A$ aliquot. Multiply $A$ in total purified sample by 10 (because only 10% of total synthesis reaction was purified) to obtain $A$ units of entire isolable product. Compare this yield with calcd value (1 μmole × theoretical yield [see above] × molar $A$ of oligonucleotide synthesized × $10^{-3}$) to det. yield of isolable product. Molar $A$ is calcd by adding number of purines (dA plus dG) times 14 000 plus number of pyrimidines (dC plus T) times 7000. These factors are molar extinction coefficients and $10^{-3}$ is used to convert molar $A$ to μmoles/mL which is a millimolar concn.

### *I. End-Labeling of Synthetic DNA*

Synthetic oligodeoxyribonucleotides are rehydrated to ca 5–10 $A_{260}$ units (ca 150–350 μg/mL) to serve as stock soln (**j**). One $A_{260}$ unit corresponds to ca 33 μg/mL single-stranded DNA. Molecular wt of 22-base, single-stranded DNA molecule is ca 7260. Prep. 10μM working soln for each DNA probe (10 pmoles/μL, 72.6 μg/mL). If desired, STH and STP synthetic DNA probes can be combined into single soln, 5μM in each probe, (**bb**).

Mix 5 μL DNA probe soln, (**bb**), 2.5 μL 10X kinase buffer, (**p**), 15 μL $H_2O$, 1.5 μL ($\gamma$-$^{32}$P) ATP, (**q**), and 1 μL T4 kinase, (**r**), in 500 μL plastic conical centrf. tube, (**a**), on ice. Add kinase, (**r**), last and return enzyme immediately to −20° because it is quite heat-labile. Centrf., (**m**), 2–3 s to adequately mix reagents. Incubate at 37° in $H_2O$ bath or dry block heater, (**b**), 1 h. Add 2 μL 0.5M EDTA, (**f**), to terminate reaction. Add 1.6 μL 4.0M ammonium acetate soln, (**y**), to bring ammonium acetate concn to 0.25M before applying sample to NACS PREPAC column.

Unincorporated $^{32}$P is removed by binding DNA to NACS PREPAC column, (**h**). Equilibrate column with 0.25M ammonium acetate, (**x**), 2 h. Load reaction mixt. onto column and wash, using gravity or very gentle pressure, with ca 4 mL loading buffer, (**x**), to remove free ATP. Elute bound DNA with 200 μL aliquots of eluting buffer, (**y**). Do not force liq. thru column rapidly. Collect three 200 μL fractions in 500 μL plastic tubes, (**a**). Spot 2 μL of each fraction onto ca 2 × 2 cm paper (e.g., Whatman 3MM), dry, add ca 5 mL scintillation fluid, (**aa**), and assay radioactivity by scintillation counting. Geiger-Mueller counter, (**i**), may suffice if properly calibrated and used. Most labeled DNA is eluted from column in fractions 1 and 2. Pool fractions and count triplicate 2 μL portions as described above. Est. total vol. of prepn by carefully drawing into plastic 1 mL pipet. Calc. total amt of radioactivity recovered in prepn. Usually, 1–2 × $10^8$ cpm is obtained if specific activity of ATP, (**q**), is 3000–7000 Ci/mmol. Store at −20°.

### *J. Colony Hybridization*

Freshly prep. 50 mL hybridization mixt., (**c**). Boil 1.0 mL sonicated calf thymus DNA, (**g**), 5 min in $H_2O$ bath and add to hybridization mixt., (**c**). Dispense 10 mL sonicated calf thymus DNA-hybridization mixt. into 100 × 15 or 20 mm *plastic* petri dish and insert cellulose filter contg lysed colony array. To use std amt of probe for each hybridization, det. vol. of probe DNA soln required to contain 1 × $10^6$ cpm after correcting for 14.2 day half-life of $^{32}$P. Add 1 × $10^6$ cpm probe DNA to soln contg filter. Mix briefly and incubate plate overnight at 40°.

Wash hybridized filters free of $^{32}$P-labeled DNA not specifically bound to DNA from colonies on filter by removing filter from hybridization mixt. and rinsing 5–10 s in plastic petri dish contg 10 mL 6X SSC, (**h**). Drain and recover filter with 6X SSC. Incubate 1 h at 50°. Again, drain plate, recover with 6X SSC, and incubate 1 h at 50°. Finally, rinse filter 5–10 s at room temp. in 2X SSC, (**i**). Air-dry on absorbent paper at room temp. to prevent curling. Mount filter to 8 × 10 in. stiff paper (e.g., Whatman 3MM) using small pieces of tape. Cover with plastic or glassine sheet (such as document or neg. holder) to prevent contamination of intensifying screens in X-ray film holders.

### *K. Autoradiography*

Exposure time is dictated by amt of radioactive DNA bound to filter. If increase above background exceeds 10 cps when Geiger-Mueller counter is held over filter, it is likely that pos. reaction will be visible after 4 h exposure at room temp. However, if increase of 2–3 cps is observed, enclose loaded film cassette in sealed plastic bag and expose film overnight, preferably at −70° or at least −20°. If −70° is not available, cassette can be sandwiched between slabs of dry ice to reduce exposure time.

In darkroom, place X-ray film onto plastic-covered filter in cassette film holder with intensifying screens. Expose film for appropriate length of time as detd above. After exposure, let cassette equilibrate at room temp. (to prevent moisture accumulation) before removing plastic bag. Develop X-ray film by following manufacturer's instructions. If spots are too faint or too intense for analysis, expose new film for appropriate length of time.

### *L. Reporting of Results*

Lysed colonies of *E. coli* strains contg DNA coding for heat-stable enterotoxins will bind radioactively labeled oligonucleotide probe for ST. These radioactive lysed colonies will expose X-ray film, and dark spots will be evident after development. Det. if each unknown culture is pos. or neg. by comparing spot intensity to that of pos. and neg. culture controls. However, many factors can influence quality of these results: size of colonies, amt of cellular debris, amt of DNA per lysed colony, hybridization and washing temps, hybridization time, specific activity of probe, and length of autoradiogram exposure. Well documented pos. and neg. controls must be present on every filter to ensure that the procedure has been performed correctly and that compensation for non-specific binding of labeled probe DNA (neg. colonies that may be seen as faint spots) has been made.

If neg. control cultures exhibit faint spots, and pos. culture spots are intense, re-wash filter(s) in 6X-SSC, (**h**), at 52–55° twice for 1 h each time. Dry filters and re-expose autoradiogram. Take care because thermal stability of oligonucleotide hybrids is much less than that of longer DNA molecules.

### *M. Troubleshooting*

Unsatisfactory autoradiograms can result from several factors, some of which have been listed in the previous section. False-neg. results can be due to spontaneous loss of plasmids, especially when strains are cultivated excessively under non-selective laboratory conditions (i.e., re-isolation or further subculture). Also, hybridization and/or washes at excessively high temps can result in decreased DNA probe binding which in turn can lead to neg. observation. Occasionally, very large colonies do not become affixed to filters and cellular material is lost from hybridization filters. False-pos. results can be observed if either hybridization or washing temp. is too low. Nonspecific DNA probe binding will occur. Autoradiogram exposures of excessive time can result in overemphasis of limited, nonspecific binding of probe to neg. cultures; this may be falsely reported as pos. results. Other possible sources of error and their remedies have been discussed (**984.34N**; JAOAC **67**, 801(1984)).

Finally, it is essential to note that resulting autoradiogram spot arrays are mirror images of plate inoculation patterns. This is not the case with **984.34**. Results are accurately read if autoradiograms are reversed (left to right) before interpretation. Films must be marked so that they can be unambiguously oriented with recorded location of each test culture.

Ref.: JAOAC **69,** 531, 151A(1986).

## 984.35 *Escherichia coli* Enterotoxins

### Mouse Adrenal Cell and Suckling Mouse Assays

**First Action 1984**
**Final Action 1987**

### *A. Principle*

When exposed to cholera toxin or heat-labile enterotoxin of *Escherichia coli,* mouse adrenal cell line, designated Y1, responds by change in morphology from flat to round. Response is mediated by adenyl cyclase and is irreversible. Intragastric administration of heat-stable enterotoxin of *E. coli* to suckling mouse causes fluid accumulation in intestinal lumen. This measurable response is mediated by guanyl cyclase.

### *B. Media and Reagents*

(**a**) *Casamino acids-yeast extract* (*CAYE*) *broth.*—*Soln a:* Casamino acids, 20 g; yeast ext, 6 g; NaCl, 2.5 g; $K_2HPO_4$, 8.71 g; adjust to pH 8.5 with 0.1N NaOH, and to final vol. of 1 L. *Soln b:* $MgSO_4$, 50 g; $MnCl_2$, 5 g; $FeCl_2$, 5 g; dissolve in min. amt of 0.01N $H_2SO_4$, and adjust to final vol. of 1 L with $H_2O$.

Add 1 mL soln b to soln a before sterilizing; autoclave 15 min at 121°C after dispensing.

(**b**) *Trypticase soy-yeast extract* (*TSYE*) *broth.*—Com. trypticase soy broth rehydrated as directed with 0.6% yeast ext added.

(**c**) *Tissue culture media.*—(*1*) *Growth medium:* Ham's F-10 with glutamine and $NaHCO_3$ (Flow Laboratories), 100 mL; newborn calf serum, 10 mL; antibiotic conc. (5000 IU penicillin G, and 5000 μg streptomycin/mL), 1 mL. (*2*) *Maintenance medium:* Same as (*1*) except serum level is 1%.

(**d**) *Dulbecco's PBS, pH 7.5.*—NaCl, 8 g; KCl, 200 mg; $Na_2HPO_4.7H_2O$, 2.16 g; $KH_2PO_4$, 200 mg; make up to 1 L with $H_2O$ and autoclave 15 min at 121°.

(**e**) *Trypsin.*—0.25% in Dulbecco's PBS.

(**f**) *Cholera enterotoxin.*—1 mg/mL when reconstituted as directed (Schwartz/Mann).

(**g**) *Mice.*—Outbred white Swiss mice, 3–5 days old.

(**h**) *Evans blue.*—2% soln.

### *C. Equipment and Materials*

(**a**) *Serological pipets.*—1 and 5 mL, small tip.

(**b**) *Pipets.*—25 μL.

(**c**) *Swinnex filters.*—25 mm, 0.45 μm membrane.

(**d**) *Disposable syringes.*—5 mL, accommodating Swinnex filters.

(**e**) *Tissue culture flasks.*—Plastic, 75 sq. cm.

(**f**) *Vertical laminar flow hood.*—Biological containment, equipped with HEPA filters.

(**g**) *Incubator.*—$CO_2$, set at 35° and 5% $CO_2$.

(**h**) *Microtiter tissue culture plates.*—96 wells, with lids, sterile.

(**i**) *Syringe.*—1 mL, disposable.

(**j**) *Animal feeding needle.*—24 gage, 1 in., straight.

(**k**) *Needle.*—27 gage. Not needed if *per os* procedure is followed.

### *Labile Toxin (LT)*

### *D. Day 1*

(**a**) Inoculate control cultures and cultures to be assayed into TSYE broth in 16 × 125 mm screw-cap tubes. Incubate in shaker incubator overnight at 37°. Both known enterotoxin-pos. and enterotoxin-neg. *E. coli* cultures should be used as controls, in addition to cholera toxin-pos. control.

(**b**) Remove growth medium from confluent layer of Y1 cells in 75 sq. cm flask. (One flask will provide enough cells for 2 microtiter assay plates.) Wash cell layer with PBS. Remove PBS wash and add 5 mL trypsin. After 1 min exposure, remove 4.5 mL trypsin and place flask in 35° incubator. Observe at 5 min intervals for cell detachment. When cell sheet has detached, add 5 mL growth medium and pipet repeatedly to break up cell clumps.

(**c**) Add cells from Day 1 (**b**) to 35 mL growth medium (total vol. is 40 mL) in small beaker. Stir this suspension while pipeting 0.2 mL into each well of two 96-well microtiter plates, using macroliter pipet. Cover finished plates and incubate ca 48 h at 35° in $CO_2$ incubator.

### *E. Day 2*

(**d**) Add 2 drops of previously prepd starter culture, Day 1 (**a**), to 10 mL CAYE broth in 50 mL erlenmeyer and incubate 24 h at 37° in shaker incubator at 250 rpm.

### *F. Day 3*

(**e**) Centrf. 24 h culture from Day 2 (**d**). (Twenty min at 2500 rpm will clarify most cultures of *E. coli.*). Filter supernate thru 0.45 μm membrane in Swinnex syringe-end filter holder.

(**f**) Divide filtrate into 2 portions. Heat one portion 30 min at 80°; leave other portion unheated. Both heated and unheated portions are assayed. Heated portion serves as neg. control. Store both at 4°.

(**g**) Prep. cholera toxin soln of 1 ng CT/mL in PBS. Soln is used as pos. control for cell reactivity. Note: CT is unstable at this concn, even at 4°; prep. daily from stock soln.

(**h**) Remove microtiter plates prepd in Day 1 (**c**) and replace growth medium with maintenance medium, 0.2 mL/well.

(**i**) Add 0.025 mL assay and control solns to one or more wells (4/test substance recommended) of microtiter plate, using microtiter syringe. Incubate microtiter plates 30 min at 35° in $CO_2$ incubator.

Replace maintenance medium, 0.2 mL/well. Incubate microtiter plates overnight at 35° in $CO_2$ incubator.

### *G. Day 4*

(**j**) Examine microtiter plates for degree of rounding, starting with controls. Score rounding as follows:

0 = no rounding
1 = ca 25% rounding
2 = ca 50% rounding
3 = ca 75% rounding
4 = 100% rounding

Score ≥2 is reported as pos. for LT. Score <2 is recorded as neg. Neg. controls should show <10% rounding.

### Stable Toxin (ST)

#### H. Day 1

**(a)** Inoculate starter culture. Procedure is identical to Day 1 **(a)**, and need not be repeated when both assays (LT and ST) are done concurrently.

#### I. Day 2

**(b)** Inoculate CAYE assay culture. Procedure is identical to Day 2 **(d)**, and need not be repeated when both assays (LT and ST) are done concurrently.

#### J. Day 3

**(c)** Prep. cultures for assay. Procedure is same as Day 3 **(e)** and **(f)**, and need not be repeated if both assays (LT and ST) are done concurrently. Heated portion only is used in ST assay (suckling mouse). Material for ST assay may be stored at 4° for several days without noticeable loss of activity.

#### K. Day 4

**(d)** Add 2 drops of sterile Evans blue to 1 mL filtrate to be assayed.

**(e)** Inject suckling mice precutaneously with 0.1 mL filtrate into milk-filled stomach. Use tuberculin syringe and 27 gage needle. Inject min. of 4 mice for each filtrate. Discard all injections in which blue filtrate is not confined to stomach (immediate visual inspection) *or*

**(f)** Inject *per os* 0.1 mL filtrate into stomach of each mouse, using tuberculin syringe equipped with 24 gage feeding needle. This procedure may be used instead of precutaneous injection described in Day 4 **(e)**. Either method works and preference is based on analyst's familiarity. Both methods yield equiv. results.

**(g)** Hold mice 3 h at *room temp*. Sacrifice mice by $CO_2$ inhalation. Open each abdomen and remove intestinal tract with exception of stomach and liver. Pool intestines treated with same filtrate in tared weighing vessel. Pool remainder of carcasses in another tared weighing vessel. Weigh both vessels on balance accurate to 0.01 g. Compute ratio of intestine wt/carcass wt.

#### L. Interpretation

Report ratio ≥0.083 as pos. for ST. Report ratio ≤0.074 as neg. for ST. Ratio 0.075–0.082 calls for re-examination of filtrate involved.

Ref.: JAOAC **67**, 946(1984).

## STAPHYLOCOCCUS

## 980.37 *Staphylococcus aureus* in Foods

### Microbiological Method

### Final Action 1984
### Repealed First Action 1987

(Applicable to detection and enumeration of small numbers of *S. aureus* in raw food ingredients and non-processed foods expected to contain large population of competing species.)

Inoculate 3 tubes of trypticase soy broth with 10% NaCl, **966.23A(f)**, at each test diln with 1 mL aliquots of decimal dilns of sample. Max. diln of sample must be high enough to yield neg. end point. Incubate 48 hr at 35–37°.

Using 3 mm loop, transfer 1 loopful from each growth-pos. tube to dried Baird-Parker medium plates, **966.23A(e)**(*3*). Streak so as to obtain isolated colonies. Incubate 45–48 hr at 35–37°.

From each plate showing growth, pick ≥1 colony suspected to be *S. aureus*, **966.23A(e)**(*4*). Transfer colonies to tubes contg 0.2 mL brain heart infusion (BHI) broth, **967.25A(r)**, and emulsify thoroly. Withdraw 1 loopful of resulting culture suspension and transfer to agar slant contg any suitable maintenance medium, e.g., trypticase soy agar: Suspend 40 g powder in 1 L $H_2O$. Let stand 5 min and mix thoroly. Heat gently with occasional agitation and boil ca 1 min or until soln is complete. Autoclave 15 min at 121°. Incubate BHI culture suspensions and slants 18–24 hr at 35–37°. Retain slant cultures at room temp. for ancillary or repeat tests, in case coagulase test results are questionable.

To BHI cultures add 0.5 mL reconstituted coagulase plasma with EDTA, **966.23A(i)**, and mix thoroly. Incubate at 35–37° and examine periodically over 6 hr interval for clot formation. Any degree of clot formation is considered pos. reaction. Small or poorly organized clots may be observed by gently tipping tube so that liq. portion of reaction mixt. approaches lip of tube; clots will protrude above liq. surface. Coagulase-pos. cultures are considered to be *S. aureus*. Test pos. and neg. controls simultaneously with cultures of unknown coagulase reactivity. Recheck doubtful coagulase test results on BHI cultures which have been incubated at 35–37° for >18 but ≤48 hr.

Report most probable number (MPN) of *S. aureus*/g from tables of MPN values, Table **966.24**.

## 987.09 *Staphylococcus aureus* in Foods

### Most Probable Number Method for Isolation and Enumeration

### First Action 1987

(Applicable to detection and enumeration of small numbers of *S. aureus* in food ingredients and food expected to contain large population of competing species)

#### A. Apparatus

**(a)** *Pipets*.—1.0 mL with 0.1 mL graduations; 5.0 mL and 10.0 mL with 0.5 and 1.0 mL graduations.

**(b)** *Blender*.—Waring Blendor, or equiv., 2-speed model, with high-speed operation at 16 000–18 000 rpm, and 1 L glass or metal blender jars with covers. One jar is required for each analytical unit.

**(c)** *Mixer*.—Vortex Genie, or equiv.

**(d)** *Water bath*.—Maintained at 35–37°.

**(e)** *Incubator*.—Maintained at 35°.

#### B. Media and Reagents

**(a)** *Trypticase (tryptic) soy broth with 10% sodium chloride and 1% sodium pyruvate*.—Add 95 g NaCl to 1 L soln of 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g $K_2HPO_4$, 2.5 g dextrose (dehydrated trypticase or tryptic soy broth is satisfactory), and 10 g sodium pyruvate. Adjust to pH 7.3. Heat gently if necessary. Dispense 10 mL into 16 × 150 mm tubes. Autoclave 15 min at 121°. Final pH, 7.3 ± 0.2. Store ≤1 month at 4 ± 1°.

**(b)** *Physiological salt soln*.—Dissolve 8.5 g NaCl in 1 L $H_2O$. Autoclave 15 min at 121° and cool to room temp.

**(c)** *Baird-Parker medium (egg tellurite glycine pyruvate agar, ETGPA)*.—(*1*) *Basal medium*.—Suspend 10.0 g tryptone, 5.0 g beef ext, 1.0 g yeast ext, 10.0 g Na pyruvate, 12.0 g glycine, 5.0 g $LiCl.6H_2O$, and 20.0 g agar in 950 mL $H_2O$. Heat to bp with frequent agitation to dissolve ingredients completely.

Dispense 95 mL portions into screw-cap bottles. Autoclave 15 min at 121°. Final pH, 7.0 ± 0.2 at 25°. Store ≤1 month at 4 ± 1°.

(*2*) *Enrichment*.—Bacto EY tellurite enrichment (Difco Laboratories) or prep. as follows: Soak fresh eggs ca 1 min in diln of satd $HgCl_2$ soln (1 + 1000). Aseptically crack eggs and sep. yolks from whites. Blend yolk and physiological saline soln, (**b**), (3 + 7, v/v) in high-speed blender ca 5 s. To 50 mL egg yolk emulsion add 10 mL filter-sterilized 1% K tellurite soln. Mix and store at 4 ± 1°.

(*3*) *Complete medium*.—Add 5 mL warmed enrichment to 95 mL molten basal medium cooled to 45–50°. Mix well, avoiding bubbles, and pour 15–18 mL into sterile 100 × 15 mm petri dishes. Store plates at room temp. (≤25°) for ≤5 days before use. Medium should be densely opaque; do not use nonopaque plates. Dry plates before use by 1 of following methods: (*a*) in convection oven or incubator 30 min at 50° with lids removed and agar surface downward; (*b*) in forced-draft oven or incubator 2 h at 50° with lids on and agar surface upward; (*c*) in incubator 4 h at 35° with lids on and agar surface upward; or (*d*) on laboratory bench 16–18 h at room temp. with lids on and agar surface upward.

(**d**) *Brain-heart infusion (BHI) broth*.—Dissolve infusion from 200 g calf brain and from 250 g beef heart, 10.0 g proteose peptone or gelysate, 5.0 g NaCl, 2.5 g $Na_2HPO_4.12H_2O$, and 2.0 g glucose in 1 L $H_2O$, heating gently if necessary. Dispense 5 mL portions into 16 × 150 mm test tubes and autoclave 15 min at 121°. Final pH, 7.4 ± 0.2.

(**e**) *Desiccated coagulase plasma (rabbit) with EDTA*.—Reconstitute according to manufacturer's directions. If not available, reconstitute *desiccated coagulase plasma (rabbit)* and add $Na_2H_2EDTA$ to final concn of 0.1% in reconstituted plasma.

(**f**) *Butterfield's buffered phosphate diluent*.—(*1*) *Stock soln*.—Dissolve 34.0 g $KH_2PO_4$ in 500 mL $H_2O$, adjust to pH 7.2 with ca 175 mL 1N NaOH, and dil. to 1 L. Store in refrigerator. (*2*) *Diluent*.—Dil. 1.25 mL stock soln to 1 L with $H_2O$. Prep. diln blanks with this soln, dispensing enough to allow for losses during autoclaving. Autoclave 15 min at 121°.

### C. Preparation of Food Homogenate

Aseptically weigh 50 g unthawed food sample into sterile blender jar. Add 450 mL phosphate-buffered diln $H_2O$ and homogenize 2 min at high speed (16 000–18 000 rpm). Use this 1:10 diln to prep. serial dilns from $10^{-2}$ to $10^{-6}$ by transferring 10 mL of 1:10 diln to 90 mL diln blank, mixing well with vigorous shaking, and continuing until $10^{-6}$ is reached.

### D. Most Probable Number Technique

Inoculate 3 tubes of trypticase soy broth with 10% NaCl and 1% sodium pyruvate, (**a**), at each test diln with 1 mL aliquots of decimal dilns of sample. Max. diln of sample must be high enough to yield neg. end point. Incubate 48 h at 35°.

Using 3 mm loop, transfer 1 loopful from each growth-pos. tube to dried Baird-Parker medium plates, (**c**)(*3*). Vortex-mix tubes before streaking if growth is visible only on bottom or sides of tubes. Streak so as to obtain isolated colonies. Incubate 48 h at 35–37°.

### E. Interpretation

Colonies of *S. aureus* are typically circular, smooth, convex, moist, 2–3 mm in diam. on uncrowded plates, gray-black to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone (ppt), and frequently with outer clear zone; colonies have buttery to gummy consistency when touched with inoculating needle. Occasional non-lipolytic strains may be encountered which have same appearance, except that surrounding opaque and clear zones are absent. Colonies isolated from frozen or desiccated foods which have been stored for extended periods are frequently less black than typical colonies and may have rough appearance and dry texture.

### F. Confirmation Technique

For each plate showing growth, pick ≥1 colony suspected to be *S. aureus*. With sterile needle transfer colonies to tubes contg 0.2 mL BHI broth, (**d**), and to agar slants contg any suitable maintenance medium, e.g., trypticase soy agar, std plate count agar, etc. Incubate BHI culture suspensions and slants 18–24 h at 35°. Retain slant cultures at room temp. for ancillary or repeat tests, in case coagulase test results are questionable.

To BHI cultures add 0.5 mL reconstituted coagulase plasma with EDTA, (**e**), and mix thoroly. Incubate at 35–37° and examine periodically over 6 h interval for clot formation. Any degree of clot formation is considered pos. reaction. Small or poorly organized clots may be observed by gently tipping tube so that liq. portion of reaction mixt. approaches lip of tube; clots will protrude above liq. surface. Coagulase-pos. cultures are considered to be *S. aureus*. Test pos. and neg. controls simultaneously with cultures of unknown coagulase reactivity. Recheck doubtful coagulase test results on BHI cultures which have been incubated at 35–37° for >18 but ≤48 h.

Report most probable number (MPN) of *S. aureus*/g from tables of MPN values, Table **966.24**.

Ref.: JAOAC **70**, 35(1987).

## 975.55 *Staphylococcus aureus* in Foods

### Surface Plating Method for Isolation and Enumeration

**First Action 1975**
**Final Action 1976**

(Applicable for general purpose use in testing foods expected to contain ≥10 cells of *S. aureus*/g. For small numbers, *see* **987.09**.)

### A. Apparatus

*Sterile, bent glass streaking rods*.—Hockey stick or hoe-shape, with fire-polished ends, 3–4 mm diam., 15–20 cm long, with angled spreading surface 45–55 mm long.

### B. Determination

At each diln plated, aseptically transfer 1 mL of sample suspension, **987.09C**, to triplicate plates of Baird-Parker medium, **987.09B(c)**(*3*), and equitably distribute the 1 mL inoculum over the triplicate plates (e.g., 0.4 mL–0.3 mL–0.3 mL). Spread inoculum over surface of agar using sterile, bent glass streaking rods. Avoid extreme edges of plate. Retain plates in upright position until inoculum is absorbed by medium (ca 10 min on properly dried plates). If inoculum is not readily absorbed, plates may be placed in incubator in upright position ca 1 hr before inverting. Invert plates and incubate 45–48 hr at 35–37°. Select plates contg 20–200 colonies, unless only plates at lower dilns (>200 colonies) have colonies with typical appearance of *S. aureus*, **987.09E**. If several types of colonies are observed which appear to be *S. aureus*, count number of colonies of each type and record counts sep. When plates at lowest diln plated contain <20 colonies, these may be used. If plates contg >200 colonies have colonies with typical appearance of *S. aureus* and typical colonies do not appear at higher dilns, use these plates for enumeration of *S. aureus*, but do not count non-typical colonies. Select ≥1 colony of each type counted and test for coagulase production, **987.09F**. Add number of colonies on triplicate plates represented by colonies giving pos. coagulase test and multiply by sample diln factor. Report this number as number of *S. aureus*/g of food tested.

Ref.: JAOAC **58,** 1154(1975).

## 976.31 Staphylococcal Enterotoxin in Foods

### Microslide Gel Double Diffusion Test

**First Action 1976**
**Final Action 1977**

(Detects 0.1–0.01 µg enterotoxin/mL and is applicable to detection of enterotoxin in culture fluids and concd food exts)

#### A. Principle

Pptn line occurs when serological type of enterotoxin diffuses thru gel and reacts with its specific antibody. Coalescence with ref. pptn line which results from serological reactivity of enterotoxin serotype and specific antibody confirms identity.

#### B. Apparatus

(**a**) *Debubblers*.—Fine glass rods. Prep. by pulling glass tubing very fine, as in making capillary pipets. Break into ca 6 cm lengths and seal ends in flame.

(**b**) *Electrical tape*.—Insulating tape, 0.25 × 19.1 mm (Temflex 1700, 3M Co., Electrical Products Div., Bldg 225-4N-05, 3M Center, St Paul, MN 55144-1000, or equiv.)

(**c**) *Microscope slides*.—Plain glass, pre-cleaned, 7.62 × 2.54 cm (3 × 1″), 0.96–1.06 mm thick.

(**d**) *Pasteur pipets*.—Prep. by drawing out ca 7 mm od glass tubing or use disposable 30 or 40 µL pipets (Kensington Scientific Corp., 1399 64th St, Emeryville, CA 94608, or equiv.).

(**e**) *Petri dishes*.—20 × 150 mm and 15 × 100 mm.

(**f**) *Plastic templates*.—*See* Fig. **976.31A**. (Available from Toxin Technology, 845 E. Johnson St, Madison, WI 53703.)

(**g**) *Silicone lubricant*.—High vac. grease (Dow Corning Corp., or equiv.).

(**h**) *Staining jars*.—Coplin or Wheaton jars.

(**i**) *Sterile bent glass spreaders*.—Bend glass rods like hockey sticks and fire polish.

(**j**) *Water-saturated synthetic sponge strips*.—Approx. 1.5 × 1.5 × 6.5 cm $H_2O$-satd absorbent cotton is also satisfactory.

#### C. Media and Reagents

(**a**) *Agar soln for coating slides*.—0.2%. Add 2 g bacteriological grade agar to 1 L boiling $H_2O$ and heat until agar dissolves. Pour 20–30 mL portions agar into 180 mL (6 oz) prescription bottles or equiv. containers and store at room temp. Remelt when needed for coating slides.

(**b**) *Brain-heart infusion (BHI) agar*.—0.7% (w/v). Adjust BHI broth to pH 5.3; add bacteriological grade agar to prep. 0.7% concn and dissolve by boiling gently. Distribute in 25 mL portions into 25 × 200 mm test tubes, and autoclave 10 min at 121°. Immediately before use, aseptically empty tubes of sterile medium into 15 × 100 mm petri dishes.

(**c**) *Enterotoxin antisera*.—Dil. lyophilized sera (Toxin Technology) with normal physiological saline according to specific instructions of supplier. Store liq. stocks (highly concd) and working dilns of antisera at 4°; for long term storage, freeze-drying or freezing is recommended.

(**d**) *Enterotoxin references*.—Rehydrate lyophilized enterotoxin prepns, (**c**), according to specific instructions of supplier.

(**e**) *Gel diffusion agar*.—Add 1.2% purified agar (Noble special agar, Difco Laboratories) to boiling fluid base (0.85% NaCl–0.80% Na barbital with final concn of 1:10,000 merthiolate (Eli Lilly and Co., Pharmaceutical Div., Lilly Corporate Center, Indianapolis, IN 46285, or equiv.) adjusted to pH 7.4). Filter hot agar thru 2 layers of anal. grade paper and store in 15–25 mL portions in screw-cap bottles.

(**f**) *Staining soln*.—0.1% Thiazine Red R stain (Fluka Chemical Corp., or equiv.) in 1% HOAc.

(**g**) *Sterile distilled water*.—Dispense 5 mL distd $H_2O$ into tubes and autoclave 15 min at 121°. Normal physiological saline may be substituted for $H_2O$.

(**h**) *Turbidity std*.—1% $BaCl_2$–1% $H_2SO_4$ (1 + 99) (No. 1 of McFarland nephelometer scale).

#### D. Preparation of Sample

Select ≥4 isolated staphylococcal colonies from enumeration and recovery media, and streak nutrient media agar slants, or equiv. Incubate slants 18–24 hr at 35–37°. Add loopful of growth from agar slants to 5.0 mL sterile distd $H_2O$ or saline and prep. aq. suspension of organisms from each slant which is equiv. to turbidity of No. 1 tube of McFarland nephelometer scale (ca 3 × $10^8$ organisms/mL). Inoculate surface of semisolid BHI agar with 4 drops aq. suspension of organisms de-

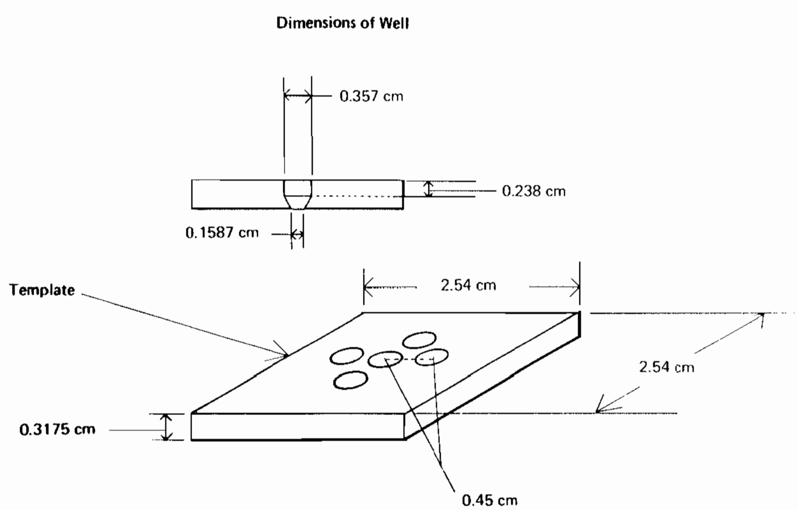
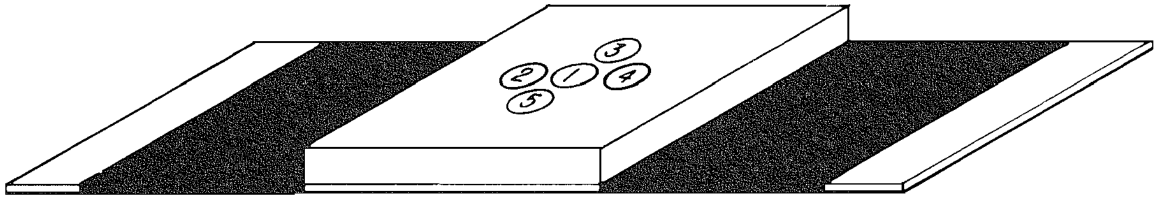



**FIG. 976.31A—Plastic template schematic for microslide assembly**




(1) Bivalent
1. Combination Antisera (e.g., Anti A and B)
2. Prepn under test
3. Ref. enterotoxin (e.g., Type A)
4. Prepn under test
5. Ref. enterotoxin (e.g., Type B)

(2) Monovalent
1. Antiserum (e.g., Anti A)
2. Dilns of prepn under test
3. Ref. enterotoxin (e.g., Type A)
4. Dilns of prepn under test
5. Dilns of prepn under test

**FIG. 976.31B—Arrangement of antisera and homologous reference enterotoxins (*1*) when assaying preparation(s) under test for presence of 2 staphylococcal enterotoxins simultaneously (bivalent detection system) or (*2*) when assaying dilutions of preparation under test with apparent enterotoxin excess (monovalent detection system)**

livered from sterile 1.0 mL pipet. Spread drops of aq. culture suspension over entire surface of semisolid agar with sterile glass rod and incubate plates upright 48 hr at 35–37°. Transfer contents of petri dish to 50 mL centrf. tube with aid of wood applicator stick and centrf. 10 min at 32,800 *g* to remove agar and organisms. Examine culture fluid for presence of serologically identifiable enterotoxins.

### *E. Preparation of Slides*

Wrap double layer of elec. tape around pre-cleaned microscope slide, leaving 2.0 cm space in center, as follows: Start piece of tape ca 9.5–10 cm long ca 0.5 cm from edge of bottom surface of slide and wrap tightly around slide twice. Wipe area between tapes with cheesecloth soaked with alcohol, and dry with dry cheesecloth. Coat upper surface area between tapes with 0.2% bacteriological grade agar as follows: Melt 0.2% agar, and maintain at ≥55° in screw-cap bottle. Hold slide over beaker on hot plate adjusted to 65–85° and pour or brush 0.2% agar over slide between 2 pieces of tape. Let excess agar drain off, wipe bottom surface of slide, and collect agar in beaker for reuse. Place slide on tray and dry in dust-free atm. (e.g., incubator). If slides are not clean, agar will not coat slides uniformly.

### *F. Preparation of Slide Assemblies*

Prep. plastic templates according to specifications in Fig. **976.31A**. Spread *thin* film of silicone grease on side of template that will be placed next to agar (i.e., side with smaller holes). Place ca 0.4 mL melted and cooled (55–60°) 1.2% gel diffusion agar between tapes. Immediately lay silicone-coated template on melted agar and edges of bordering tapes. Place 1 edge of template on 1 piece of tape, and bring opposite edge to rest gently on other piece. Sat. strips of synthetic sponge (ca 1.5 × 1.5 × 6.5 cm) with $H_2O$, and place 2 strips on periphery of each 20 × 150 mm petri dish. Place slide in prepd petri dish (2–4 slide assemblies/dish) soon after agar hardens, and label slide.

### *G. Slide Gel Diffusion Test*

To prep. record of assay, draw hole pattern of template on record sheet and indicate number (same as that used for slide) and contents of each well. Place suitable diln of antiserum or sera in central well, homologous ref. enterotoxin in peripheral well(s), and material under examination in well adjacent to that contg ref. enterotoxin. See Fig. **976.31B**(*1*) for reagent arrangement for simultaneous detection of 2 enterotoxin types (bivalent detection system). Prep. control slide with only ref. toxin and antienterotoxin serum to det. proper reactivity of reagents. Fill wells to convexity with reagents, using Pasteur or disposable 30 or 40 μL pipet. Partially fill capillary pipet with soln and remove excess liq. by touching pipet to edge of sample tube. Slowly lower pipet into well until it touches agar surface, and fill to convexity. Remove trapped air bubbles from *all* wells by probing with debubbler, (**a**), against dark background. Let slides incubate 48–72 hr at room temp. in covered petri dishes contg moist sponge strips (24 hr slide incubation at 35° is generally sufficient for testing of culture fluids). Carefully remove template by sliding it to 1 side. If necessary, clean slide by dipping in $H_2O$ and wiping bottom of slide. Enhance lines of pptn by immersing slide in staining soln, (**f**), 5–10 min. To preserve slide as permanent record, rinse any reactant liq. remaining on slide by dipping in $H_2O$ and then immerse slide in each of following baths 10 min: staining soln, 1% HOAc, 1% HOAc, and 1% HOAc contg 1% glycerol. Drain excess fluid from slide and dry in 35° incubator. After prolonged storage, lines of pptn may not be visible until slide is immersed in $H_2O$.

### *H. Interpretation*

Examine slide for lines of pptn by holding at oblique angle to light source against dark background. Coalescence of test sample lines of pptn with ref. line(s) of pptn indicates pos. reaction. Fig. **976.31C** shows microslide gel diffusion test as bivalent detection system: Antisera to enterotoxins A and B are in well 1; known ref. enterotoxins A and B are in wells 3 and 5, resp., to produce ref. lines of A and B; prepns under test are in wells 2 and 4. Interpret 4 reactions as follows: (1) No line development between test prepns—absence of enterotoxins A and B; (2) coalescence of test prepn line from well 4 with enterotoxin A ref. line (intersection of test prepn line with enterotoxin B ref. line)—absence of enterotoxins A and B in well 2, presence of enterotoxin A and absence of enterotoxin B in well 4; (3) presence of enterotoxin A and absence of enterotoxin B in both test prepns; and (4) absence of enterotoxins A and B in test prepn in well 2, presence of enterotoxins A and B in well 4. Operator can simplify assay by

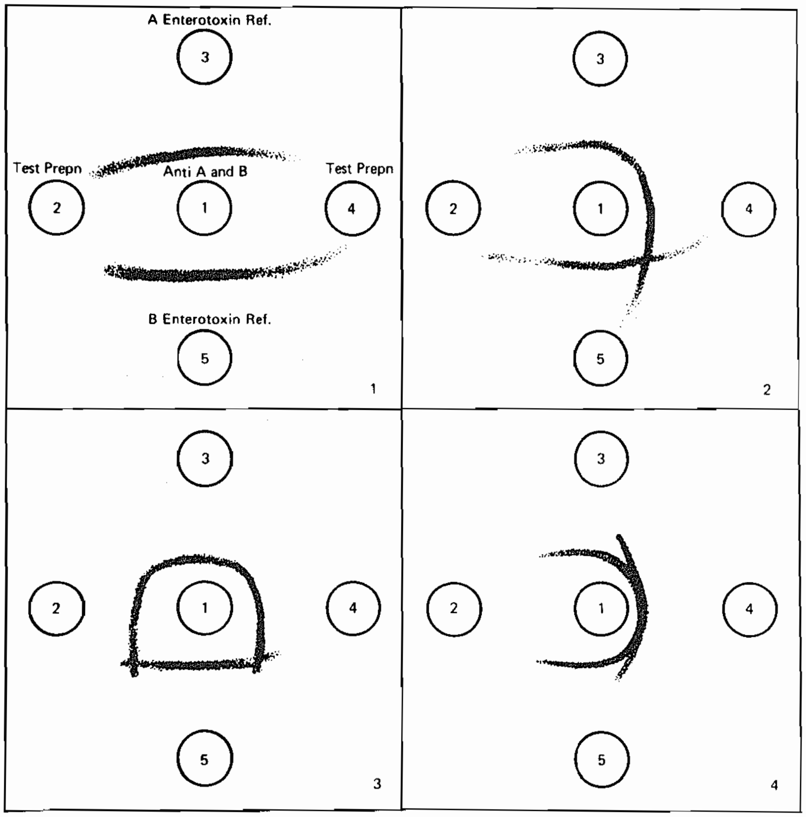



**FIG. 976.31C—Examples of 4 possible reactions in bivalent detection system.** ***See*** **976.31H for explanation of reactions**

testing only 1 prepn for presence of 2 different enterotoxins on same set of slides.

If concn of enterotoxin in test material is excessive, formation of ref. line will be inhibited because of fast migration of toxin thru gel, thus localizing antibody in its well. Fig. **976.31D**(*A*) shows this inhibition of ref. line formation when 10 and 5 μg enterotoxin/mL, resp., are used. Figs. **976.31D**(*B*)–(*F*) show ppt patterns when successively less enterotoxin is used. If test prepn inhibits formation of ref. line as in Fig. **976.31D**(*A*), dil. test material, utilizing monovalent system shown in Fig. **976.31E**. Reactant arrangement for assaying dilns of prepn under test is shown in Fig. **976.31B**(*2*). Figure **976.31E** shows microslide gel diffusion test as monovalent system in which antiserum is placed in well 1; ref. enterotoxin in well 3; and dilns of test prepn in wells 2, 4, and 5. Do not make starting diln of culture fluid (test material) so high as to dil. beyond reactive concn of enterotoxin.

Occasionally, atypical ppt patterns form which may be difficult for inexperienced analysts to interpret. One of most common atypical reactions is formation of lines not related to toxin, but caused by other antigens in test material. Examples of such patterns are given in Fig. **976.31F**, which shows microslide

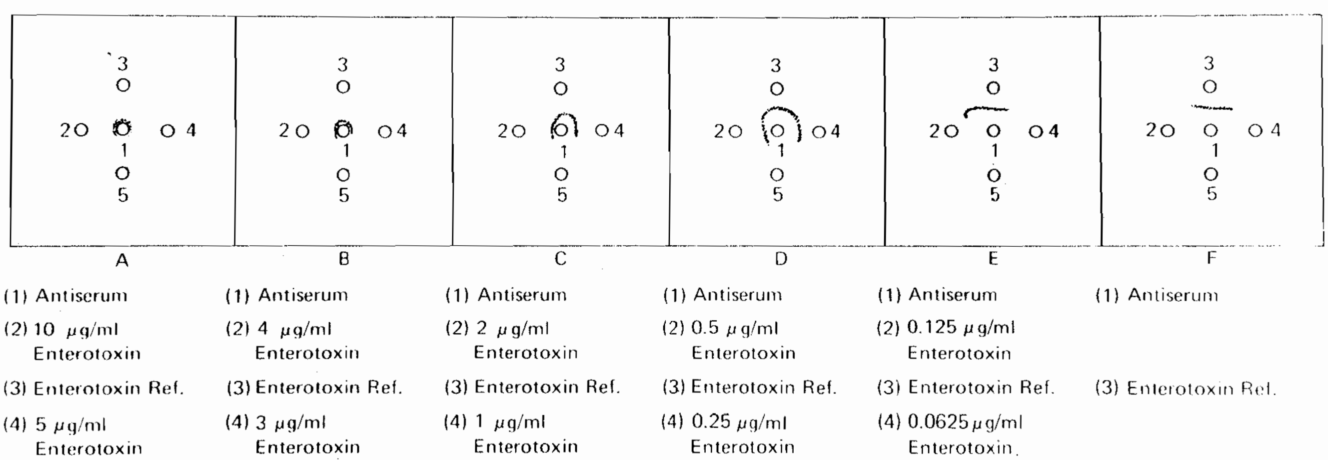



**FIG. 976.31D—Effect of amount of enterotoxin in test preparation on development of reference line of precipitation.** ***See*** **976.31H for explanation of reactions**

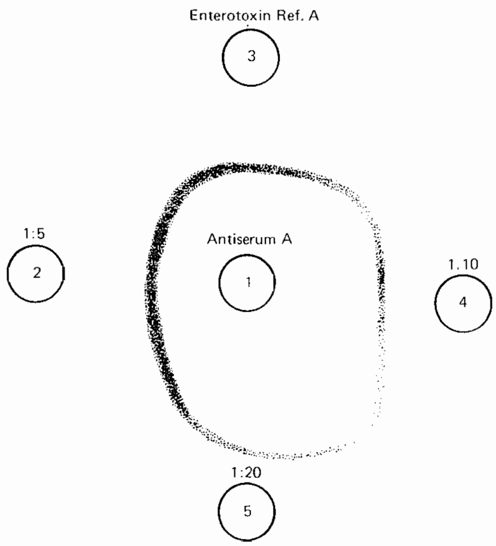



**FIG. 976.31E—Appearance of microslide gel diffusion test as monovalent system**

gel diffusion test as bivalent detection system. (*See* reactant arrangement in Fig. **976.31B**(*1*).) In ppt pattern **976.31F**(*1*), test prepn in well 4 produced atypical reaction indicated by nonspecific line of pptn (lines of nonidentity with enterotoxin refs A and B), which intersects enterotoxin ref. lines. In ppt pattern **976.31F**(*2*), both test prepns (wells 2 and 4) are neg. for enterotoxins A and B but produce nonspecific lines of pptn which intersect enterotoxin A and B ref. lines of pptn.

### *I. Slide and Template Recovery*

To recover slides for reuse, clean without removing tape. Rinse slides with tap $H_2O$ to remove agar gel, boil 3–5 min in tap $H_2O$ contg mild detergent, rinse in tap $H_2O$ and then in distd $H_2O$, immerse momentarily in alcohol, and wipe dry with cheesecloth. Wash templates with hot (not boiling) $H_2O$ contg moderately strong detergent, using cheesecloth to remove silicone film. Rinse templates with tap $H_2O$, distd $H_2O$, and alcohol; dry with cheesecloth, and tap alcohol out of wells. In cleaning plastic templates, avoid exposure to excessive heat or plastic-dissolving solvs. Templates and especially wells must be dry before reuse.

Ref.: JAOAC **59,** 594(1976).

## 980.32 Staphylococcal Enterotoxin in Foods

### Extraction and Separation Methods

**First Action 1980**
**Final Action 1981**

### *A. Apparatus*

(**a**) *Centrifuge*.—High-speed, preferably refrigerated, with 285 mL stainless steel bottles, or equiv.

(**b**) *Dialysis sac*.—1.25 in. (32 mm) flat width tubing, av. pore diam. 4.8 μm. Cut piece of tubing long enough to accommodate vol. of food to be extd. Soak tubing in 2 changes of $H_2O$ to remove glycerol coating. Tie 1 end with 2 knots close together. Test for leaks by filling sac with $H_2O$ and squeezing, while untied end is held tightly with fingers. Empty sac and place in $H_2O$ until ready for use.

(**c**) *Chromatographic columns*.—400 × 20 (id) mm, with stopcock (or use rubber tube attachment with finger clamp), packed with carboxymethyl cellulose (CMC), Whatman CM 22, 0.6 meq/g, or equiv. Pack as follows: Suspend 1 g CMC in 100 mL 0.005*M* Na phosphate buffer, pH 5.7, in 250 mL beaker, and adjust to pH 5.7 with 0.005*M* $H_3PO_4$. Stir intermittently 15 min, recheck pH, and adjust, if necessary. Pour suspension into tube containing plug of glass wool, and let settle. Withdraw liq. from column to within ca 25 mm of surface of settled CMC in column. Place loosely packed plug of glass wool on column. Pass 0.005*M* Na phosphate buffer, pH 5.7, thru column until washing is clear and pH is 5.7 (150–200 mL). Leave enough buffer in column to cover glass wool to prevent column from drying out.

(**d**) *Reservoir*.—Attach ca 60 cm latex tubing to stem of separator of appropriate size and attach other end of tube to piece of glass tubing inserted thru No. 3 rubber stopper to fit chromatgc column. Suspend separator from ring stand above chromatgc tube.

### *B. Reagents*

(**a**) *Polyethylene glycol (PEG) soln*.—30%. *See* **974.38B**(**f**).

(**b**) *Sodium phosphate buffer solns*.—(*1*) *pH 5.7, 0.2M*.—Add 0.2 *M* $NaH_2PO_4$ (27.60 g $1H_2O$/L) to 0.2*M* $Na_2HPO_4$ (53.61 g $7H_2O$/L) to pH 5.7. (*2*) *pH 5.7, 0.005M*.—Dil. 0.2*M*, pH 5.7, buffer with $H_2O$ (1 + 39). Adjust to pH 5.7 with 0.005*M* $H_3PO_4$. (*3*) *pH 6.4, 0.2M*.—Add 0.2*M* $Na_2HPO_4$ to 0.2*M* $NaH_2PO_4$ to pH 6.4. (*4*) *pH 6.5, 0.05M Na phosphate-NaCl*.—Add NaCl (11.69 g/L) to pH 6.4, 0.2*M* soln to give 0.2*M* NaCl (pH is ca 6.3). Dil. with $H_2O$ (1 + 3) and adjust to pH 6.5 with 0.05*M* $H_3PO_4$ or 0.05*M* $Na_2HPO_4$.

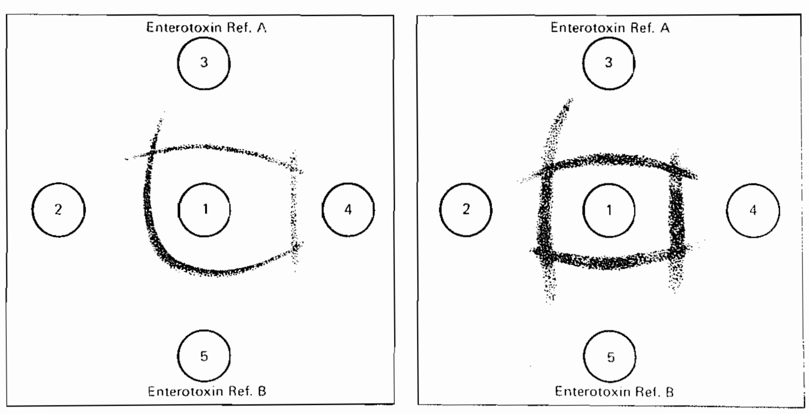



**FIG. 976.31F—Precipitate patterns in microslide gel diffusion test demonstrating nonspecific (atypical) lines of precipitation**

### C. Extraction of Toxin

Homogenize 100 g sample in 500 mL (or 20 g with 100 mL) 0.2 *M* NaCl 3 min in high-speed blender to very fine consistency. Adjust to pH 7.5 with 1*N* NaOH or HCl if food is highly buffered or with 0.1*N* if weakly buffered. Let stand 10–15 min, recheck pH, and readjust to pH 7.5, if necessary. Transfer homogenate to two 285 mL stainless steel bottles and centrf. 20–30 min at 27,300 × *g* at 5° in refrigerated centrf. (Lower speeds for longer times may be used.) If refrigerated centrf. is not available, centrf. at room temp., but chill supernate 1 h at 4° before filtering.

Decant supernate into beaker thru fine mesh screen (or other filtering material (e.g., miracloth) placed in funnel). Re-homogenize solids left in centrf. bottles with 125 mL (for 100 g sample; 25 mL for 20 g) 0.2 *M* NaCl as above. Centrf., filter and combine filtrate with original supernate.

### D. Purification of Toxin

Place combined exts in dialysis sac, immerse sac in 30% PEG soln and let conc. at 5° to ≤15 mL. Remove sac from soln and wash outside thoroly with tap $H_2O$. Soak sac in distd $H_2O$ 1–2 min and let stand in 0.2*M* NaCl few min. Pour contents of sac into 50 mL beaker. Rinse inside of sac with 2–3 mL portions 0.2*M* NaCl by running fingers up and down outside of sac to remove material adhering to insides. Add rinsings to beaker. Repeat rinsings until clear, keeping vol. at min.

Quant. transfer ext to separator, add $^1/_4$–$^1/_2$ vol. $CHCl_3$, and shake vigorously 10 times thru arc of 90°. Centrf. 10 min at 32,800 × *g* at 5°. Return mixt. to separator. Slowly drain lower $CHCl_3$ layer and discard. Repeat extn at least once (twice with high protein foods). After final extn, measure vol. of aq. phase, and dil. with 40 vols pH 5.7, 0.005*M* Na phosphate buffer. Adjust pH to 5.7 with 0.005*M* $H_3PO_4$ or $Na_2HPO_4$. Place adjusted soln in separator large enough to accommodate vol. for percolation thru CMC column.

Place stopper (attached thru tubing to separator) loosely into top of chromatgc column and slowly fill tube nearly to top with dild ext from separator. Tighten stopper in tube and open stopcock of separator. Let liq. percolate thru column at 5° at 1–2 mL/min by adjusting flow rate with stopcock at bottom of tube. Stop flow when liq. reaches top of glass wool layer. (If liq. has passed, rehydrate column with 25 mL $H_2O$.)

Wash column with 100 mL pH 5.7 0.005*M* Na phosphate buffer at same flow rate, stopping flow when liq. level reaches top of glass wool. Discard wash.

Elute enterotoxin from CMC column with 200 mL pH 6.5 0.05*M* Na phosphate-NaCl buffer at rate of 1–2 mL/min at room temp. Force last of liq. from column by applying air pressure to top of tube.

### E. Concentration of Toxin

Place eluate in dialysis sac. Place sac in 30% PEG at 5° and conc. to almost dryness. Remove sac and wash thoroly with tap $H_2O$. Soak sac in pH 6.5, 0.05*M* phosphate-NaCl buffer, and remove conc. from sac by rinsing with five 2–3 mL portions pH 6.5, 0.05*M* phosphate-NaCl buffer.

Transfer soln to separator, add $^1/_4$–$^1/_2$ vol. $CHCl_3$, and shake vigorously 10 times thru arc of 90°. Centrf. 10 min at 32,800 × *g* at 5°. Return mixt. to separator. Slowly drain lower $CHCl_3$ layer and discard.

Place ext in short dialysis sac (ca 16 cm). Place sac in 30% PEG and let stand until all liq. has been removed from inside of sac. Remove sac from soln and wash outside thoroly with tap $H_2O$. Place sac in distd $H_2O$ 1–2 min. Remove contents of sac by rinsing inside with 1 mL portions distd $H_2O$ until rinse is clear, keeping vol. to min. Place rinsings in 18 × 100 mm test tube or other container (e.g., 2–3 dram vial), and freeze-dry. Dissolve freeze-dried sample in as small vol. saline soln. **974.38B(e)** as possible (0.15–0.1 mL).

Det presence of enterotoxin as in **976.31**.

Ref.: JAOAC **63,** 1205(1980).

## STERILITY (COMMERCIAL) OF FOODS (CANNED, LOW ACID)

### 972.44 Microbiological Method

**First Action 1972**
**Final Action 1978**

(Personnel with beards, mustaches, or sideburns below ear lobe should not perform sterility examination unless these are completely covered with sterile caps and masks. Wear clean laboratory coat for examination.)

### A. Principle

"Low acid foods" means any food with finished equilibrium pH value >4.6. Method applies only to containers which show no distention of either end. Incubate containers ≥10 days at 21–35° before examination.

Com. sterility is defined as that condition achieved by application of heat which renders food free of viable forms of microorganisms having public health significance, as well as microorganisms not of health significance capable of reproducing in the food under normal non-refrigerated conditions of storage and distribution.

### B. Media and Reagents

*See also* **966.23A**.

(**a**) *Tryptone broth*.—(Aerobic medium.) Dissolve 10.0 g tryptone or trypticase, 5.0 g glucose, 1.25 g $K_2HPO_4$, 1.0 g yeast ext, and 2.0 mL 2% alc. soln of bromocresol purple in 1 L $H_2O$ with gentle heat, if necessary. Dispense 10 mL portions into 20 × 150 mm screw-cap test tubes and autoclave 20 min at 121°. Do not exhaust before using.

(**b**) *Modified PE-2 medium*.— (Anaerobic medium.) Dissolve 20.0 g peptone, 3.0 g yeast ext, and 2.0 mL 2% alc. soln of bromocresol purple in 1 L $H_2O$ with gentle heat, if necessary. Dispense 19 mL portions into 20 × 150 mm screw-cap test tubes contg 8–10 *untreated* Alaska seed peas (Rogers Brothers Co., Seed Div., PO Box 2188, Idaho Falls, ID 83401, No. 423; or hardware store). Autoclave 30 min at 121°. If not freshly prepd, heat to 100° and cool to 55° before using.

(**c**) *Glucose starch agar*.— (Aerobic medium.) Dissolve 15.0 g proteose peptone No. 3, 2.0 g glucose, 10.0 g sol. starch, 5.0 g NaCl, 3.0 g $Na_2HPO_4$, 20.0 g gelatin, and 10.0 g agar in 1 L $H_2O$, heat to bp, and autoclave 15 min at 121° in erlenmeyer. Aseptically pour into sterile petri dishes and allow to solidify.

(**d**) *Nutrient agar*.—(Aerobic medium for spore production; Difco dehydrated, or equiv.) Dissolve 3.0 g beef ext, 5.0 g peptone, and 15.0 g agar in 1 L $H_2O$, heat to bp, and autoclave 30 min at 121°.

(**e**) *Detergent sanitizer soln*.—pHisoHex (3% hexachlorophene), or equiv.

### C. Apparatus

(**a**) *Can opener*.—Bacti-Disc Cutter (Wilkens-Anderson Co., 4525 W Division St, Chicago, IL 60651, No. 10810-01), bacteriological can opener (Marmora Machine Co., 1956 N Latrobe Ave, Chicago, IL 60639), or equiv.

(**b**) *Caps*.—Disposable, operating room-type (Baxter Hospital Supply Div., 1450 Waukegan Rd, McGaw Park, IL 60085, or equiv.).

(**c**) *Pipets.*—Straight wall, 200–250 mm long × 7 mm id, 9 mm od (Scientific Products, Inc., No. G6100–9, cut and fire polished, or equiv.).

***D. Sampling***

Conduct test in clean room. (If necessary, open room may be used but outside windows must be closed and direct drafts across work area must be eliminated.) If available, use laminar flow cabinet. Strip labels from cans, examine cans for external defects, and record descriptions. Wash cans with soap (or detergent sanitizer soln) and $H_2O$, and dry with clean paper towels. Wipe counter top with 100 ppm Cl soln (e.g., Clorox or dild NaOCl soln) immediately before placing washed and dried can on it. Place code end of can in down position and number cans in ink or with $CuSO_4$ marking soln to right of side seam.

Wash hands and face with soap, and rewash hands and face with detergent sanitizer soln. Completely cover hair with clean disposable operating room cap.

Hold noncoded end of can over large Meker burner, just above blue portion of flame. Heat this end of can until all condensation is evapd; then return can to table in former position. Clean handle and blade of special can opener, (**a**), with paper towel moistened with 70% alcohol, flame metal portion enough to destroy all microorganisms, and use it to make 4 cm (1.5″) diam. hole in noncoded, heated end of can. Immediately and without moving can, use straight-wall sterile glass pipet, (**c**), to transfer ca 2 g food to sep. tubes, 2 each of aerobic and 2 of anaerobic media (4 total). (No other transferring tool may be substituted.) Preloosen screw cap and hold it between little and ring fingers while transfer is being made. Flame lips of media tubes both before and after addn of food. When transferring food to anaerobic tubes, food must be inoculated into lower portion of medium. Tighten screw caps after inoculation, incubate tubes 72 hr at 35°, and observe daily. Record results for each tube sep.

Remove addnl ≥10 g food sample from each container with sterile pipet and place in sterile 25 × 200 mm screw-cap test tube. Use pipet-like spatula, if necessary, for this operation (thermophilic contamination unlikely). Number tube to correspond to can and refrigerate for later testing, if necessary.

***E. Contamination Control***

Use sterile loop or glass rod to streak plate of glucose starch agar, (**c**). On table, open plate of glucose starch agar for time equal to longest duration that any medium tube or plate is exposed. Incubate plates 72 hr at 35°, and observe daily.

***F. Microscopic Examination***

With pair of metal cutting shears, enlarge hole in can and record odor. Microscopically (oil immersion) examine heat-fixed thin smear of food, stained 10 sec with 1% gentian (or crystal) violet and washed in running tap $H_2O$, or, alternatively, examine wet mounts with phase contrast microscope. If food contains appreciable fat, xylol should be dripped across food smear while it is still hot from heat fixing. Compare stained smear with one made from normal product, if possible.

***G. pH Determination***

Det. pH with pH meter, using ref. buffer near normal pH of food. Record both ref. buffer pH and sample pH. Compare to normal can of food, if available.

***H. Confirmation of Results***

If there is any abnormal odor, abnormal appearance, abnormal pH, numbers of bacteria on microscopic examination, and/or growth in media from any can of food, subculture corresponding refrigerated tube as follows: Flame lip of tube and, with straightwall sterile glass pipet, (**c**), transfer ca 2 g food to 2 tubes each of aerobic and anaerobic media (4 total). Flame lips of media tubes both before and after addn of food. Tighten caps after inoculation, incubate tubes 72 hr at 55°, and observe daily. Record results for each tube sep.

Any organisms isolated from normal cans having obvious vac. which produce gas in anaerobic medium at 35° should immediately be suspected as being from laboratory contamination. Aseptically inoculate growing organism into another normal can, close hole with solder, and incubate 14 days at 35°. Any swelling of container indicates that organism was not in original sample. Record as laboratory contamination and review results of addnl cans to verify finding of contamination.

Growth in aerobic medium at 35° from normal cans indicates either non-com. sterility or laboratory contamination. Unless there is abnormal odor, abnormal appearance, abnormal pH, and/or numbers of bacteria on microscopic examination from product in original can, record results as laboratory contamination and review results of addnl cans to verify finding of contamination. Otherwise, observe subculture results at 55°. Growth at 35° and absence of growth at 55° confirm nonsterility of original container. Check growth under aerobic conditions on nutrient agar plates, (**d**), at 55° and confirm for spores after 72 hr. Confirmation indicates nonsterility due to flat sour spoilage. Record growth at 55° under anaerobic conditions with gas production as com. sterile. Growth is caused by dormant spores incapable of growth at normal temps of storage and distribution.

If only one of duplicate tubes is pos. after incubation and streaked glucose starch agar is also neg., record as laboratory contamination. Growth on air control plate of glucose starch agar also indicates potential laboratory contamination.

Ref.: JAOAC **55**, 613(1972).

## 984.36 Microleak Detection in Low-Acid Canned Food Containers

**Helium Leak Test**

**First Action 1984**
**Final Action 1987**

***A. Principle***

(This test does not detect bacterial contamination.)

He is inert gas with small MW that can be forced through micron size openings and be easily detected by gas chromatgc analysis. After can is pierced aseptically, and sample is taken for microbiological analysis, can is sealed with rubber disc and subjected to He at 45 psi for 30 min. Headspace sample is then taken and analyzed for He.

***B. Apparatus***

(**a**) *Gas chromatograph.*—Instrument capable of sepg He from N, O, H, and $CO_2$ as described, or equiv., with strip chart recorder, gas partitioner (Model 1200, Fisher Scientific) with dual thermal conductivity cells and dual in-line columns. Column 1: 6½ ft × ⅛ in. Al packed with 80–100 mesh Columpak™ PQ. Column 2: 11 ft × 3/16 in. Al packed with 60–80 mesh Molecular Sieve 13×.

*Operating conditions:* column temperature 75°; attenuation 128; Ar carrier gas inlet pressure 40 psi, flow rate 26 mL/min thru gas partitioner; bridge current 125 mA; column mode 1 & 2; temperature mode, column; injector temp., off.

(**b**) *Puncturing press* (*Fig. **984.36***).—Made from drill press for electric hand drill with internal spring reversed to push head

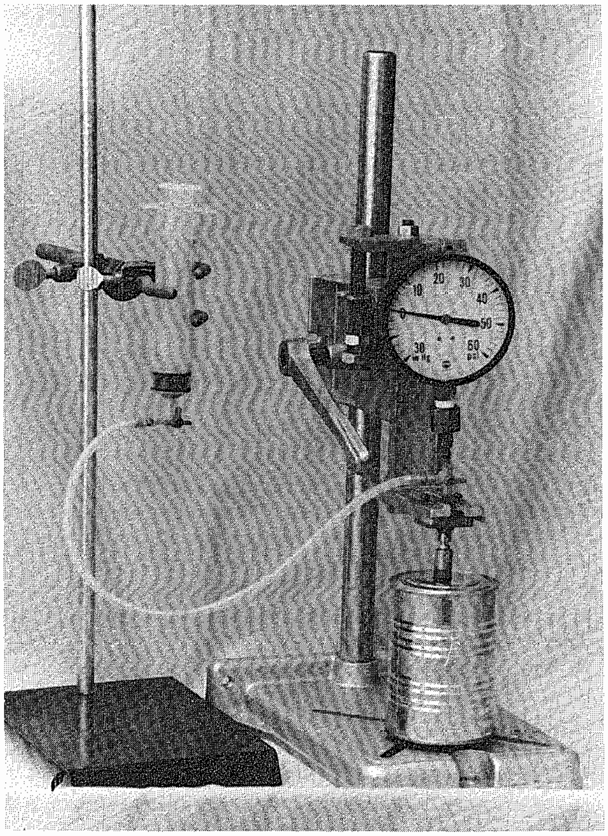


FIG. 984.36—Puncturing press

down. Metal valve, 3-way (stopcock No. 3161 Becton-Dickinson & Co., Stanley St, Rutherford, NJ 07070); vac. pressure gage, 30 in. Hg/0-60 psi, $2\frac{1}{2}$ in. face (Ametek, US Gauge Div., PO Box 152, Sellerville, PA 18960). Stainless steel piercer $1\frac{1}{2}$ in. × $\frac{1}{2}$ in. (machined in local machine shop) with No. 2 taper in piercer top, beveled $\frac{1}{8}$ in. × $\frac{3}{16}$ in. at bottom. $\frac{1}{4}$ in. × $\frac{1}{2}$ in. silicone rubber gasket around beveled $\frac{1}{8}$ in. piercer to maintain seal.

(**c**) *Helium exposure tank*.—ASME paint tank, 10 gal., tested to 100 psi, equipped with inlet and outlet microcontrol valves (Harrison Rubber and Supply Co., Court and Race Sts, Cincinnati, OH 45202).

(**d**) *Pressurized helium tank*.—With 2 stage regulator.

(**e**) *Timer and solenoid*.—To automate release of He from exposure tank.

(**f**) *Helium gas standards*.—Scott Specialty Gasses, 2330 Hamilton Blvd, South Plainfield, NJ 07090.

(**g**) *Cyanoacrylate glue*.—SuperGlue (3M, AC&S Div., 3M Center, St Paul, MN 55144-1000, or equiv.).

(**h**) *Can opener*.—Bacteriological, **972.44C(a)**.

(**i**) *Rubber discs*.—$2\frac{3}{8}$ in. × $\frac{1}{8}$ in. and 70 durometer (Netherland Rubber Co., 629 Burbank, Cincinnati, OH 45206).

### C. Calibration Test Procedure

For gas chromatographs equipped with side port loop (0.5 mL), inject 5.0 mL calibrated He stds (suggested range of 5, 15, 25, 50, and 75% He). For instruments not equipped with side port loop, inject appropriate vol. of stds. Use same vol. for analysis of headspace gas samples. Plot percent He vs He peak ht at attenuation used. Depending on qual. of instrument, plot should approximate a straight line.

Check gage on can piercer against known pressure and vac. Test resealing procedure, **984.36E**, on control cans.

### D. Helium Exposure Tank

Control introduction rate of He into exposure tank, and time cans are exposed to He pressure at 45 ± 2 psi. Timer, solenoid, and microvalves with vernier scales can facilitate procedure. Connect He source to exposure tank. Turn timer on to close outlet solenoid valve. Approx. 15–20 min are needed to reach 45 psi in tank. Make minor adjustments if necessary. Adjust timer to expose cans to He pressure at 45 psi for 30 min (30 min exposure period is in addition to time necessary to reach 45 psi.). Tank pressure should be reduced to 0 psi within 5–10 min.

### E. Preparation of Can for Helium Test

If sample is to be taken for microbiological testing, proceed as in **972.44D**.

For nonsterile opening of can, use opener, **972.44C(a)** to cut $1\frac{1}{2}$ in. hole in can lid. Remove and discard portion of contents.

Push down any sharp metal projections around 1.5 in. hole. Wipe lid dry and lightly sand area where rubber disc will be inserted. Pool cyanoacrylate glue around surface covered by edge of rubber disc. Place disc over hole and smooth edges with fingers to remove air bubbles. Place wt (>500 g) on disc ≥1 h.

### F. Collection and Analysis of Headspace Gas

Can piercing assembly is shown in Fig. **984.36**. Before piercing can, close gage valve and pull plunger on syringe to remove air from silicone tubing. Close syringe valve and expel air from syringe. Puncture can and open gage valve to read vac. or pressure. Turn gage valve and syringe valve to release gas into syringe. If gas sample is >5.0 mL, withdraw this amt (as shown in Fig. **984.36**) and inject into port of gas chromatograph. If gas sample is <5.0 mL, force collected gas back into can. Close syringe valve to retain gas in tubing and can. Use syringe to add 40 mL room air to can, and pump syringe twice to mix gas. Let syringe equilibrate to atm. pressure and record syringe vol. From this dil. gas, sample may be obtained for gas chromatograph. Percent He measured should be divided by diln factor to obtain correct percent He in headspace gas. Use following formula to det. diln factor:

$$\text{Diln factor} = (\text{equilibrated syringe vol.} - 40\text{ mL air} + \text{headspace vol.})/(\text{equilibrated syringe vol.} + \text{headspace vol.})$$

For example: $(43-40+9)/(43+9) = 12/52 = 0.23$ diln factor.

$$\%\text{ He in can} = \%\text{ He measured/diln factor}$$

For example: 5% He/0.23 = 22% He in can.

Headspace vol. may be measured by piercing control can that still has vac. Assume sample and control can same volume. Measure amt of vac. (in. Hg) and vol. air pulled in from syringe.

$$\text{Headspace vol.} = \text{measured vol. from syringe} \times 30\text{ in Hg/measured vac. in can (in. Hg)}$$

For example, if 6 mL air is pulled into can and vac. is 20 in. Hg, then,

$$\text{Headspace vol.} = 6\text{ mL} \times 30\text{ in Hg}/20\text{ in. Hg} = 9\text{ mL}$$

To perform addnl work on can, collected gas may be stored in capped syringe 2–3 h without appreciable change in its composition.

### G. Interpretation of Results

Report can as leaker if, after exposure to pressurized He, can internal pressure is ≥8 psi or percentage He is ≥1%. Report can as nonleaker if, after exposure to pressurized He, can internal vac. is ≥5 in., or percentage He is <1%.

Ref.: JAOAC **67,** 942(1984).

## 972.45 Thermophilic Bacterial Spores in Sugars

**Microbiological Method**

**First Action 1972**
**Final Action 1989**

(Sugar, both beet and cane, may carry spores of all 3 groups of thermophilic bacteria that are important as spoilage agents in low-acid canned foods, i.e., flat sour bacteria (*Bacillus stearothermophilus*), thermophilic anaerobes not producing $H_2S$ (*Clostridium thermosaccharolyticum*), and sulfide spoilage bacteria or thermophilic anaerobes producing $H_2S$ (*C. nigrificans*). These bacteria are not of health significance, but excessive numbers may survive com. heat processes.)

### A. Sampling

Take 225 g (0.5 lb) samples from 5 sep. bags or barrels of shipment or lot, place in clean containers, and seal.

Sample liq. sugar by drawing 5 sep. 200–250 mL (6–8 oz) portions during pumping transfer from tank trucks to storage tanks or at refinery during filling of tank trucks.

Number of samples will vary in relation to size of shipment or lot. If there is significant variability in lot, this fact will become evident, in majority of cases, thru individual tests on the 5 samples.

### B. Preparation of Sample

(**a**) *Dry sugar*.—Place 20 g sample in sterile 150–250 mL erlenmeyer marked to indicate 100 mL. Add sterile $H_2O$ to 100 mL mark. Bring rapidly to bp, and boil 5 min. Replace liq. evapd with sterile $H_2O$.

(**b**) *Liquid sugar*.—Add sample contg 20 g dry sugar, detd on basis of °Brix (e.g., 29.41 g 68° Brix (%) liq. sugar is equiv. to 20 g dry sugar), to sterile 250 mL flask and proceed as in **972.45D(a)**.

### C. Culture Media

(**a**) *Glucose tryptone agar*.—For detection of flat sour bacteria. Use com. stdzd dehydrated medium (Bacto-Dextrose Tryptone Agar) preferably, or prep. as follows: Suspend 10.0 g tryptone, 5.0 g glucose, 15.0 g agar, and 0.04 g bromocresol purple in 1 L $H_2O$, and mix thoroly. Final pH, 6.7 ± 0.1. Autoclave 30 min at 121° and cool to 55°.

(**b**) *Liver broth*.—For detection of thermophilic anaerobes not producing $H_2S$ (*C. thermosaccharolyticum*). Mix 500 g chopped beef liver with 1 L $H_2O$. Slowly boil mixt. 1 hr, adjust to ca pH 7.0, and boil addnl 10 min. Press boiled material thru cheesecloth and dil. liq. to 1 L. To broth, add 10.0 g peptone and 1.0 g $K_2HPO_4$, and adjust to pH 7.0. To test tube, add 1–2 cm previously boiled ground beef liver and 10–12 mL broth. Sterilize 20 min at 121°. Before using medium, unless freshly prepd, exhaust by subjecting to flowing steam ≥20 min, and, after inoculation, stratify with 5–6 cm layer of plain nutrient agar (common formula) that has been cooled to 50°.

(**c**) *Sulfite agar, modified*.—For detection of sulfide spoilage bacteria. Suspend 10.0 g tryptone, 1.0 g $Na_2SO_3$, and 20.0 g agar in 1 L $H_2O$, and mix thoroly. At time agar is added to tube, place clean iron strip or nail in each tube. No adjustment of reaction is necessary. Prep. medium and $Na_2SO_3$ soln, if used in place of solid $Na_2SO_3$, fresh weekly. Autoclave medium 20 min at 121° and cool to 55°.

### D. Culture Technic

(**a**) *Flat sour spores*.—Into 5 sep. petri dishes, pipet 2 mL boiled sugar soln. Cover, and mix inoculum with glucose tryptone agar. Incubate plates 35–48 hr at 55° and, to prevent drying of agar, humidify incubator. Combined count from 5 plates represents number of spores in 2 g original sugar. Multiply this count by 5 to express results in terms of number of spores/10 g sugar.

Characteristic colonies are round, 1–5 mm in diam., with typical opaque central "spot," and, usually, surrounded by yellow halo in field of purple. This halo may be insignificant or missing with certain low acid-producing types or if plate is so thickly seeded that entire plate has yellow tinge. Typical subsurface colonies are compact and may approach "pin point" conditions.

If identity of subsurface colonies is doubtful, observe nature of surface colonies. If they show reasonable purity of formed flora, assume that subsurface colonies have been formed by similar bacterial groups. If plate is heavily seeded, counts may not be accurate and colony structure and size may be atypical. If plates are so heavily seeded that counting is impractical, dil. original soln and repeat procedure.

To det. if typical subsurface colonies are flat sour organisms, apply streak from colonies to agar plates to det. surface characteristics.

(**b**) *Thermophilic anaerobes not producing hydrogen sulfide*.—Divide 20 mL boiled sugar soln equally among 6 liver broth tubes and stratify liq. medium with plain nutrient agar. After agar has solidified, preheat to 55° and incubate 72 hr at that temp.

Thermophilic anaerobes not producing $H_2S$ are identified by splitting of agar, presence of acid, and, occasionally, cheesy odor. Method is suitable as qual. test but provides only rough estn; results cannot be expressed as number of spores/unit wt sugar.

(**c**) *Sulfide spoilage bacteria*.—Divide 20 mL boiled sugar soln equally among 6 freshly exhausted tubes contg modified sulfite agar. Incubate 48 hr at 55°.

In sulfite agar, sulfide spoilage bacteria form characteristic blackened spherical areas. Due to solubility of $H_2S$ and its fixation by Fe, no gas is noted. Some thermophilic anaerobes not producing $H_2S$ generate relatively large amts of H, which splits agar and reduces sulfite, thereby causing general blackening of medium. This condition, however, is readily distinguishable from restricted blackened area mentioned above. Count blackened areas to obtain quant. results.

### E. Reporting Results

Report flat sour and sulfide spoilage results as number of spores/10 g sugar. Report thermophilic anaerobes not producing $H_2S$ as number of tubes pos. or neg. (+ or −).

Refs.: JAOAC **19,** 438(1936); **21,** 457(1938); **55,** 445(1972).

## 985.41 Sporeformers in Low-Acid Canned Foods

**Gas Chromatographic Method**

**First Action 1985**
**Final Action 1989**

### A. Principle

Two org. compds produced by sporeforming organisms in low-acid canned foods, D-(−)-2,3-butanediol (BD) and butyric acid (BA), but not produced by nonsporeformers, are measured by gas chromatgy. Identification of BD and BA is based on relative retention times (RRTs) to internal std, propionic acid. Identification of sporeforming organisms as cause of spoilage is based on ratio of peak hts for BD and butyric acid (in external std) and BA and butyric acid (in external std).

### B. Apparatus

(**a**) *Gas chromatograph.*—Suitable for use with 2 heated flash vaporizer injectors contg glass sleeves; equipped with flame ionization detectors (FID); linked to data processor (if available) with printer/plotter (Sigma Series Instrument with console and printer/plotter, Perkin-Elmer, or equiv.).

(**b**) *Gas chromatographic columns.*—*(1)* 1.8 m (6 ft) × 2 mm id glass column packed with 15% SP 1220/1% $H_2PO_4$ on 100–120 mesh Chromosorb W(AW); *(2)* 1.8 m × 2 mm (id) glass column packed with 0.3% CW 20M/0.1% $H_3PO_4$ on 60–80 mesh Carbopack C (Supelco Inc.). Condition both columns, using recommended procedures: purge column at ambient temp. ≥30 min with carrier gas at 20 mL/min; then program from 50° to 150° at 2°/min and hold overnight. Cool column 1, attach to detector, and set column temp. at 118°. Inject twenty 10 μL portions of freshly boiled $H_2O$ on column 2 at 150°. Then cool, attach to detector, and set column temp. at 125°.

(**c**) *Operating conditions.*—Select 2 methods from available chromatgc data systems software (if data processor is available) that provide relative retention time (RRT) = 1.00 for internal std. Column 1: injector 200°, detector 240°, column 118° for 12 min isothermal run; He carrier flow rate 24 mL/min; propionic acid elution time 2.70–3.30 min; electrometer range 10, attenuation 4; chart speed 5 mm/min; injection vol. 1 μL. For butyric acid, retention time = ca 6 min, RRT = 1.7, and peak ht = 60% FSD (10 cm). Column 2: injector 200°, detector 240°, column 125° for 25 min isothermal run; N carrier flow rate 15 mL/min; propionic acid elution time 2.25–2.45 min; electrometer range 10, attenuation 2; chart speed 10 mm/min; injection vol. 0.3 μL. For butyric acid, retention time = ca 6.5 min, RRT = 2.7, and peak ht = 60% FSD (10 cm). Theoretical plates for each column ≥1600.

(**d**) *Syringes.*—1 and 5 μL (Hamilton 7001-N and 1705-N, or equiv.).

(**e**) *Centrifuge.*—With adapters suitable to accept 5 mL minivials, 1.0 and 5.0 mL capacity, with silicone stopper and screw cap (95010 and 95050, Alltech Associates, Inc., 2051 Waukegan Rd, Deerfield, IL 66015), or equiv.

(**f**) *Disposable Pasteur pipets.*—7760-F 30 series (Thomas Scientific), or equiv.

### C. Reagents

(**a**) *Water.*—Distd $H_2O$ that elutes with no detectable peaks on 2 columns used for assay.

(**b**) *External std solns.*—*(1)* For column 1, WSFA-2 (Supelco Inc.,), or equiv. It must contain aq. soln of propionic, isobutyric, and butyric acids. Concn of butyric acid should be 0.1%. Concns of propionic and isobutyric acids should be sufficient to produce peak hts ranging from detectable to 100% scale deflection (SD).

*(2)* For column 2, dil. WSFA-2 with equal vol. $H_2O$ or prep. equiv. soln. It must contain aq. soln of propionic and butyric acids. Concn of butyric acid should be 0.05%. Concn of propionic acid should be sufficient to produce peak ht ranging from detectable to 100% SD.

(**c**) *Internal std solns.*—*(1)* For column 1, dil. 5 μL reagent grade propionic acid to 5 mL with $H_2O$. For injection, take up 1 μL $H_2O$ into 5 μL syringe, followed by 0.1 μL soln. Modify proportions of 2 components, if necessary, to give ca 50% FSD for propionic acid peak eluting in 2–3 min. *(2)* For column 2, dil. protion of internal std for column 1 with equal vol. $H_2O$. For injection, take up 0.3 μL $H_2O$ into 1 μL syringe, followed by 0.02 μL of this diln. Modify proportions of 2 components, if necessary, to give ca 50% FSD for propionic acid peak eluting in 2–3 min.

### D. Assay

Pierce each can aseptically by any microbiologically acceptable technic (see **972.44C(a)**, (**c**) and **972.44D**). Transfer portion of liq. can contents to 5 mL mini-vial, using sterile Pasteur disposable pipet, or equiv. and store at ca −20° overnight or longer. Warm 5 mL vials to room temp. and then centrf. 15 min at 1000 × *g* or until phases sep. Transfer portion of clear upper phase to 1 mL mini-vial, using sterile Pasteur disposable pipet, or equiv. Store this upper phase at −20° overnight or until day of assay.

Let 1 mL mini-vials of upper phase of centrfd can contents warm to room temp. Use syringe to remove (and discard) any particulate matter from bottom of cone; then mix sample, using syringe. For assays on either column, assay external std to ensure optimal instrumental conditions; propionic acid must be eluted in time range such that RRT = 1.000; theoretical plates should be ca ≥1600; and butyric acid peak should be ca 60% FSD. Then replace glass liner in injector and inject sample of $H_2O$, using sample syringe. Repeat $H_2O$ injections, if necessary, until chromatogram has no ghost peaks. For assays on column 1, take up 1 μL portion of sample into 5 μL syringe, followed by 0.1 μL internal std soln 1 and inject into gas chromatograph. For assays on column 2, take up 0.3 μL portion of sample into 1 μL syringe followed by 0.02 μL internal std soln 2 and inject into gas chromatograph.

### E. Interpretation

For each sample, examine chromatograms from both columns, together with chromatograms for external std assayed same day. On column 1, BD elutes as tailing peak with retention time between those for isobutyric and butyric acids; it will not elute on column 2 in 25 min run. Sample contg BA gives peak eluting in both sample chromatograms in retention time range for butyric acid. Measure peak ht for BD peak and divide by peak ht for butyric acid in external std 1 assayed on column 1. Measure BA peak ht from column 2 and divide by peak ht for butyric acid in external std 2 assayed on column 2. Peak ht ratio for BD ≥0.39 or peak ht ratio for BA ≥0.30 indicates sporeformers as cause of spoilage. Record cause of spoilage as from either sporeformer or nonsporeformer.

Ref.: JAOAC **68,** 626(1985).

## CLOSTRIDIUM

## 977.26 *Clostridium botulinum* and Its Toxins in Foods

### Microbiological Method

**First Action 1977**
**Final Action 1979**

(*Caution:* See safety notes on pipets.)

### A. Principle

Mice injected intraperitoneally (IP) with food ext contg ≥1 min. lethal dose (MLD) of botulinum toxin die within 72 hr after exhibiting sequence of symptoms characteristic of botulinum intoxication. Homologous antitoxin will protect mice from symptoms while other antitoxins will not, thus detg serological type. Viable spores in food will grow in suitable culture medium and produce toxin, which is detected and typed.

### B. Apparatus

(**a**) *Can opener.—See* **972.44C(a)**.

(**b**) *Anaerobic jars.*—GasPak (BBL) or Case-nitrogen replacement.

(**c**) *Petri dishes.*—100 mm diam. Dry prepd plates ca 24 hr at 35° before streaking.

(**d**) *Centrifuge.*—High-speed, refrigerated.

(**e**) *Syringes.*—1.0 or 3.0 mL with 25 gage $^5/_8$" needles for inoculating mice.

### C. Media and Reagents

(**a**) *Cooked meat broth.*—Use either liver or heart medium. (*1*) *Chopped liver broth.*—Grind 500 g fresh beef liver into 800 mL $H_2O$. Heat to bp and simmer 1 hr. Cool, adjust to pH 7.0, and boil 10 min. Filter thru cheesecloth, pressing out excess liq. To broth add 10 g peptone, 1 g $K_2HPO_4$, and 1 g sol. starch. Adjust to pH 7.0 and dil. to 1 L with $H_2O$. Filter thru coarse paper. (If desired, broth and liver may be stored sep. in freezer for future use.) To 18 or 20 × 150 mm test tubes, add liver to ht of 1–2 cm and 10–12 mL liq. Autoclave 20 min at 121°. (*2*) *Cooked meat medium.*—Use com. medium of following formula: beef heart 454 g, proteose peptone 20 g, dextrose 2 g, and NaCl 5 g. Suspend 12.5 g medium in 100 mL cold $H_2O$. Mix thoroly and let stand until particles are thoroly wetted (ca 15 min). (Alternatively, add 1.25 g solid medium into test tubes, add 10 mL cold $H_2O$, and mix thoroly to wet all particles.) Autoclave 15 min at 121°. Final pH, 7.2 ± 0.1.

(**b**) *Trypticase-peptone-glucose-yeast extract broth with trypsin* (*TPGYT*).—Dissolve 50 g trypticase, 5 g Bacto-peptone, 20 g yeast ext, 4 g dextrose, and 1 g Na thioglycollate in 1 L $H_2O$, and dispense 15 mL portions into 20× 150 mm culture tubes or 100 mL portions into 6 fl oz prescription bottles. Autoclave 10 min (tubes) or 15 min (bottles) at 121°. Final pH, 7.0 ± 0.1. Refrigerate, and discard if not used within 2 weeks. Immediately before use, steam or boil 10–15 min to remove O, cool quickly, and aseptically add 1.0 mL trypsin soln/15 mL broth.

Prep. trypsin soln by dissolving 1.5 g trypsin (Difco 1:250) in 100 mL $H_2O$. Sterilize by filtering thru 0.45 μm Millipore or equiv. filter, and refrigerate.

(**c**) *Liver-veal-egg yolk agar or anaerobic egg yolk agar.*—(*1*) *Liver-veal-egg yolk agar* (*LVEY*).—Wash 2 or 3 eggs with stiff brush, and drain. Soak eggs in 0.1% $HgCl_2$ soln 1 hr. Drain $HgCl_2$ soln and replace with 70% alcohol, soaking 30 min. Remove eggs, crack aseptically, and discard whites. Remove yolk with syringe, place in sterile container, and add equal vol. sterile 0.85% NaCl soln. Mix thoroly. To each 500 mL prepd sterile com. dehydrated liver veal agar at 50°, add 40 mL egg yolk-NaCl suspension. Mix thoroly and pour plates. Dry plates 2 days at room temp. or 24 hr at 35°. Discard contaminated plates, and store sterile plates in refrigerator. (*2*) *Anaerobic egg agar.*—Dissolve 5 g yeast ext, 5 g tryptone, 20 g proteose peptone, 5 g NaCl, and 20 g agar in 1 L $H_2O$. Adjust to pH 7.0, dispense 500 mL into 1 L flask, and autoclave 20 min at 121°. To 500 mL melted agar at 45–50°, add 40 mL egg yolk-NaCl suspension, prepd as in (*1*). Mix, and pour plates immediately. Dry and store sterile plates as in (*1*).

(**d**) *Gel-phosphate buffer.*—pH 6.2. Dissolve 2 g gelatin and 4 g $Na_2HPO_4$ in 1 L $H_2O$ with gentle heat. Dispense into 100 mL milk diln bottle. Autoclave 20 min at 121°.

(**e**) *Clostridium botulinum antitoxin preparations.*—Types A thru F or polyvalent A–F. Available from Centers for Disease Control, CID, Office of Official Services, Atlanta, GA 30333.

### D. Preparation of Sample

(**a**) *Preliminary examination.*—Keep samples refrigerated. Unopened canned foods, unless badly swollen and in danger of bursting, need not be refrigerated. Record code and condition of container. Clean and identify container.

(**b**) *Solid foods.*—Aseptically transfer portion, with little or no free liq., to sterile mortar. Add equal amt sterile gel-phosphate buffer, (**d**), and grind with sterile pestle. Alternatively, inoculate small pieces of sample with sterile forceps directly into enrichment broth.

(**c**) *Liquid foods.*—Inoculate with sterile pipets directly into enrichment broth.

(**d**) *Canned foods.*—Prep., disinfect with alc. I soln, and open cans as in **972.44D**. If can has swelled, position can so vertical side seam is away from operator. If can has buckled ends, chill before opening, and flame cautiously to avoid bursting can.

(**e**) *Visual examination.*—Note appearance, odor, and any evidence of decomposition. DO NOT TASTE PRODUCT under any circumstances.

(**f**) *Reserve sample.*—After culturing, aseptically remove portion to sterile sample jars for further tests which may be needed later.

### E. Detection of Viable C. botulinum

(**a**) *Enrichment.*—Remove dissolved O from media before inoculation by steaming 10–15 min and cooling quickly without agitation. Inoculate 2 tubes of cooked meat broth, (**a**), with 1–2 g solid or 1–2 mL liq. food or ext/15 mL broth, introducing inoculum slowly beneath surface of broth. Incubate at 35°. Similarly inoculate 2 tubes of TPGYT broth, **977.26C(b)**, and incubate at 26°.

(**b**) *Examination.*—After 5 days, examine cultures for turbidity, gas production, digestion of meat particles, and odor. Also examine microscopically by wet mount under high power phase contrast or by bright field illumination of smear stained by Gram stain, crystal violet, or methylene blue. Observe morphology of organisms and note existence of typical clostridial cells, occurrence and relative extent of sporulation, and location of spores within cells.

(**c**) *Further treatment.*—Usually 5 day incubation produces active growth and highest concn of toxin, as well as peak sporulation. Retain culture in refrigerator for pure culture isolation. If there is no growth after 5 days, incubate addnl 10 days to detect possible delayed germination of *C. botulinum* spores before discarding culture as sterile.

### F. Isolation of Pure Cultures

If good sporulation has occurred, *C. botulinum* is more readily isolated from mixed flora in enrichment culture or from original sample.

(**a**) *Pretreatment.*—Add equal vol. filter-sterilized absolute alcohol to 1–2 mL culture or sample in sterile screw-cap tube. Mix well and incubate at room temp. 1 hr. Alternatively, heat 1–2 mL enrichment culture 10–15 min at 80° to destroy vegetative cells. (Do not use heat treatment for nonproteolytic type *C. botulinum.*)

(**b**) *Plating.*—With inoculating loop, streak 1 or 2 loopfuls of alcohol or heat-treated cultures, dild if necessary, to either or both liver-veal-egg yolk agar or anaerobic egg yolk agar dried plates in manner to obtain isolated colonies. Incubate plates ca 48 hr at 35° under anaerobic conditions of Case anaerobic jar or Gas-Pak systems, or equivs.

(**c**) *Selection of colonies.*—Typical colonies are raised or flat, smooth or rough, and commonly show some spreading and have irregular edge. On egg yolk media, colonies usually exhibit surface iridescence when examined by oblique light. This luster zone is referred to as "pearly layer." Zone usually extends beyond and follows irregular contour of colony. Besides pearly zone, colonies of types C, D, and E are ordinarily surrounded by wide (2–4 mm) zone of yellow ppt. Colonies of types A and B generally show smaller zone of pptn. Not all typical colonies will produce toxin. Some members of genus *Clostridium* have typical morphological characteristics but do not produce toxins.

(**d**) *Cultures*.—With sterile transfer loop, inoculate each of 10 selected colonies into tube of sterile medium: (*1*) TPGYT broth for *C. botulinum* Type E, incubating 5 days at 26°; and (*2*) cooked meat broth for other toxin types, incubating 5 days at 35°. Use cultures for confirmation as in (**e**) and for detection and identification of toxin as in **977.26G**.

(**e**) *Confirmation*.—Streak culture from (**d**) in duplicate on egg yolk agar plates, incubating 1 plate anaerobically and other plate aerobically at 35°. If colonies typical of *C. botulinum* are found on anaerobic plate and no growth is found on aerobic plate, culture may be pure. Failure to isolate *C. botulinum* from ≥1 of selected colonies may indicate that its population relative to mixed flora is low. Repeated serial transfers thru addnl enrichment steps, **977.26E(a)**, may increase numbers sufficiently to permit isolation. Store pure culture, (**d**), either under refrigeration, on glass beads, or lyophilized.

### G. Detection of Toxin

(**a**) *Preparation of sample*.—Ext solid foods with equal vol. gel-phosphate buffer, **977.26C(d)**, macerating with sterile, prechilled mortar and pestle. Centrf. ext and liq. foods contg suspended solids under refrigeration. Rinse empty containers suspected of having held toxic foods with few mL gel-phosphate buffer. Use min. vol. to avoid diln of toxin.

(**b**) *Trypsin treatment*.—Toxins of nonproteolytic types, if present, may need trypsin activation to be detected. Do not use trypsin treatment with TPGYT culture which already contains trypsin. Further treatment may degrade any fully activated toxin present in culture.

Adjust portion of food supernate, (**a**), liq. food, or cooked meat culture, if necessary, to pH 6.2 with 1*N* NaOH or HCl. Prep. satd trypsin soln by dispersing 1 g trypsin (Difco 1:250) in 10 mL $H_2O$ in clean culture tube. Mix 0.2 mL trypsin soln with 1.8 mL liq. to be tested. Incubate 1 hr at 37° with occasional gentle agitation.

(**c**) *Toxicity testing*.—Conduct each test in duplicate, *i.e.*, on trypsin treated and untreated materials. Dil. portions of untreated and treated food supernate, liq. food, or culture 1:2, 1:10, and 1:100, resp., with gel-phosphate buffer. Inject sep. pairs of mice, ca 15–20 g, IP with original and dild fluids, treated and untreated, using syringe, (**e**). Heat 1.5 mL original untreated fluid 10 min at 100° for control. Cool, and inject pair of mice each with 0.5 mL heated fluid. These mice should not die because botulinum toxin, if present, is inactivated by this heat treatment.

Observe mice periodically for 72 hr, recording symptoms and time of deaths. Typical symptoms of botulism usually begin within 24 hr with ruffling of fur, followed in sequence by labored breathing, weakness of limbs, and finally total paralysis with gasping for breath, followed by death due to respiratory failure. Death without symptoms of botulism is not sufficient evidence that injected material contained botulinum toxin. Deaths may occur from chems present in fluid or from trauma.

If after 72 hr, all but mice receiving heated prepn have died, repeat toxicity test, using higher dilns of fluids. It is necessary to have dilns that kill as well as dilns that do not kill to establish an end point or MLD (min. lethal dose) as est. of amt of toxin present. MLD is contained in highest diln killing both (or all) mice inoculated. Calc. MLD/mL.

### H. Typing of Toxin

Dil. monovalent antitoxins to types A, B, E, and F in 0.85% NaCl soln to concn of 1 International Unit/0.5 mL. Prep. enough dild antitoxin to inject 0.5 mL into each of 2 mice for each diln of prepn to be tested.

Use toxic prepn which gave greatest number of MLD, either treated or untreated. If untreated, same prepn can be used as was used for toxicity testing; if trypsinized prepn was most lethal, prep. freshly trypsinized fluid since continued action of trypsin may destroy toxin. Prep. dilns to cover range of at least 10, 100, and 1000 MLD below previously detd end point of toxicity.

Inject several groups of mice IP, each mouse receiving 0.5 mL of 1 of dild antitoxins, 30–60 min before challenging them with IP injection of toxic prepns.

Inject pairs of mice protected by specific monovalent antitoxin injection IP with each diln of toxic prepn. Also inject pair of unprotected mice (no injection of antitoxin) with each toxic diln as control. (This protocol requires 30 mice: 3 pairs for each of the 4 monovalent antitoxins (A, B, E, and F), each pair to receive challenge of 1 of the 3 dilns of toxic prepn ($2 \times 3 \times 4 = 24$) plus 1 pair of unprotected mice for each diln of toxic material as control ($2 \times 3 = 6$).

Observe mice 72 hr for symptoms of botulism and record time of deaths. If results indicate that toxin was not neutzd, repeat test, using monovalent antitoxins to types C and D, plus polyvalent antitoxin pool of types A thru F.

### I. Interpretation

Toxin in food means that product, if consumed without thoro heating, could cause botulism. Presence of toxin in food is required for botulism to occur. Viable *C. botulinum* but no toxin in food is not proof that food in question caused botulism. Ingested organisms may be found in alimentary tract, but are considered to be unable to multiply and produce toxin *in vivo*.

Presence of botulinum toxin and/or organisms in low-acid (pH >4.6) canned foods means that items were underprocessed or were contaminated thru post-processing leakage. Swollen cans are more likely than flat cans to contain botulinum toxin since organism produces gas during growth. Presence of toxin in flat can may imply that seams were loose enough to let gas escape. Toxin in canned foods is usually of type A or of proteolytic type B strain, since spores of proteolytics can be among more heat resistant bacterial spores. Spores of nonproteolytics, types B, E, and F, generally are of low heat resistance and would not normally survive even mild heat treatment.

Protection of mice from botulism and death with 1 of monovalent botulinum antitoxins confirms presence of botulinum toxin and dets serological type of toxin in sample.

If mice are not protected by 1 of monovalent antitoxins, there may be too much toxin in sample, there may be more than 1 kind of toxin present, or deaths may be due to some other cause. In such cases, retesting at higher dilns of test fluids is required and mixts of antitoxins must be used in place of monovalent antiserum. If mice are still not protected, some other toxic material, which is not heat labile, could be responsible if both heated and unheated fluids cause death. It is also possible that heat stable toxic substance could mask botulinum toxin.

Ref.: JAOAC **60,** 541(1977).

## 976.30 *Clostridium perfringens* in Foods

**Microbiological Method**

**First Action 1976**
**Final Action 1979**

(Applicable to examination of outbreak foods in which relatively small numbers of vegetative cells are expected to be present)

### *A. Apparatus*

(**a**) *Pipets*.—1.0 mL serological with 0.1 mL graduations and 10.0 mL with 1.0 mL graduations.

(**b**) *Colony counter*.—Quebec, or equiv., dark field model.

(**c**) *High-speed blender*.—Waring Blendor, or equiv., multispeed model, with low-speed operation at 13,000 rpm, and 1 L glass or metal blender jars with covers. One jar is required for each sample.

(**d**) *Anaerobic jars*.—BBL GasPak jars equipped with GasPak H + $CO_2$ generator envelopes are recommended. Anaerojar (Pfizer Diagnostics, 1407 N Dayton St, Chicago, IL 60622) with replacement of air by purified N or N-$CO_2$ (9 + 1) is satisfactory.

(**e**) *Freezer, ultra-low temperature*.—REVCO Model ULT-107 (Revco Scientific, Inc., 275 Aiken Rd, Asheville, NC 28804) or equiv., capable of maintaining temp. of −68°.

(**f**) *Shipping container*.—Heavy duty styrofoam, including hermetically sealable metal canister (friction-fit paint can is satisfactory).

### *B. Reagents*

(**a**) *Peptone dilution water*.—Dissolve 2.0 g peptone (Difco B118) in 2 L $H_2O$ for each sample, and adjust to pH 7.0 ± 0.1. Dispense enough vol. in 175 mL (6 oz) bottles to give 90 ± 1 mL and in 750 mL erlenmeyers to give 450 ± 5 mL after autoclaving 15 min at 121°.

(**b**) *Nitrite test reagents*.—(*1*) *Reagent A*.—Dissolve 8 g sulfanilic acid in 1 L 5*N* HOAc (2 + 5). (*2*) *Reagent B*.—Dissolve 5 g α-naphthol in 1 L 5*N* HOAc.

(**c**) *Buffered glycerol-salt soln*.—Dissolve 4.2 g NaCl in 900 mL $H_2O$. Add 12.4 g anhyd. $K_2HPO_4$, 4.0 g anhyd. $KH_2PO_4$, and 100 mL glycerol. Mix well to dissolve, and adjust pH to 7.2. Autoclave 15 min at 121°. For double-strength glycerol soln (20%), use 200 mL glycerol and 800 mL $H_2O$.

### *C. Culture Media*

(Sizes of culture media containers (test tubes, flasks, and petri dishes) are specified for each medium. All media except tryptose-sulfite-cycloserine (TSC) agar are incubated in air at 35°. Media not used ≤4 hr after prepn must be heated 10 min in boiling $H_2O$ or flowing steam to expel O and cooled rapidly in tap $H_2O$ without agitation just before use.)

(**a**) *Tryptose-sulfite-cycloserine agar*.—15.0 g tryptose, 20.0 g agar, 5.0 g soytone, 5.0 g yeast ext, 1.0 g Na metabisulfite, and 1.0 g ferric ammonium citrate (NF Brown Pearls) dild to 1 L with $H_2O$ (SFP agar base, Difco 0811-01, is satisfactory). Adjust to pH 7.6 ± 0.1, dispense 250 mL portions into 500 mL flasks, and sterilize 15 min at 121°. Before plating, add 20.0 mL 0.5% filter-sterilized soln of D-cycloserine to each 250 mL sterile melted medium at 50°. To make egg yolk-contg plates, add 20 mL 50% egg yolk emulsion, (**c**), to 250 mL sterile medium contg D-cycloserine. Dispense 15 mL portions into 100 × 15 mm sterile petri dishes. Cover plates with towel and let dry overnight at room temp. before use.

(**b**) D-*Cycloserine soln*.—Dissolve 1 g D-cycloserine (Sigma Chemical Co. or Serva Feinbiochemicia, Heidelberg, West Germany) without heating in 200 mL 0.05*M* phosphate buffer (pH 8.0 ± 0.1) and sterilize by filtering thru 0.45 μm membrane filter.

(**c**) *Egg yolk emulsion*.—Wash fresh eggs with stiff brush and drain. Soak 1 hr in 70% alcohol. Aseptically remove yolk and mix with equal vol. sterile 0.85% NaCl soln. Store at 4°.

(**d**) *Buffered motility-nitrate medium*.—3.0 g beef ext, 5.0 g peptone, 5.0 g $KNO_3$, 2.5 g $Na_2HPO_4$, 3.0 g agar, 5.0 g galactose, and 5.0 g glycerol dild to 1 L with $H_2O$. Adjust to pH 7.3 ± 0.1, dispense 11 mL portions into 150 × 16 mm tubes, and sterilize 15 min at 121°.

(**e**) *Lactose-gelatin medium*.—15.0 g tryptose, 10.0 g yeast ext, 10.0 g lactose, 5.0 g $Na_2HPO_4$, 0.05 g phenol red, and 120.0 g gelatin dild to 1 L with $H_2O$. Adjust to pH 7.5 ± 0.1 before adding lactose and phenol red. Dispense 10 mL portions into 150 × 16 mm screw-cap tubes and sterilize 15 min at 121°.

(**f**) *Sporulation broth*.—15.0 g polypeptone, 3.0 g yeast ext, 3.0 g sol. starch, 0.1 g $MgSO_4$, 1.0 g Na thioglycollate, and 11.0 g $Na_2HPO_4$ dild to 1 L with $H_2O$. Adjust to pH 7.8 ± 0.1, dispense 15 mL portions into 150 × 20 mm screw-cap tubes, and sterilize 15 min at 121°.

(**g**) *Polypeptone-yeast extract* (*PY*) *medium*.—20.0 g polypeptone, 5.0 g yeast ext, and 5.0 g NaCl dild to 1 L with $H_2O$. Adjust to pH 6.9 ± 0.1, dispense 9 mL portions into 125 × 16 mm screw-cap tubes, and sterilize 15 min at 121°.

(**h**) *Fluid thioglycollate medium*.—(BBL No. 11260 or Difco No. 0256). Dispense 10 mL portions into 150 × 16 mm screw-cap tubes. Sterilize 15 min at 121°, and cool quickly. Final pH is 7.1 ± 0.1.

### *D. Preparation of Sample*

(**a**) *For storage and shipping*.—Using aseptic technic, transfer 50 g sample to sterile container such as Whirl-Pak plastic bag and add 50 g sterile buffered glycerol-salt soln. Mix well by kneading bag or stirring with sterile pipet. Let soln penetrate solid foods 10 min before freezing. Treat liq. samples such as beef juice or gravy with double-strength (20% glycerol) soln to obtain final concn of 10% glycerol. Freeze samples as quickly as possible in ultra-low temp. freezer at −68° or, alternatively, by placing in sealable metal canister and storing with solid $CO_2$ in insulated shipping container. To ship samples, place in sealable metal canister and pack in well insulated styrofoam shipping carton with sufficient solid $CO_2$ to keep samples frozen during transit. Ship by most rapid means possible. Upon receipt, transfer samples to ultra-low temp. freezer at −68° or replenish solid $CO_2$ in shipping carton to maintain temp. at ca −56° until samples can be examined. Thaw samples and proceed as in (**b**) without delay.

(**b**) *For analysis*.—Using aseptic technic, weigh 50 g food sample into sterile blender jar. Add 450 mL peptone diln $H_2O$ and homogenize 2 min at low speed (13,000 rpm). Use this 1:10 diln to prep. serial dilns from $10^{-2}$ to $10^{-6}$ by transferring 10 mL of 1:10 diln to 90 mL diln blank, mixing well with gentle shaking, and continuing until $10^{-6}$ diln is reached.

### *E. Plate Count Technic*

Pour ca 5 mL TSC agar without egg yolk into each of ten 100 × 15 mm petri dishes and spread evenly by rapidly rotating dish. When agar has solidified, label plates and aseptically pipet 1 mL of each diln of homogenate in duplicate onto agar surface in center of dish. Pour addnl 15 mL TSC agar without egg yolk into dish and mix well with inoculum by gently rotating dish.

Alternatively, with sterile glass rod spreader, spread 0.1 mL diln over previously poured plates of TSC agar contg egg yolk emulsion. Let plates absorb inoculum 5–10 min; then overlay with 10 mL TSC agar without egg yolk. (TSC agar contg egg yolk is preferred for foods which may also contain other sulfite-reducing *Clostridium* sp.)

When agar has solidified, place plates in upright position in anaerobic jar. Produce anaerobic conditions, and incubate jar 20 hr at 35° for TSC agar without egg yolk and 24 hr at 35° for TSC agar with egg yolk. After incubation, remove plates from jar and observe macroscopically for growth and black colony production. Select plates showing estd 20–200 black colonies. Using Quebec colony counter with piece of white tissue paper over counting area, count black colonies and calc. number of *Clostridium* sp./g food. *C. perfringens* colonies in medium contg egg yolk are black and usually surrounded by 2–4 mm zone of white ppt due to lecithinase activity. However, since a few strains are weak or neg. for lecithinase, count any black colonies suspected to be *C. perfringens* and confirm identity as in **976.30F**.

### *F. Confirmation Technic*

Select 10 characteristic colonies from countable plates (20–200 colonies), inoculate each into tube of fluid thioglycollate medium, and incubate 18–24 hr at 35°. Make Gram-stained smear of fluid thioglycollate cultures and check for purity and presence of short, thick, Gram-pos. bacillus characteristic of *C. perfringens*. Streak contaminated cultures on TSC agar contg egg yolk and incubate plates anaerobically 24 hr at 35° to obtain pure cultures. Stab-inoculate buffered motility-nitrate and lactose gelatin media with 2 mm loopfuls of pure fluid thioglycollate culture or portion of isolated colony from TSC agar plate. Inoculate sporulation broth with 1 mL fluid thioglycollate culture and incubate 24 hr at 35°. Examine tubes of buffered motility-nitrate medium by transmitted light for type of growth along stab. Nonmotile organisms produce growth only in and along line of stab. Motile organisms produce diffuse growth out into medium away from stab.

Test buffered motility-nitrate medium for presence of nitrite by adding 0.5 mL Reagent A and 0.2 mL Reagent B. Orange which develops within 15 min indicates presence of nitrites. If no color develops, add few grains of powd Zn metal, and let stand 10 min. No color change after addn of Zn indicates that nitrates are completely reduced; change to orange indicates that organism is incapable of reducing nitrates.

Examine lactose-gelatin medium for gas and color change from red to yellow, indicating that lactose is fermented with production of acid. Chill tubes 1 hr at 5° and check for gelatin liquefaction. If medium solidifies, reincubate addnl 24 hr at 35° and repeat test for gelatin liquefaction. Make Gram-stained smear from sporulation broth and examine microscopically for spores. Report whether or not spores are produced. Store sporulated cultures at 4° if further testing of isolates is desired.

Nonmotile, Gram-pos. bacilli which produce black colonies in TSC agar, reduce nitrates to nitrites, produce acid and gas from lactose, and liquefy gelatin within 48 hr are provisionally identified as *C. perfringens*.

Organisms suspected to be *C. perfringens* that do not meet criteria stated above must be confirmed by further testing. Subculture into fluid thioglycollate medium isolates that do not liquefy gelatin or which are atypical in other respects. Incubate 24 hr at 35°, make Gram-stained smear, and check for purity. Inoculate 1 tube of PY medium, (**g**), contg 1% salicin and 1 tube contg 1% raffinose with 0.1 mL fluid thioglycollate culture. Incubate media 24 hr at 35° andj check PY-salicin for acid and gas. Transfer 1.0 mL culture to test tube and add 1–2 drops 0.04% phenol red. Yellow indicates acid is produced from salicin. (Salicin usually is not fermented by *C. perfringens* but is rapidly fermented with production of acid and gas by closely related species.) Reincubate media addnl 48 hr and test both media for production of acid. Acid is usually produced from raffinose by *C. perfringens* but not by closely related species. Acid is produced from salicin in PY medium by a few strains of *C. perfringens*.

Calc. number of *C. perfringens* in sample on basis of % colonies tested that are confirmed as *C. perfringens*. (*Example:* If av. plate count of $10^{-4}$ diln was 85, and 8 of 10 colonies tested were confirmed as *C. perfringens*, number of *C. perfringens*/g food is 85 × (8/10) × 10,000 = 680,000.) (*Note:* Diln factor with plates contg egg yolk is 10-fold higher than diln plated.)

Refs.: JAOAC **59**, 606(1976); **61**, 785(1978).

## 974.38 *Clostridium perfringens* in Foods

### alpha-Toxin Estimation Method

**First Action 1974**
**Final Action 1979**

(Applicable to examination of outbreak foods in which presence of large numbers of vegetative cells are suspected but which may no longer be viable)

### *A. Apparatus*

(**a**) *Centrifuge*.—High-speed, preferably refrigerated, with 250 mL bottles.

(**b**) *Seitz filter*.—100–250 mL with sterilizing filter pads.

(**c**) *High-speed blender*.—Waring Blendor or Omni-Mixer homogenizer (DuPont Co., Sorvall Operations, Newtown, CT 06470), with blending vessels.

(**d**) *Vacuum flask*.—Sidearm 1 L erlenmeyer fitted with 1-hole rubber stopper to receive 200 mm glass tubing with 125 cm of 6 mm od (3 mm id) rubber tubing attached.

(**e**) *Tubing*.—Stainless steel thin wall (No. 9 surgical), 3 (od) × 180 mm (Tubesales, 175 Tubeway St, Forrest Park, GA 30051).

(**f**) *Dialysis tubing*.—1.21" flat width (Fisher Scientific Co., No. 8667C).

### *B. Reagents*

(**a**) *N-2-Hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES) buffer soln*.—Dissolve 6.0 g HEPES (Calbiochem Corp.) and 11.7 g NaCl in 500 mL $H_2O$. Adjust to pH 8.0 with 3*N* NaOH and store at 4°.

(**b**) *Lecithovitellin soln*.—Mix 1 egg yolk with 250 mL saline soln, (**e**), and clarify by centrfg 20 min at 14,000×*g* at 4°. Filter-sterilize supernate with Seitz filter and store at 4°.

(**c**) *Saline agar base*.—Add 15.0 g purified agar (Difco Laboratories) and 8.5 g NaCl to 1 L $H_2O$. Adjust to pH 7.0, heat to dissolve agar, dispense in 100 mL portions, and autoclave 15 min at 121°.

(**d**) *Washed red blood cells*.—Wash packed human red blood cells 3 times by mixing with 4 vols saline soln, (**e**). Centrf. 10 min at low speed (2500 rpm) to sediment cells. Remove supernate with vac. flask. Resuspend cells in addnl saline soln and repeat these steps twice. After final wash, mix cells with equal vol. saline soln. Use sterile precautions.

(**e**) *Sterile saline soln*.—Dissolve 8.5 g NaCl in 1 L $H_2O$. Adjust to pH 7.0, dispense 250 mL portions into Pyrex containers, and autoclave 15 min at 121°.

(**f**) *Polyethylene glycol soln*.—30%. Dissolve 120 g polyethylene glycol (Carbowax Compound 20M, Union Carbide Corp., PO Box 8361, S Charleston, WV 25303) in 400 mL $H_2O$.

(**g**) *Antiserum*.—*Clostridium perfringens* Type A diagnostic serum (Coopers Animal Health Inc., PO Box 41967, Kansas City, MO 64141-6167).

### C. Preparation of Hemolysin Plates

Melt 100 mL saline agar base, (**c**), cool to 50°, and add 11 mL washed red cells, (**d**). Mix thoroly and dispense 7 mL into 15 × 100 mm sterile plastic petri dishes. Dry plates overnight at room temp. and store at 4°. Just before use, cut test wells by applying vac. to sterile stainless steel tube, (**e**), and plunging tube into agar. Using template, space 9 test wells 3 cm apart and 2 cm from edge, and place 2 addnl wells 3 cm apart near center of plate.

### D. Toxin Extraction

Homogenize 25 g food (do not include fat) in 100 mL HEPES buffer soln, (**a**), 1 min in high-speed blender. Centrf. homogenate 20 min at 14,000–20,000×*g* at 5°. Filter supernate thru Whatman No. 31 paper, or equiv., to remove fat (chill ext centrfd without refrigeration 1 hr at 4° before filtering). Discard solids. Rinse Seitz filter pad with 15 mL saline soln. Discard saline soln and filter-sterilize ext, rinsing filter pad with 10 mL saline soln.

### E. Concentration

Soak 90 cm dialysis tubing 1 hr in $H_2O$. Tie one end and fill with saline soln. Check for leaks and rinse out twice with saline soln. Transfer sterile ext to dialysis sack and conc. to <10 mL by dialyzing 4–5 hr against 400 mL 30% polyethylene glycol, (**f**), at 4°. Rinse outside of sack with tap $H_2O$ and collect concd ext in sterile tube.

### F. Toxin Testing

Adjust vol. of concd ext to 10 ± 0.5 mL with saline soln. Set up 10 sterile 13 × 100 mm test tubes and add 0.5 mL saline soln to all tubes except first and last. Add 0.5 mL ext to first and second tubes. Mix ext and saline soln in second tube and transfer 0.5 mL to third tube, etc., to serially dil. ext from 0 to 1 + 255. Change pipet after 3 dilns to prevent excessive carry-over. Mix 0.25 mL ext, 0.25 mL saline soln, and 0.1 mL antiserum, (**g**), in last tube. Fill 1 peripheral well of duplicate hemolysin plates with each diln of ext, using fine-tipped Pasteur pipet. Fill 1 center well of each plate with ext-antiserum mixt. and the other with saline soln. Add 0.5 mL lecithovitellin soln, (**b**), to remainder of dild ext in each tube, including ext-antiserum mixt. Mix well, and incubate tubes and plates (in plastic bag) 24 hr at 35°.

### G. alpha-Toxin Titer

After incubation, refrigerate plates 2 hr at 4°. Measure hemolytic zone (width from edge of well in mm). Last 3 dilns before end point should exhibit ca 1 mm reduction in width for each 2-fold diln. If not, repeat α-toxin test. Hemolytic zone 1 mm in width is end point of titrn.

Examine ext-lecithovitellin mixt. in tubes for lecithinase activity and record results. Max. reaction (++++) is white pellicle 4–5 mm thick over clear liq. Activity decreases with diln to (+) reaction (opaque soln with no pellicle). This diln is end point of lecithovitellin test. Hemolytic and lecithinase activities neutzd by antiserum are due to α-toxin.

### H. Population Estimate

Compare titer of α-toxin present in ext with data in Table **974.38** to est. population of *C. perfringens*. Hemolysin (HI) plate titer is preferred for this because lecithovitellin (LV) test is less sensitive with some food exts.

Ref.: JAOAC **57**, 91(1974).

**Table 974.38 Correlation Between Population Levels of *C. perfringens* and Amount alpha-Toxin Produced in Food[a]**

| α-Toxin Titer[b] | | Estd *C. perfringens* Population/g × $10^6$ |
|---|---|---|
| HI Plate | LV Test | |
| Undild | | 1.2 |
| 1+1 | Undild | 2.5 |
| 1+3 | 1+1 | 6.5 |
| 1+7 | 1+3 | 9.5 |
| 1+15 | 1+7 | 25 |
| 1+31 | 1+15 | 55 |
| 1+63 | 1+31 | 80 |
| 1+127 | 1+127 | 150 |
| 1+255 | 1+255 | 210 |

[a] Based on viable counts obtained with 6 strains in chicken broth.
[b] Diln which produces 1 mm zone of hemolysis in HI plate or one + reaction in LV test.

## BACILLUS

## 980.31 *Bacillus cereus* in Foods

### Enumeration and Confirmation Microbiological Methods

**First Action 1980**
**Final Action 1981**

### A. Apparatus

(**a**) *Pipets*.—1.0 mL with 0.1 mL graduations; also 5.0 mL and 10.0 mL with 1.0 mL graduations.

(**b**) *Colony counter*.—Quebec, or equiv., dark field model.

(**c**) *High-speed blender*.—Waring blender, or equiv. 2 speed model with high-speed operation at 18,000–21,000 rpm, and 1 L glass or metal blender jars with covers. One jar is required for each sample.

(**d**) *Anaerobic jar*.—BBL GasPak jar equipped with GasPak H + $CO_2$ generator envelopes, or equiv.

(**e**) *Vortex mixer*.—Vortex Genie, or equiv.

(**f**) *Sterile bent glass spreading rods*.—Hockey stick or hoe shape with fire polished ends; 3–4 mm diam. with 45–55 mm spreading surface.

(**g**) *Inoculating loops*.—One each, 26 gage nichrome wire with loop 2 mm id and one 24 gage nichrome wire loop 3 mm id.

(**h**) *Staining rack*.—Rack must be accessible from below for heating slides.

### B. Media and Reagents

(**a**) *Mannitol-egg yolk-polymyxin (MYP) agar*.—1.0 g beef ext, 10.0 g peptone, 10.0 g D-mannitol, 10.0 g NaCl, 0.025 g phenol red (as soln), and 15.0 g agar dild to 900 mL with $H_2O$. Adjust to pH 7.2 ± 0.1, heat to dissolve, and dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Cool to 50° in $H_2O$ bath and add 12.5 mL egg yolk emulsion, (**b**), and 2.5 mL polymyxin B soln, (**c**), to each 225 mL medium. Mix well and dispense 18 mL portions into 100 × 15 mm sterile petri dishes. Dry plates 24 h at room temp. before use.

(**b**) *Egg yolk emulsion*.—50%. Wash fresh eggs with stiff brush and drain. Soak 1 h in 70% alcohol. Aseptically remove yolk and mix (1 + 1) with sterile 0.85% NaCl soln. (Difco egg yolk enrichment 50% is satisfactory).

(**c**) *Polymyxin B soln*.—Dissolve 500,000 units sterile polymyxin B sulfate in 50 mL sterile $H_2O$.

(**d**) *Trypticase-soy-polymyxin broth*.—17.0 g trypticase, 3.0 g phytone peptone, 5.0 g NaCl, 2.5 g $K_2HPO_4$, and 2.5 g dextrose dild to 1 L with $H_2O$. (Rehydrated trypticase soy broth is satisfactory.) Boil 2 min. Dispense 15 mL portions into 150 × 20 mm tubes and autoclave 15 min at 121°. Final pH 7.3 ± 0.1. Just prior to use, add 0.1 mL 0.15% polymyxin B soln to each tube of medium and mix well. To make polymyxin B soln, dissolve 500,000 units sterile polymyxin B sulfate in 33.3 mL sterile $H_2O$.

(**e**) *Phenol red-dextrose broth*.—10.0 g proteose peptone No. 3, 1.0 g beef ext, 5.0 g NaCl, 0.018 g phenol red (as soln), and 5.0 g dextrose dild to 1 L with $H_2O$. (Phenol red dextrose broth, Difco 0093, is satisfactory). Dispense 3 mL portions into 100 × 13 mm tubes and autoclave 10 min at 121°. Final pH 7.4 ± 0.1.

(**f**) *Nitrate broth*.—3.0 g beef extract, 5.0 g peptone, and 1.0 g $KNO_3$ dild to 1 L with $H_2O$. (Rehydrated nitrate broth is satisfactory.) Adjust to pH 7.0 ± 0.1 and dispense 5 mL portions into 125 × 16 mm tubes. Autoclave 15 min at 121°.

(**g**) *Nutrient agar slants and plates*.—3.0 g beef ext, 5.0 g peptone, and 15.0 g agar dild to 1 L with $H_2O$ (dehydrated nutrient agar is satisfactory). Heat to dissolve, and dispense 6.5 mL portions into 125 × 16 mm screw-cap tubes. Autoclave 15 min at 121° and slant tubes until medium solidifies. Final pH 6.8 ± 0.1. For plates, dispense 100–500 mL portions in bottles or flasks and autoclave 15 min at 121°. Cool to 50° in $H_2O$ bath and dispense 18–20 mL portions in 100 × 15 mm sterile petri dishes. Dry plates 24–48 h at room temp. before use.

(**h**) *Nutrient agar with* L*-tyrosine*.—Prep. nutrient agar as in (**g**) and dispense 100 mL portions into bottles. Autoclave 15 min at 121°. Cool to 45° in $H_2O$ bath and add 0.5 g sterile L-tyrosine suspended in 10 mL $H_2O$ to each 100 mL of medium. Mix thoroly by rotating or inverting bottle and aseptically dispense 3.5 mL portions of complete medium into sterile 100 × 13 mm tubes. Slant tubes and cool rapidly to prevent sepn of tyrosine. To prep. L-tyrosine suspension, add 0.5 g to 150 × 20 mm tube and suspend in 10 mL $H_2O$ with Vortex mixer. Autoclave 15 min at 121°.

(**i**) *Nutrient broth with lysozyme*.—3.0 g beef ext and 5.0 g peptone dild to 1 L with $H_2O$. (Rehydrated nutrient broth, Difco 0003, is satisfactory.) Dispense 99 mL portions in bottles and autoclave 15 min at 121°. Final pH 6.8 ± 0.1. Mix 1.0 mL 0.1% lysozyme soln with 99 mL broth and aseptically dispense 2.5 mL complete medium into sterile 100 × 13 mm tubes. To make lysozyme soln, dissolve 0.1 g lysozyme in 65 mL sterile 0.01*N* HCl, boil for 20 min, and dil. to 100 mL with sterile 0.01*N* HCl. Alternatively, dissolve 0.1 g lysozyme hydrochloride in 100 mL $H_2O$ and sterilize with 0.45 μm membrane filter.

(**j**) *Modified Voges-Proskauer* (*VP*) *medium*.—7.0 g proteose peptone, 5.0 g dextrose, and 5.0 g NaCl dild to 1 L with $H_2O$. Dispense 5 mL portions into 150 × 20 mm tubes. Autoclave 10 min at 121°. Final pH 6.5 ± 0.1.

(**k**) *Motility medium*.—10.0 g trypticase, 2.5 g yeast ext, 5.0 g dextrose, 2.5 g $Na_2HPO_4$, and 3.0 g agar dild to 1 L with $H_2O$. Heat to dissolve. Dispense 2 mL portions into 13 × 100 mm tubes, and autoclave 10 min at 121°. Final pH 7.4 ± 0.2. Alternatively, dispense 100 mL amts in 150 mL bottles and autoclave 15 min at 121°. Cool at 50° and aseptically dispense 2 mL into sterile 13 × 100 mm tubes. For best results, store at room temp. 2–4 days before use to prevent growth along side of medium.

(**l**) *Trypticase-soy-sheep blood* (*TSSB*) *agar*.—Dil. 15.0 g trypticase, 5.0 g phytone peptone, 5.0 g NaCl, and 15.0 g agar to 1 L with $H_2O$. Adjust pH to 7.0 ± 0.2. Heat to boiling to dissolve, and dispense 100–500 mL portions in bottles or flasks. Autoclave 15 min at 121° and cool to 48° in $H_2O$ bath. Add 5 mL sterile defibrinated sheep blood per 100 mL medium. Mix well, and dispense 18–20 mL portions into 100 × 15 mm petri dishes. (Trypticase-soy or tryptic-soy agar plates contg 5% sheep blood are satisfactory.)

(**m**) *Butterfield's buffered phosphate diluent*.—(*1*) *Stock soln*.—Dissolve 34.0 g $KH_2PO_4$ in 500 mL $H_2O$, adjust to pH 7.2 with ca 175 mL 1*N* NaOH, and dil. to 1 L with $H_2O$. Store in refrigerator. (*2*) *Diluent*.—Dil. 1.25 mL stock soln to 1 L with $H_2O$. Prep. 90 ± 1 mL diln blanks with this soln and autoclave 15 min at 121°.

(**n**) *Nitrite test reagents*.—(*1*) *Reagent A*.—Dissolve 8 g sulfanilic acid in 1 L 5*N* HOAc (2 + 5). (*2*) *Reagent B*.—Dissolve 2.5 g α-naphthol in 1 L 5*N* HOAc.

(**o**) *Voges-Proskauer* (*VP*) *test reagents*.—(*1*) *Alpha-naphthol soln*.—5%. Dissolve 5.0 g α-naphthol in 100 mL absolute alcohol. (*2*) *Potassium hydroxide soln*.—40%. Dissolve 40 g KOH in $H_2O$ and dil. to 100 mL. (*3*) *Creatine crystals*.

(**p**) *Basic fuchsin stain*.—Dissolve 0.5 g basic fuchsin in 20 mL alcohol and dil. to 100 mL with $H_2O$. Filter soln if necessary thru fine paper to remove excess dye particles. Store in tightly stoppered container. (TB Carbol-fuchsin ZN stain is satisfactory.)

### C. Preparation of Food Homogenate

Using aseptic technic, weigh 50 g food sample into sterile blender jar. Add 450 mL phosphate buffered diln $H_2O$ and homogenize 2 min at high speed (ca 20,000 rpm). Use this 1:10 diln to prep. serial dilns from $10^{-2}$ to $10^{-6}$ by transferring 10 mL of 1:10 diln to 90 mL diln blank, mixing well with vigorous shaking, and continuing until $10^{-6}$ is reached.

### D. Plate Count Technic

Inoculate duplicate MYP agar plates with each diln of homogenate by spreading 0.1 mL evenly onto each plate with sterile glass rod spreader. Incubate plates 24 h at 30° and check for colonies surrounded by ppt zone indicating lecithinase is produced. *B. cereus* colonies usually are pink which becomes more intense after addnl incubation. If reactions are not clear, incubate plates for addnl 24 h before counting.

Select plates showing estd 15–150 eosin pink colonies surrounded by lecithinase zones. Mark bottom of plates into zones with black felt pen to facilitate counting and count colonies. This is the presumptive count of *B. cereus*/g of food. Pick 5 or more colonies from plates counted and transfer to nutrient agar slants for confirmation tests.

### E. Most Probable Number Technic

(For foods containing $\leq 10^3$ *B. cereus*/g)

Inoculate 3-tube most probable number (MPN) series in trypticase-soy-polymyxin broth, (**d**), using 1 mL inocula of 1:10, 1:100, and 1:1000 dilns with triplicate tubes at each diln. Incubate 48 ± 2 h at 30° and examine tubes for dense growth typical of *B. cereus*. Streak pos. tubes on sep. MYP agar plates, (**a**), and incubate 24–48 h at 30°. Pick 1 or more eosin pink colonies surrounded by ppt zone due to lecithinase from each plate and transfer to nutrient agar slants for confirmation tests. Confirm as *B. cereus* and compute MPN of *B. cereus*/g using Table **966.24** on basis of number of tubes in which *B. cereus* was present.

### F. Confirmation Technic

Pick ≥5 presumptive pos. colonies from MYP agar plates and transfer to nutrient agar slants. Incubate 24 h at 30°. Make Gram stained smears from slants and examine microscopically. *B. cereus* will appear as large Gram pos. bacilli in short to long chains; spores are ellipsoidal, central to subterminal, and do not swell sporangium.

Transfer 3 mm loopful culture from each slant to 100 × 13 mm tube contg 0.5 mL sterile phosphate buffered diln $H_2O$ and suspend culture in diluent with Vortex mixer. Inoculate following media with suspended culture:

(a) *Phenol red dextrose broth,* (*e*).—Inoculate broth with 2 mm loopful culture and incubate anaerobically 24 h at 35° in Gas-Pak anaerobic jar. Shake tubes and check for growth. Change from red to yellow indicates acid was produced from dextrose anaerobically.

(b) *Nitrate broth,* (*f*).—Inoculate with 3 mm loopful culture and incubate 24 h at 35°. Test for presence of nitrite by adding 0.25 mL nitrite test reagent A and 0.25 mL reagent B. Orange which develops within 10 min indicates presence of nitrites.

(c) *Modified VP medium,* (*j*).—Inoculate with 3 mm loopful of culture and incubate 48 h at 35°. Transfer 1 mL culture to empty tube to test for acetylmethylcarbinol. Add 0.2 mL 40% KOH soln, 0.6 mL 5% alc. α-naphthol soln, and few crystals creatine. Let stand 1 h. Test is pos. if eosin pink develops.

(d) *Nutrient agar with* L*-tyrosine,* (*h*).—Inoculate entire surface of slant with 3 mm loopful of culture. Incubate 48 h at 35°. Check for clearing of medium near growth indicating tyrosine is decomposed. Check neg. tubes for growth and incubate addnl 72 h before discarding.

(e) *Nutrient broth with lysozyme,* (*i*).—Inoculate nutrient broth contg 0.001% lysozyme with 2 mm loopful of culture; also inoculate control tube of plain nutrient broth. Incubate 24 h at 35° and record growth as + or −. Incubate neg. tubes addnl 24 h before discarding.

(f) *MYP agar,* (*a*).—(Test may be omitted if reactions of all isolates on MYP agar plates were typical.) Inoculate premarked 4 sq. cm area of MYP agar plate by gently touching surface with 2 mm loopful of culture. Let inoculum be absorbed and incubate 24 h at 35°. Check for lecithinase production as indicated by zone of ppt surrounding growth. Mannitol fermentation is neg. if growth and surrounding medium are eosin pink.

Large Gram pos. bacilli which produce lecithinase and are neg. for mannitol fermentation on MYP agar, grow and produce acid from dextrose anaerobically, reduce nitrate to nitrite, produce acetylmethylcarbinol, decompose L-tyrosine, and grow in the presence of 0.001% lysozyme are provisionally identified as *B. cereus*. (These characteristics are shared by all members of *B. cereus* group. See Differentiation of Members of *Bacillus cereus* Group, **983.26**.)

Calc. number of *B. cereus* in sample on basis of % colonies tested that are confirmed as *B. cereus*. (*Example:* If av. plate count with $10^{-4}$ diln of sample was 65 and 4 of 5 colonies tested were confirmed as *B. cereus*, number of *B. cereus*/g food is 65 × (4/5) × 10,000 × 10 = 5,200,000.) (Diln factor is 10-fold higher than sample diln because only 0.1 mL was tested.)

Ref.: JAOAC **63,** 581(1980).

## 983.26 Differentiation of Members of *Bacillus cereus* Group

**Microbiological Method**

**First Action 1983**
**Final Action 1984**

(Typical strains of *B. cereus* isolated from foods by **980.31** can be differentiated from other members of *B. cereus* group including: (1) insect pathogen *B. thuringiensis*, (2) mammalian pathogen *B. anthracis*, and (3) rhizoid strains of *B. cereus* var. *mycoides*.)

### *A. Apparatus*

(a) *Staining rack.*—Rack must be accessible from below for heating slides.

(b) *Inoculating loops.*—One each, 26 gage nichrome wire with loop 2 mm id and one 24 gage nichrome wire loop 3 mm id.

### *B. Media and Reagents*

(a) *Mannitol-egg yolk-polymyxin* (*MYP*) *agar.*—1.0 g beef ext, 10.0 g peptone, 10.0 g D-mannitol, 10.0 g NaCl, 0.025 g phenol red (as soln), and 15.0 g agar dild to 900 mL with $H_2O$. Adjust to pH 7.2 ± 0.1, heat to dissolve, and dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Cool to 50° in $H_2O$ bath and add 12.5 mL sterile 50% egg yolk emulsion (**b**) and 2.5 mL polymyxin B soln contg 10 000 units per mL (if available) to each 225 mL medium. (Addn of polymyxin B soln is optional when medium is to be used for testing reactions of pure cultures.) Mix well and dispense 18 mL portions into 100 × 15 mm sterile petri dishes. Dry plates 24 h at room temp. before use. (Dehydrated mannitol-egg yolk-polymyxin (MYP) agar contg 50% egg yolk enrichment is satisfactory.)

(b) *Egg yolk emulsion.*—50%. Wash fresh eggs with stiff brush and drain. Soak 1 h in 70% alcohol. Aseptically remove yolk and mix (1 + 1) with sterile 0.85% NaCl soln. (50% egg yolk enrichment is satisfactory.)

(c) *Nutrient agar slants and plates.*—3.0 g beef ext, 5.0 g peptone, and 15.0 g agar dild to 1 L with $H_2O$ (dehydrated nutrient agar is satisfactory). Heat to dissolve, and dispense 6.5 mL portions into 125 × 16 mm screw-cap tubes. Autoclave 15 min at 121° and slant tubes until medium solidifies. Final pH 6.8 ± 0.2. For plates, dispense 100–500 mL portions in bottles or flasks and autoclave 15 min at 121°. Cool to 50° in $H_2O$ bath and dispense 18–20 mL portions in 100 × 15 mm sterile petri dishes. Dry plates 24–48 h at room temp. before use.

(d) *Motility medium.*—10.0 g trypticase, 2.5 g yeast ext, 5.0 g dextrose, 2.5 g $Na_2HPO_4$, and 3.0 g agar dild to 1 L with $H_2O$. Heat to dissolve. Dispense 2 mL portions into 13 × 100 mm tubes, and autoclave 10 min at 121°. Final pH 7.4 ± 0.2. Alternatively, dispense 100 mL amts in 150 mL bottles and autoclave 15 min at 121°. Cool to 50° and aseptically dispense 2 mL into sterile 13 × 100 mm tubes. For best results, store at room temp. 2–4 days before use to prevent growth along side of medium.

(e) *Trypticase-soy-sheep blood* (*TSSB*) *agar.*—Dil. 15.0 g trypticase, 5.0 g phytone peptone, 5.0 g NaCl, and 15.0 g agar to 1 L with $H_2O$. Adjust pH to 7.0 ± 0.2. Heat to boiling to dissolve, and dispense 100–500 mL portions in bottles or flasks. Autoclave 15 min at 121° and cool to 48° in $H_2O$ bath. Add 5 mL sterile defibrinated sheep blood per 100 mL medium. Mix well, dispense 18–20 mL portions into 100 × 15 mm petri dishes. (Trypticase-soy or tryptic-soy agar plates contg. 5% sheep blood are satisfactory.)

(f) *Basic fuchsin stain.*—Dissolve 0.5 g basic fuchsin in 20 mL alcohol and dil. to 100 mL with $H_2O$. Filter soln if necessary thru fine paper to remove excess dye particles. Store in tightly stoppered container. (TB Carbol-fuchsin ZN stain is satisfactory.)

(g) *Butterfield's buffered phosphate diluent.*—(*1*) *Stock soln.*—Dissolve 34.0 g $KH_2PO_4$ in 500 mL $H_2O$, adjust to pH 7.2 with ca 175 mL 1N NaOH, and dil. to 1 L with $H_2O$. Store in refrigerator. (*2*) *Diluent.*—Dilute 1.25 mL stock soln to 1 L with $H_2O$. Prep. 90 ± 1 mL diln blanks with this soln and autoclave 15 min at 121°. Dispense 0.5 mL portions sterile diluent into sterile 13 × 100 mm tubes for preparing suspension of cultures to be tested.

(**h**) *Methanol fixative*.—Dispense undild methanol in plastic squeeze bottle for use in fixing slides.

### *C. Differential Tests*

(**a**) *Preparing test inoculum*.—Inoculate sep. nutrient agar slants with each culture to be tested. Incubate slants 18–24 h at 30° and transfer 3 mm loopful of culture from each slant to 100 × 13 mm tube contg 0.5 mL sterile phosphate buffered diluent. Suspend culture in diluent with vortex mixer. Alternatively, inoculate 5 mL trypticase-soy broth and incubate tubes 18 h at 30°. Mix culture well and use for performing differential tests. Latter procedure is preferred for rhizoid strains and other strains which do not disperse well in phosphate buffer.

(**b**) *Reaction on MYP agar*.—Mark bottom of MYP agar plate into 6–8 equal segments with black felt pen as indicated in Fig. **983.26** and label each section. Place plate in upright position on piece of white paper and inoculate one or more of the prelabeled sections by gently touching surface of agar with 2 mm loopful of culture. Let inoculum be absorbed and incubate plates in upright position 24–48 h at 30–35°. Check for lecithinase production as indicated by zone of ppt surrounding growth. Mannitol fermentation is neg. if growth and surrounding medium are eosin pink. These reactions should be observed with all organisms of *B. cereus* group except rare lecithinase-neg. variants.

(**c**) *Motility tests*.—Inoculate BC motility medium by stabbing down center with 3 mm loopful of culture. Incubate 18–20 h at 30° and examine for type of growth along stab. Motile strains produce diffuse growth into medium away from stab. Nonmotile strains except *B. cereus* var. *mycoides* grow only in and along stab. Strains of *B. cereus* var. *mycoides* often produce "fuzzy" growth in semisolid media resulting from cellular expansion but are not motile by means of flagella. Recheck doubtful results by alternative microscopic motility test as follows: Add 0.2 mL sterile $H_2O$ to nutrient agar slant and inoculate with 3 mm loopful of culture. Incubate slant 6–8 h at 30°, and mix small loopful of liq. culture from base of slant with drop of sterile $H_2O$ on microscope slide. Apply cover glass and examine immediately for signs of motility. *B. cereus* and *B. thuringiensis* cultures are usually actively motile by means of peritrichous flagella. *B. anthracis* and typically rhizoid strains of *B. cereus* var. *mycoides* are nonmotile.

(**d**) *Rhizoid growth*.—Inoculate predried nutrient agar plate by touching medium surface near center with 2 mm loopful of culture. Let inoculum be absorbed, and incubate plate in upright position 24–48 h at 30°. Check plate for rhizoid growth characterized by root or hairlike structures which may extend several cm from point of inoculation. Many *B. cereus* strains produce rough irregular colonies that should not be confused with rhizoid growth. This property is characteristic only of strains which are classified as *B. cereus* var. *mycoides*.

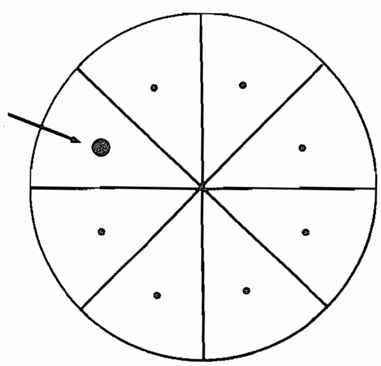


**FIG. 983.26—Diagram of template for marking and inoculating *B. cereus* confirmatory plates. Each section is labeled and inoculated in the center, as indicated by arrow**

(**e**) *Hemolytic activity*.—Mark bottom of trypticase-soy-sheep blood agar plate into 6–8 equal segments (see Fig. **983.26**) with black felt marking pen. Label each segment and inoculate one or more segments near center by gently touching agar surface with 2 mm loopful of culture. Let inoculum be absorbed, and incubate plates 24 h at 30–32°. Check plates for hemolytic activity as indicated by 2–4 mm zone of complete (beta) hemolysis surrounding growth. *B. cereus* is usually strongly hemolytic, whereas *B. thuringiensis* and *B. cereus* var. *mycoides* are often weakly hemolytic and produce complete hemolysis only underneath colonies. *B. anthracis* is usually nonhemolytic after 24 h of incubation. *Caution:* Nonmotile, nonhemolytic cultures could be *B. anthracis*. See precautions under *interpreting test results*, (**g**).

(**f**) *Detection of toxin crystals*.—Inoculate nutrient agar slant with loopful of culture. Incubate slant 24 h at 30° and hold at room temp. 2–3 days. Make smear on microscope slide with sterile $H_2O$. Air-dry and briefly heat-fix by passing slide slowly over burner flame; let cool, and place slide on staining rack. Flood slide with methanol, wait 30 s, and pour off methanol. Dry thoroughly by passing through burner flame. Return slide to staining rack, and flood completely with 0.5% aq. soln of basic fuchsin or TB Carbol-fuchsin ZN stain. Heat slide gently from below with micro burner until steam is seen. Wait 1–2 min and repeat this step. Let stand 30 s, pour off stain, and rinse slide thoroughly in 1 L clean tap $H_2O$. Dry slide without blotting and examine microscopically under oil immersion for presence of free spores and darkly stained tetragonal (diamond-shaped) toxin crystals. Free toxin crystals are usually abundant after 3 days but will not be detectable unless sporangia have lysed. Therefore, if free spores are not seen, leave cultures at room temp. for a few more days and repeat test. *B. thuringiensis* produces protein toxin crystals that usually can be detected by staining, but are not produced by other members of *B. cereus* group.

(**g**) *Interpreting test results*.—On basis of test results, identify as *B. cereus* those isolates which are actively motile, strongly hemolytic, and do not produce rhizoid growth or protein toxin crystals. Nonmotile strains of *B. cereus* may be encountered and a few are weakly hemolytic. These strains can be differentiated from *B. anthracis* by their resistance to penicillin and to gamma bacteriophage. *Caution:* Nonmotile, nonhemolytic strains could be *B. anthracis*, and should be handled with special care and submitted to pathology laboratory such as Centers for Disease Control for identification or destroyed by autoclaving. Noncrystalliferous variants of *B. thuringiensis* and nonrhizoid strains derived from *B. cereus* var. *mycoides* cannot be differentiated from *B. cereus* by tests described.

Ref.: JAOAC **65**, 1134(1982).

## SALMONELLA

### 967.25 *Salmonella* in Foods

**Preparation of Culture Media and Reagents**

**First Action 1967**
**Final Action 1970**

(Applicable to the detection and identification of *Salmonella* from dried active yeast, dried whole egg, dried egg yolk, and dried egg white, edible casein, milk chocolate, nonfat dry milk and dry whole milk, and onion and garlic powders. Method described is minimal. Depending upon history of sample, addnl types of examinations may be applied. Use *Edwards and Ewing's Identification of Enterobacteriaceae*, W. H. Ewing, Elsevier Science Publishing Co., Inc., New York, NY 10017, 4th ed., 1986, as guide for further study of isolated microorganisms. For food sampling plans and initial sample handling, refer to Chapter 1, *Bacteriological Analytical Manual*, 6th ed., 1984.)

### A. Preparation

(Sizes of culture media containers (test tubes, flasks, and petri dishes) are specified in prepn of each medium. Different size containers may be used if they give identical results. All media containers must have covers, caps, or plugs which prevent contamination but maintain aerobic conditions unless otherwise directed.)

(**a**) *Lactose broth*.—*See* **940.36A(f)**. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust, if necessary, to 225 mL. Final pH, 6.7 ± 0.2.

(**b**)(*1*) *Selenite cystine broth*.—Suspend 5.0 g tryptone or polypeptone, 4.0 g lactose, 10.0 g $Na_2HPO_4$, 4.0 g $NaHSeO_3$, and 0.01 g L-cystine in 1 L $H_2O$ and mix thoroly. Heat with frequent agitation. Dispense 10 mL portions into sterile 16 × 150 mm test tubes. Heat 10 min in flowing steam. *Do not autoclave*. Final pH, 7.0 ± 0.2. Medium is not sterile. Use same day as prepd.

(*2*) *Selenite cystine broth* (*North and Bartram*).—Prep. as in (*1*), using 5.0 g polypeptone or 4.0 g tryptone, 4.0 g lactose, 4.0 g $NaHSeO_3$, 5.5 g $Na_2HPO_4$, 4.5 g $KH_2PO_4$, and 1 mL 1% L-cystine (10 mg) soln prepd by dissolving 1.0 g L-cystine in 15 mL 1*N* NaOH and dilg to 100 mL with sterile $H_2O$.

(**c**) *Tetrathionate broth* (*with iodine and brilliant green*).—Suspend 5.0 g polypeptone, 1.0 g bile salts, 10 g $CaCO_3$, and 30 g $Na_2S_2O_3.5H_2O$ in 1 L $H_2O$, mix thoroly, and heat to bp. (Ppt will not dissolve completely.) Cool to <45° and store at 5–8°. Prep. I-KI soln by dissolving 5 g KI in 5 mL sterile $H_2O$, adding 6 g resublimed I, dissolving, and dilg to 20 mL with sterile $H_2O$. Prep. brilliant green soln by dissolving 0.1 g dye in sterile $H_2O$ and dilg to 100 mL. On day medium is used, add 20 mL I-KI soln and 10 mL brilliant green soln per 1 L basal broth. Resuspend ppt by gentle agitation and aseptically dispense 10 mL portions into 20 or 16 × 150 mm sterile test tubes. *Do not heat medium after addn of I-KI and dye solns*.

(**d**) *Xylose lysine desoxycholate agar* (*XLD*).—Suspend ingredients (*1*) or (*2*) (varies with mfgr of formula) in 1 L $H_2O$ and mix thoroly. Heat with frequent agitation just until medium boils. Do not overheat. Cool in $H_2O$ bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 7.4 ± 0.2. Do not autoclave.

(*1*) 3.5 g xylose, 5.0 g L-lysine, 7.5 g lactose, 7.5 g sucrose, 5.0 g NaCl, 3.0 g yeast ext, 0.08 g phenol red, 2.5 g Na desoxycholate, 6.8 g Na thiosulfate, 0.8 g ferric ammonium citrate, and 13.5 g agar.

(*2*) 3.75 g xylose, 5.0 g L-lysine, 7.5 g lactose, 7.5 g sucrose, 5.0 g NaCl, 3.0 g yeast ext, 0.08 g phenol red, 2.5 g Na desoxycholate, 6.8 g Na thiosulfate, 0.8 g ferric ammonium citrate, and 15 g agar.

(**e**) *Hektoen enteric agar* (*HE*).—Suspend ingredients (*1*) or (*2*) (varies with mfgr of formula) in 1 L $H_2O$ and mix thoroly. Heat to boiling with frequent agitation and let boil few moments. Do not overheat. Cool in $H_2O$ bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 7.6 ± 0.2. Do not autoclave.

(*1*) 12.0 g thiotone peptone, 3.0 g yeast ext, 9.0 g bile salts, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.064 g bromthymol blue, 0.1 g acid fuchsin, and 13.5 g agar.

(*2*) 12.0 g proteose peptone, 3.0 g yeast ext, 9.0 g bile salts No. 3, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.065 g thymol blue, 0.1 g acid fuchsin, and 14.0 g agar.

(**f**) *Bismuth sulfite (BS) agar* (*Wilson and Blair*).—Suspend 10 g polypeptone or peptone, 5.0 g beef ext, 5.0 g glucose, 4.0 g $Na_2HPO_4$, 0.3 g $FeSO_4$, 8.0 g $Bi_2(SO_3)_3$ indicator, 0.025 g brilliant green, and 20 g agar in 1 L $H_2O$, mix thoroly, and heat with occasional agitation. Boil ca 1 min to obtain uniform suspension. (Ppt will not dissolve.) Cool to 45–50°. Suspend ppt by gentle agitation and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 hr with covers partially removed; then close plates. Final pH, 7.6 ± 0.2. *Do not autoclave*. Prepare plates day before streaking and store in dark at room temp. Selectivity of plates decreases 48 hr after prepn.

(**g**) *Triple sugar iron agar* (*TSI agar*).—Suspend ingredients (*1*) or (*2*) in 1 L $H_2O$, mix thoroly, and heat with occasional agitation. Boil ca 1 min until ingredients dissolve. Fill 16 × 150 mm tubes 1/3 full and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 3 cm) and adequate slants (ca 5 cm) are formed on solidification.

(*1*) 20 g polypeptone, 5.0 g NaCl, 10 g lactose, 10 g sucrose, 1 g glucose, 0.2 g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.2 g $Na_2S_2O_3$, 0.025 g phenol red, and 13 g agar. Final pH, 7.3 ± 0.2.

(*2*) 3.0 g beef ext, 3.0 g yeast ext, 15 g peptone, 5.0 g proteosepeptone, 1.0 g glucose, 10 g lactose, 10 g sucrose, 0.2 g $FeSO_4$, 5.0 g NaCl, 0.3 g $Na_2S_2O_3$, 0.024 g phenol red, and 12 g agar. Final pH, 7.4 ± 0.2.

(**h**) *Tryptophane broth*.—*See* **940.36A(h)**. Use 16 or 20 × 150 mm test tubes.

(**i**) *Buffered glucose broth* (*MR-VP medium*).—*See* **940.36A(b)**. Use 16 or 20 × 150 mm test tubes.

(**j**) *Simmons' citrate agar*.—Dissolve 2.0 g Na citrate, 5.0 g NaCl, 1.0 g $K_2HPO_4$, 1.0 g $NH_4H_2PO_4$, 0.2 g $MgSO_4$, 0.08 g bromothymol blue, and 15 g agar in 1 L $H_2O$, and heat gently with occasional agitation. Boil 1–2 min until ingredients dissolve. Final pH, 6.9 ± 0.2. Fill 13 × 100 or 16 × 150 mm test tubes 1/3 full and cap or plug so that aerobic conditions are maintained during use. Autoclave 15 min at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 2 or 3 cm, resp.) and adequate slants (ca 4 or 5 cm, resp.) are formed on solidification.

(**k**)(*1*) *Urea broth*.—Dissolve 20 g urea, 0.1 g yeast ext, 9.1 g $KH_2PO_4$, 9.5 g $Na_2HPO_4$, and 4.0 mL 0.25% phenol red (10 mg) soln in 1 L $H_2O$. *Do not heat*. Sterilize by filtration and aseptically dispense 1.5–3 mL portions into 13 × 100 mm sterile test tubes. Final pH, 6.8 ± 0.2.

(*2*) *Rapid urea broth*.—Prep. as in (*1*), using 0.091 and 0.095 g phosphate salts, resp.

(**l**) *Malonate broth*.—Dissolve 1.0 g yeast ext, 2.0 g $(NH_4)_2SO_4$, 0.6 g $K_2HPO_4$, 0.4 g $KH_2PO_4$, 2.0 g NaCl, 3.0 g Na malonate, 0.25 g glucose, and 0.025 g bromothymol blue in 1 L $H_2O$, heating if necessary until dissolved. Dispense 3 mL portions into 13 × 100 mm test tubes and autoclave 15 min at 121°. Final pH, 6.7 ± 0.2.

(**m**)(*1*) *Lysine iron agar* (*Edwards and Fife*).—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext, 1.0 g glucose, 10 g L-lysine, 0.5 g ferric ammonium citrate, 0.04 g anhyd. $Na_2S_2O_3$, 0.02 g bromocresol purple, and 15 g agar in 1 L $H_2O$, heating until dissolved. Dispense 4 mL portions into 13 × 100 mm test tubes and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that 4 cm butts and 2.5 cm slants are formed on solidification. Final pH, 6.7 ± 0.2.

(*2*) *Lysine decarboxylase broth* (*Falkow*).—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext, 1.0 g glucose, 5.0 g L-lysine, and 0.02 g bromocresol purple in 1 L $H_2O$, heating until dissolved. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Autoclave, loosely capped, 15 min at 121°. Screw caps on tightly for storage and after inoculation. Final pH, 6.5–6.8.

(**n**) *Motility test medium* (*semisolid medium*).—Dissolve 3.0 g beef ext, 10 g peptone or gelysate, 5.0 g NaCl, and 4.0 g agar in 1 L $H_2O$ and heat gently with occasional agitation. Boil 1–2 min to dissolve. If medium is to be stored, dispense 20 mL portions into screw-cap containers, replacing caps loosely. Autoclave 15 min at 121°. Cool to 45°. To store, screw caps on tightly and refrigerate at 5–8°. To use, remelt in boiling $H_2O$ or flowing steam and cool to 45°. Aseptically dispense 20 mL portions into 15 × 100 mm petri dishes and let solidify with dish completely covered. Use plates same day as prepd. Final pH, 7.4 ± 0.2.

(**o**) *Potassium cyanide* (*KCN*) *broth*.—Dissolve 3.0 g proteose peptone No. 3 or polypeptone, 5.0 g NaCl, 0.225 g $KH_2PO_4$, and 5.64 g $Na_2HPO_4$ in 1 L $H_2O$. Autoclave 15 min at 121°. Cool and refrigerate at 5–8°. Final pH, 7.6 ± 0.2. Dissolve 0.5 g KCN in 100 mL cold (5–8°) sterile $H_2O$. Using sterile bulb pipet or sterile syringe (*do not pipet by mouth*), aseptically add 15 mL cold KCN soln per L cold, sterile basal broth. Mix thoroly with gentle agitation and aseptically dispense 1.0–1.5 mL portions into sterile 13 × 100 mm test tubes. Using aseptic technic, immediately stopper tubes with No. 2 corks impregnated with paraffin. Prep. corks by boiling in paraffin ca 5 min. Place corks in tubes so that paraffin does not flow into broth but forms good seal between rim of tube and cork. Medium stored at 5–8° is usually stable ca 2 weeks.

(**p**)(*1*) *Phenol red carbohydrate broth*.—Dissolve 10 g trypticase or proteose peptone No. 3, 5.0 g NaCl, 1.0 g beef ext (optional), and 7.2 mL 0.25% phenol red (18 mg) soln in 1 L $H_2O$ and heat with gentle agitation until dissolved. Dissolve 5 g dulcitol, 10 g lactose, or 10 g sucrose (as specified in title of test) in this basal broth. Dispense 2.5 mL portions into 13 × 100 mm test tubes contg inverted 6 × 50 mm fermentation tubes. Autoclave 10 min at 118° (12 psi). Final pH, 7.4 ± 0.2. Alternatively, dissolve ingredients, omitting carbohydrate, in 800 mL $H_2O$ with heat and occasional agitation. Dispense 2.0 mL portions into 13 × 100 mm test tubes contg inverted fermentation tubes. Autoclave 15 min at 118° and let cool. Dissolve carbohydrate in 200 mL $H_2O$ and sterilize by passing soln thru bacteria-retaining filter. Aseptically add 0.5 mL sterile filtrate to each tube of sterilized broth after cooling to <45°. Shake gently to mix. Final pH, 7.4 ± 0.2.

(*2*) *Purple carbohydrate broth*.—Prep. as in (*1*), using as basal broth 10 g proteose peptone No. 3 or gelysate, 5.0 g NaCl, and 0.015 or 0.020 g bromocresol purple. Final pH, 6.8 ± 0.2.

(**q**) *MacConkey agar*.—Suspend 3.0 g proteose peptone or polypeptone, 17 g peptone or gelysate, 10 g lactose, 1.5 g bile salts No. 3 or bile salts mixt., 5.0 g NaCl, 3.0 mL 1% neutral red (30 mg) soln, 1 mL 0.1% crystal violet (1.0 mg) soln, and 13.5 g agar in 1 L $H_2O$ and mix thoroly until homogeneous. Heat, with occasional agitation, and boil 1–2 min until ingredients dissolve. Autoclave 15 min at 121°. Cool to 45–50° and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ≥2 hr with plates covered. Do not use wet plates. Final pH, 7.1 ± 0.2.

(**r**) *Brain-heart infusion broth*.—Dissolve infusion from 200 g calf brain and from 250 g beef heart, 10.0 g proteose peptone or gelysate, 5.0 g NaCl, 2.5 g $Na_2HPO_4.12H_2O$, and 2.0 g glucose in 1 L $H_2O$, heating gently if necessary. Dispense 5 mL portions into 16 × 150 mm test tubes and autoclave 15 min at 121°. Final pH, 7.4 ± 0.2.

(**s**) *Trypticase soy-tryptose broth*.—Combine 15 g com. dehydrated trypticase soy broth medium (contg 17.0 g trypticase, 3.0 g phytone, 5.0 g NaCl, 2.5 g $K_2HPO_4$, and 2.5 g glucose), 13.5 g com. dehydrated tryptose broth medium (contg 20 g tryptose, 5 g NaCl, and 1.0 g glucose), 3 g yeast ext, and 1 L $H_2O$. Heat, if necessary, until dissolved. Dispense 5 mL portions into 16 × 150 mm test tubes and autoclave 15 min at 121°. Final pH, 7.2 ± 0.2.

(**t**) *Trypticase* (*tryptic*) *soy broth*.—Suspend 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g $K_2HPO_4$, and 2.5 g glucose in 1 L $H_2O$. Heat gently to dissolve completely. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust, if necessary, to 225 mL. Final pH, 7.3 ± 0.2.

(**u**) *Lauryl sulfate tryptose broth*.—Prep. as **966.23A(b)**, but without inverted fermentation tubes, 10 × 75 mm.

(**v**) *Reconstituted nonfat dry milk with brilliant green dye* (*NFDM-BG*).—Suspend 100 g dehydrated NFDM in 1 L $H_2O$; mix by swirling until dissolved. Autoclave 15 min at 121°. Add brilliant green dye soln, **966.24(n)**, after blending sample/broth mixture as in **978.23A(e)**.

(**w**) *Brilliant green* (*BG*) *water*.—Prep. sterile $H_2O$ as **966.24(m)** and add 2 mL 1% aq. brilliant green dye, **966.24(n)**, per L sterile $H_2O$ and mix well.

### B. Diagnostic Reagents

(**a**) *Kovacs reagent for indole test*.—Dissolve 5 g *p*-dimethylaminobenzaldehyde in 75 mL amyl alcohol and slowly add 25 mL HCl.

(**b**) *Voges-Proskauer* (*VP*) *test reagents*.—(*1*) *Alpha-naphthol soln*.—5%. Dissolve 5.0 g α-naphthol in 100 mL absolute alcohol.

(*2*) *Potassium hydroxide soln*.—40%. Dissolve 40 g KOH in $H_2O$ and dil. to 100 mL.

(**c**) *Sodium hydroxide soln*.—1*N*. Dissolve 42.11 g 95% reagent NaOH in sterile $H_2O$ and dil. to 1 L.

(**d**) *Hydrochloric acid soln*.—1*N*. Dil. 89 mL to 1 L with sterile $H_2O$.

(**e**) *Methyl red indicator*.—Dissolve 0.10 g Me red in 300 mL alcohol and dil. to 500 mL with $H_2O$.

(**f**) *Sterile physiological saline soln*.—*See* **940.36B(c)**.

(**g**) *Formalinized physiological saline soln*.—Add 6 mL HCHO soln (36–38%) to 1 L sterile saline soln, (**f**), mix, and store in tightly stoppered containers.

(**h**) *Salmonella polyvalent somatic* (*O*) *antiserum**.—("Serological Identification of the *Salmonella* Serotypes," No. 1229, Difco Laboratories, November 1977, p. 13, or equiv.) Contains agglutinins for at least somatic (O) antigens 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 19, 22, 23, 24, 25, 34, and Vi. They are agglutinins for somatic (O) groups: A, B, $C_1$, $C_2$, D, $E_1$, $E_2$, $E_3$, $E_4$, F, $G_1$, $G_2$, H, I, and Vi.

(**i**) *Salmonella individual somatic* (*O*) *antisera**.—(*See* ref.

* Conform to specifications issued by Diagnostic Products Evaluation Branch, Biological Products Div., Bureau of Laboratories, Centers for Disease Control, Atlanta, GA 30333.

in (**h**).) For at least each of the somatic (O) groups listed in (**h**).

(**j**) *Salmonella polyvalent flagellar (H) antiserum Poly a-z**.—(*See* p. 12 of ref. in (**h**).) Contains agglutinins for at least the following flagellar (H) antigens: a, b, c, d, e, f, g, h, i, k, l, m, n, p, q, r, s, t, u, v, w, x, y, z, $z_4$, $z_6$, $z_{10}$, $z_{13}$, $z_{15}$, $z_{23}$, $z_{24}$, $z_{28}$, $z_{29}$, $z_{32}$, 1, 2, 5, 6, 7.

(**k**) *Salmonella "Spicer-Edwards" flagellar (H) antisera**.—(From pp. 11 and 12 of ref. in (**h**).) Consists of 7 pooled or polyvalent antisera which react as in Table **967.25**.

(**l**) *pH Test paper*.—Min. range 6.0–7.6 with max. gradations of 0.4 pH unit per color change.

(**m**) *Sterile distilled water*.—Dispense 1 L $H_2O$ into 2 L wide-mouth flask or wide-mouth jar; plug or cap loosely. Autoclave 20 min at 121°.

(**n**) *Brilliant green dye soln*.—1%. Dissolve 1 g in sterile $H_2O$ and dil. to 100 mL. (Since some batches of dye are unusually toxic, test all batches of dye before use and use only those producing satisfactory results when tested with known pos. and neg. test organisms.)

(**o**) *Bromcresol purple soln*.—0.2%. Dissolve 0.2 g in sterile $H_2O$ and dil. to 100 mL.

Refs.: JAOAC **50,** 753(1967); **51,** 870(1968); **52,** 455(1969); **56,** 1027(1973); **59,** 731(1976); **62,** 499(1979); **64,** 893(1981); **64,** 899(1981); **65,** 356(1982).

## 967.26 *Salmonella* in Foods

### Detection

**First Action 1967**
**Final Action 1974**

### A. Preparation of Sample

(**a**) *Dried whole egg, dried egg yolk, and dried egg white*.—Aseptically open sample container and aseptically weigh 25 g sample into sterile, empty, wide-mouth, screw-cap pt (500 mL) jar. Add ca 15 mL sterile lactose broth, **967.25A(a)**. Stir with sterile glass rod, sterile spoon, or sterile tongue depressor to smooth suspension. Add 3 addnl portions lactose broth, 10, 10, and 190 mL for total of 225 mL. Stir after each addn until sample is suspended without lumps. Cap jar securely and let stand at room temp. 60 min. Mix well by shaking, and det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1*N* NaOH or HCl, **967.25B(c)** or (**d**), capping jar securely and mixing well before detg final pH. Loosen jar cap ca 1/4 turn and incubate 24 ± 2 hr at 35°.

(**b**) *Dry whole milk*.—Aseptically weigh 25 g sample into sterile, wide-mouth screw-cap 500 mL (1 pt) jar. Add 225 mL sterile $H_2O$ and mix well. Cap jar securely and let stand 60 min at room temp. Mix well by swirling and det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1*N* NaOH or HCl, **967.25B(c)** or (**d**). Add 0.45 mL of 1% aq. brilliant green dye soln, **967.25B(n)**, and mix well. Loosen jar cap ca 1/4 turn and incubate 24 ± 2 hr at 35°.

(**c**) *Dried active yeast*.—Aseptically weigh 25 g sample into sterile, empty, wide-mouth, screw-cap pt (500 mL) jar. Add 225 mL sterile trypticase (tryptic) soy broth, **967.25A(t)**, and let yeast form smooth suspension. Cap securely and let stand 60 min at room temp. Det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary to 6.8 ± 0.2 with sterile 1*N* NaOH or HCl, **967.25B(c)** or (**d**), capping jar securely and mixing well before detg final pH. (If pH is adjusted before yeast is evenly suspended, final pH will be less than desired.) Incubate 24 ± 2 hr at 35°, with jar cap loosened 1/4 turn.

(**d**) *Onion powder and garlic powder (First Action 1979; Final Action 1980)*.—Aseptically weigh 25 g sample into sterile, wide-mouth, screw-cap 500 mL (1 pt) jar. Sample is pre-enriched in trypticase (tryptic) soy broth, **967.25A(t)**, with added $K_2SO_3$ (5 g/L) for final 0.5% $K_2SO_3$ concn. Autoclave 225 mL portions in 500 mL flasks for 15 min at 121°. Aseptically det. vol. and adjust, if necessary, to 225 mL. Add 225 mL sterile trypticase (trypic) soy broth, **967.25A(t)**, with $K_2SO_3$, to sample, and mix thoroly using sterile glass rod or spoon. Let stand 60 min and det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1*N* NaOH or 1*N* HCl, **967.25B(c)** or (**d**). Incubate 24 ± 2 hr at 35°, with jar cap loosened 1/4 turn.

(**e**) *Milk chocolate and casein*.—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile reconstituted NFDM, **967.25A(v)**, to chocolate samples, and add 225 mL lactose broth, **967.25A(a)**, to casein samples. Blend each sample/broth mixt. 2 min at high speed and decant blended homogenate into sterile 500 mL jar. Cap jar securely and let stand 60 min at room temp. Mix well by shaking, and det. pH with test paper, **967.25B(l)**. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, **967.25B(c)** or (**d**), capping jar securely and mixing well before detg final pH. To chocolate-reconstituted NFDM samples, add 0.45 mL 1% aq. brilliant green dye, **967.25B(n)**, and mix well. Loosen jar caps 1/4 turn and incubate jar 24 ± 2 h at 35°.

(**f**) *Instant nonfat dry milk* (NFDM) (*First Action 1984*).—Aseptically open sample container and aseptically weigh 25 g sample into sterile beaker (250 mL) or other appropriate container. Cover with sterile foil cover or sterile cap to prevent contamination. Using sterile glass or paper (made with tape to withstand autoclaving) funnel, pour 25 g analytical unit gently and slowly over surface of 225 mL brilliant green $H_2O$, **967.25A(w)**, contained in sterile 500 mL erlenmeyer or other appropriate container. Let container with sample-pre-enrichment broth stand undisturbed 60 ± 5 min. Incubate loosely capped container, without mixing or pH adjustment, for 24 ± 2 h at 35°.

### B. Isolation

(**a**) *Growth in selective broth*.—Gently shake incubated sample mixt., **967.26A**, and transfer 1 mL to 10 mL selenite cystine broth, **967.25A(b)**(*1*) or (*2*), and addnl 1 mL to 10 mL tetrathionate broth, **967.25A(c)**. Incubate 24 ± 2 h at 35°. (For

**Table 967.25 Spicer-Edwards *Salmonella* H Antisera and H Antigens with Which Each Antiserum Reacts**

| | Spicer-Edwards *Salmonella* H Antisera | | | |
|---|---|---|---|---|
| H Antigens | 1 | 2 | 3 | 4 |
| a | + | + | + | − |
| b | + | + | − | + |
| c | + | + | − | − |
| d | + | − | + | + |
| eh | + | − | + | − |
| G Complex[a] | + | − | − | + |
| i | + | − | − | − |
| k | − | + | + | + |
| r | − | + | − | + |
| y | − | + | − | − |
| z | − | − | + | + |
| $z_4$ Complex[b] | − | − | + | − |
| $z_{10}$ | − | − | − | + |
| $z_{29}$ | − | + | + | − |

| H Antigens | *Salmonella* H Antisera |
|---|---|
| enx, $enz_{15}$ | EN complex |
| lv, lw, $lz_{13}$, $lz_{28}$ | L complex |
| 1, 2; 1, 5; 1, 6; 1, 7 | 1 complex |

[a] The G complex component of Spicer-Edwards *Salmonella* H antisera 1 and 4 reacts with antigens f, g, m, p, q, s, t, and u.
[b] The $z_4$ complex component reacts with $z_4$, $z_{23}$, $z_{24}$, and $z_{32}$.
(From Difco Laboratories)

dried active yeast, substitute lauryl sulfate tryptose broth, **967.25A(u)**, for selenite cystine broth, **967.25A(b)**(*1*) or (*2*).

Vortex-mix, and streak 3 mm loopful of incubated selenite cystine broth on selective media plates of xylose lysine desoxycholate agar, **967.25A(d)**, Hektoen enteric agar, **967.25A(e)**, and BS agar, **967.25A(f)**. Repeat with 3 mm loopful of incubated tetrathionate broth. Incubate plates 24 ± 2 h at 35°.

(**b**) *Appearance of typical Salmonella colonies*.—(*1*) *On xylose lysine desoxycholate agar*.—Pink colonies with or without black centers. Many *Salmonella* may have large, glossy black centers or may appear as almost completely black colonies. Atypically, a few *Salmonella* cultures produce yellow colonies with or without black centers.

(*2*) *On Hektoen enteric agar*.—Blue-green to blue colonies with or without black centers. Many *Salmonella* cultures may have large glossy black centers or may appear as almost completely black colonies.

(*3*) *On bismuth sulfite agar*.—Brown, gray, or black, sometimes with metallic sheen. Surrounding medium is usually brown at first, turning black with increasing incubation time. Some strains produce green colonies with little or no darkening of surrounding medium.

Examine XLD and HE agar plates for typical or suspicious *Salmonella* colonies after 24 ± 2 h incubation at 35°. BS agar plates should be examined for typical or suspicious *Salmonella* colonies after 24 ± 2 h and 48 ± 2 h incubation at 35°.

### C. Treatment of Typical or Suspicious Colonies

(**a**) *Inoculation of triple sugar iron (TSI) agar and lysine iron agar (LIA)*.—Pick with needle 2 or more typical or suspicious colonies, if present, from each xylose lysine desoxycholate, Hektoen enteric, and BS agar plates having growth. Inoculate TSI agar slant, **967.25A(g)**, with portion of each colony by streaking slant and stabbing butt. After inoculating TSI agar with needle, do not obtain more inoculum from colony and do not heat needle, but inoculate LIA, **967.25A(m)**(*1*), as in **967.27C(a)**. Store picked selective plates at 5–8° or at room temp. (ca 26°).

(**b**) *Presumptive reactions*.—Incubate TSI and LIA slants at 35° for 24 ± 2 h and 48 ± 2 h, resp. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive $H_2S$ production. *Salmonella* cultures typically have alk. (red) slant and acid (yellow) butt, with or without $H_2S$ (blackening of agar) in TSI agar. In LIA, *Salmonella* cultures typically have alk. (purple) reaction in butt. Consider only a distinct yellow coloration in butt of tube as an acidic (neg.) reaction. Do not eliminate cultures that produce discoloration in butt solely on this basis. Most *Salmonella* cultures produce $H_2S$ in LIA. Retain all presumptive pos. *Salmonella* cultures on TSI (alk. slant and acid butt) agar for biochem. and serological tests whether or not corresponding LIA reaction is pos. (alk. butt) or neg. (acid butt). Do not exclude a TSI culture that appears to be non-*Salmonella* if the reaction in LIA is typical (alk. butt) for *Salmonella*. Treat these cultures as presumptive pos. and submit them to further examination. LIA is useful in detection of *S. arizonae* and atypical *Salmonella* strains that utilize lactose and/or sucrose. Discard only apparent non-*Salmonella* TSI agar cultures (acid slant and acid butt) if corresponding LIA reactions are not typical (acid butt) for *Salmonella*. Test retained presumptive pos. TSI agar cultures as directed in **967.26C(c)** to det. if they are *Salmonella* sp., **967.27D(e)**(*1*), or *S. arizonae* organisms, **967.27D(e)**(*2*).

If TSI slants fail to give typical *Salmonella* reactions, pick addnl suspicious colonies from selective medium plate not giving presumptive pos. culture and inoculate TSI and LIA slants as in (**a**).

(**c**) *Selection for identification*.—Apply biochem. and serological identification tests to 3 presumptive pos. TSI agar cultures picked from selective agar plates streaked from selenite cystine broth and to 3 presumptive pos. TSI agar cultures picked from selective agar plates streaked from tetrathionate broth.

If 3 presumptive pos. TSI agar cultures are not isolated from 1 set of selective agar plates, test other presumptive pos. TSI agar cultures, if isolated, by biochem. and serological tests. A min. of 6 TSI cultures are examined for each 25 g sample tested.

Refs.: JAOAC **50,** 753(1967); **51,** 870(1968); **52,** 455(1969); **56,** 1027(1973); **59,** 731(1976); **61,** 401(1978); **62,** 499(1979); **64,** 893(1981); **64,** 899(1981); **65,** 356(1982); **67,** 807(1984); **69,** 277(1986).

## 967.27 *Salmonella* in Foods

### Identification

**First Action 1967**
**Final Action 1968**

### A. Cultures

Pure cultures on TSI agar are required for inoculation of biochem. test media.

(**a**) *Pure cultures*.—Proceed to **967.27B**.

(**b**) *Mixed cultures*.—Streak any culture that appears to be mixed on MacConkey agar, **967.25A(q)**, or xylose lysine desoxycholate agar, **967.25A(d)**, or Hektoen enteric agar, **967.25A(e)**. Incubate 24 ± 2 h at 35°.

(**c**) *Appearance of Salmonella colonies*.—(*1*) *On MacConkey agar*.—Typical colonies appear transparent and colorless, sometimes with dark centers. *Salmonella* will clear areas of pptd bile caused by other organisms sometimes present in medium.

(*2*) *On xylose lysine desoxycholate agar*.—See **967.26B (b)**(*1*).

(*3*) *On Hektoen enteric agar*.—See **967.26B(b)**(*2*).

Pick with needle ≥2 typical or suspicious colonies and inoculate TSI slants by streaking the slant and stabbing the butt as in **967.26C(a)**. Retest purified cultures as in **967.26C(b)**, and proceed with identification.

As alternative to conventional tube system for *Salmonella*, any one of the 4 commercial biochem. systems (API, Enterotube, Minitek, or Micro-ID) may be used for presumptive generic identification of foodborne *Salmonella*. *See* **978.24** and **989.12**.

### B. Subcultures

(**a**) *Urease test*.—Subculture small amt of growth from presumptive pos. TSI agar culture to urea broth, **967.25A(k)**(*1*), and incubate 24 ± 2 hr at 35° or inoculate rapid urea broth, **967.25A(k)**(*2*) with two 3 mm loopfuls of growth from each presumptive-pos. TSI agar slant culture, and incubate 2 hr in $H_2O$ bath at 37 ± 0.5°. Discard all cultures that give pos. test (purple-red color). Salmonellae are urease neg. (no change in orange color of medium).

(**b**) *Serological flagellar (H) screening test*.—To reduce number of presumptive pos. TSI agar cultures carried thru identification tests, perform serological flagellar (H) screening test by transferring one 3 mm loopful of each urease-neg. TSI agar culture to either:

(*1*) Brain-heart infusion broth, **967.25A(r)**, (for test on same day) and incubate at 35° until visible growth occurs (ca 4–6 hr); or

(*2*) Trypticase soy-tryptose broth, **967.25A(s)**, (for test on following day) and incubate 24 ± 2 hr at 35°.

To 5 mL of each of the 6 broth cultures add ca 2.5 mL formalinized physiological saline soln, **967.25B(g)**. Select 2 formalinized broth cultures and test with *Salmonella* flagellar (H) antisera, **967.25B(j)** or **(k)**, as in **967.28C** or **D**.

If selected formalinized broth cultures are pos., perform addnl tests on these cultures, beginning with **967.27C**, except step **967.27C(d)** may be omitted.

If both formalinized broth cultures are neg., perform serological test on the 4 additional broth cultures (**967.27B(b)**(*1*) or (*2*)) to obtain, if possible, 2 pos. cultures for addnl testing, **967.27C**.

If all urease-neg. TSI cultures from sample are *Salmonella* serological flagellar (H) test neg., then perform addnl tests, beginning with **967.27C**, on these cultures.

### *C. Testing Urease-Negative Cultures*

Using needle, transfer portion of presumptive pos. TSI agar culture to lysine iron agar medium and small amt of growth from the TSI agar culture to each of other media:

**(a)** *Lysine iron agar*, **967.25A(m)**(*1*).—Stab butt twice and then streak slant. Replace tube cap *loosely* and incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. Most salmonellae give purple color of alk. reaction thruout medium (final color is slightly darker than original purple color of medium). If $H_2S$ is produced, butt of medium is blackened. Neg. test is purple or red slant and yellow butt. If LIA test, **967.26C(a)**, was satisfactory, it need not be repeated. Use lysine decarboxylase broth for final detn of lysine decarboxylase if culture gives doubtful LIA reaction.

If liq. medium is preferred, inoculate tube of lysine decarboxylase broth, **967.25A(m)**(*2*). Close tube cap *tightly* after inoculation and incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. Salmonellae give purple color of alk. reaction thruout broth (final color is slightly darker than original purple color of medium). Sometimes tubes which have yellow color after 8–12 hr of incubation change to purple later. Neg. test is permanent yellow color thruout broth. If medium appears to be discolored (neither purple nor yellow) add few drops of 0.2% bromcresol purple dye **967.25B(o)**, and re-read tube reactions.

(LIA is incubated loosely capped so that aerobic conditions are maintained, while lysine decarboxylase broth is incubated tightly closed to exclude air.)

**(b)** *Phenol red dulcitol broth*, **967.25A(p)**(*1*).—Incubate 48 ± 2 hr at 35°. Examine at least every 24 hr. Most salmonellae give pos. test indicated by gas formation (displacement of liq. in inverted tube) and/or acid reaction (yellow). Neg. test is alk. reaction (red) and no gas formation.

(Purple broth base with dulcitol, **967.25A(p)**(*2*), may be substituted. Pos. test is acid reaction (yellow) and gas. Neg. test is alk. reaction (purple).)

**(c)** *Tryptophane broth*, **967.25A(h)**.—Incubate 24 ± 2 hr at 35° and test as follows:

(*1*) Transfer 3 mm loopful, excluding all solid particles, to KCN broth, **967.25A(o)**. Heat rim of tube to form good seal when restoppered. Incubate 48 ± 2 hr at 35°. Salmonellae do not grow in this broth as shown by lack of turbidity (neg. test).

(*2*) Transfer 3 mm loopful to malonate broth, **967.25A(l)**, and incubate 48 ± 2 hr at 35°. Salmonellae give neg. test as shown by green color (unchanged). Pos. test (alk. reaction) is shown by blue color.

(*3*) Transfer 5 mL to empty test tube and add 0.2–0.3 mL Kovacs reagent, **967.25B(a)**. Pos. test for indole is shown by deep red color in reagent on surface of broth. Most salmonellae are indole-neg.

**(d)** *Brain-heart infusion broth, **967.25A(r)**, or trypticase soy-tryptose broth, **967.25A(s)**.*—Incubate brain-heart infusion broth until visible growth occurs (ca 4–6 hr) or incubate trypticase soy-tryptose broth 24 ± 2 hr at 35°. To 5 mL broth culture add ca 2.5 mL formalized physiological saline soln, **967.25B(g)**. Refrigerate formalized broth at 5–8° if test is to be performed on another day. Perform *Salmonella* serological flagellar (H) test, **967.28C**, or "Spicer-Edwards" flagellar (H) test tube test, **967.28D**, using formalized broth culture as flagellar (H) antigen to be tested.

**(e)** *Tests indicating absence of Salmonella*.—Discard, as not *Salmonella*, cultures that show either:

(*1*) Pos. indole test (red) and neg. *Salmonella* serological flagellar (H) test.

(*2*) Pos. KCN broth test (growth) and neg. lysine decarboxylase test (yellow).

**(f)** *Testing of TSI agar cultures*.—Use *Salmonella* serological somatic (O) test, **967.28A**.

**(g)** *Classification*.—Classify as *Salmonella* sp. cultures that have all characteristics shown in Table **967.27A**. If 1 TSI culture from 25 g sample is classified as *Salmonella* sp., further testing of other TSI cultures from same 25 g sample is unnecessary.

**(h)** *Special cases*.—Cultures that contain demonstrable *Salmonella* antigens as shown by pos. *Salmonella* serological somatic (O) test and pos. flagellar (H) test but do not have biochem. characteristics of salmonellae should be purified as in **967.27A(b)** and retested, beginning with **967.27B**.

### *D. Additional Biochemical Tests*

Perform addnl tests on cultures that do not give identical test results as in Table **967.27A** and do not classify as *Salmonella* sp. Transfer 1 loopful of culture from each unclassified TSI agar slant to each of following media:

**(a)** *Phenol red lactose broth*, **967.25A(p)**(*1*).—Incubate 48 ± 2 hr at 35°. Examine inoculated broth at least every 24 hr. Pos. test is shown by gas formation (displacement of liq. in inverted tube) and acid reaction (yellow). Most salmonellae give neg. test shown by alk. reaction (red) and no gas formation.

Discard, as not *Salmonella*, cultures that give pos. phenol red lactose broth test, except: (*1*) Cultures described in **967.26C(b)**, and (*2*) cultures that also give pos. malonate broth test. Cultures that are phenol red lactose broth pos. or neg. and malonate broth pos. are tested further to det. if they are *S. arizonae*, **967.27D(e)**(*2*).

(Purple lactose broth, **967.25A(p)**(*2*), may be substituted. Pos. test is acid reaction (yellow) and gas. Neg. test is alk. reaction (purple) and no gas formation.)

**(b)** *Phenol red sucrose broth*, **967.25A(p)**(*1*).—Incubate and read as in **(a)** above. Discard, as not *Salmonella*, cultures that give pos. test, except cultures described in **967.26C(b)**. (Purple sucrose broth may be substituted and read as in **(a)** above.)

**Table 967.27A Characteristics of *Salmonella***

| Test or Substrate | Results[a] |
|---|---|
| Urease, **967.27B(a)** | Negative (orange-red) |
| Lysine decarboxylase, **967.27C(a)** | Positive (alk.; purple thruout medium) |
| Phenol red dulcitol broth, **967.27C(b)** | Positive (yellow and/or gas)[b] |
| KCN broth, **967.27C(c)**(*1*) | Negative (no growth) |
| Malonate broth, **967.27C(c)**(*2*) | Negative (unchanged green)[c] |
| Indole test, **967.27C(c)**(*3*) | Negative (no red color) |
| Polyvalent flagellar test, **967.27B(b)**, **967.27C(d)** | Positive (visible agglutination) |
| Polyvalent somatic test, **967.27C(f)** | Positive (visible agglutination) |

[a] +, ≥90% pos. in 1–2 days; −, ≥90% neg. in 1–2 days.
[b] Majority of *S. arizonae* cultures are neg.
[c] Majority of *S. arizonae* cultures are pos.

**Table 967.27B Biochemical and Serological Reactions of *Salmonella***

| Test or Substrate | Positive | Negative | *Salmonella* reaction[a] |
|---|---|---|---|
| Glucose (TSI), **967.26C(b)** | yellow butt | red butt | + |
| $H_2S$ (TSI), **967.26C(b)** | blackening | no blackening | + |
| Urease, **967.27B(a)** | purple-red | no color change | − |
| Lysine decarboxylase broth, **967.27C(a)** | purple | yellow | + |
| Phenol red dulcitol broth, **967.27C(b)** | yellow and/or gas | no gas; no color change | +[b] |
| KCN broth, **967.27C(c)**(*1*) | turbidity | no turbidity | − |
| Malonate broth, **967.27C(c)**(*2*) | blue | no color change | −[c] |
| Indole test, **967.27C(c)**(*3*) | violet at surface | yellow at surface | − |
| Polyvalent flagellar test, **967.27B(b)**, **967.27C(d)** | agglutination | no agglutination | + |
| Polyvalent somatic test, **967.27C(f)** | agglutination | no agglutination | + |
| Phenol red lactose broth, **967.27D(a)** | yellow and/or gas | no gas; no color change | −[c] |
| Phenyl red sucrose broth, **967.27D(b)** | yellow and/or gas | no gas; no color change | − |
| Voges-Proskauer test, **967.27D(c)**(*1*) | pink to red | no color change | − |
| Methyl red test, **967.27D(c)**(*2*) | diffuse red | diffuse yellow | + |
| Simmons' citrate, **967.27D(d)** | growth; blue | no growth; no color change | v |

[a] +, ≥90% pos. in 1–2 days; −, ≥90% neg. in 1–2 days; v, variable.
[b] Majority of *S. arizonae* cultures are neg.
[c] Majority of *S. arizonae* cultures are pos.

(**c**) *Buffered glucose broth* (*MR-VP medium*, **967.25A(i)**).—Incubate 48 ± 2 hr at 35°.

(*1*) Perform Voges-Proskauer (VP) test at room temp. by transferring 1 mL 48-hr culture to test tube and adding 0.6 mL α-naphthol soln, **967.25B(b)**(*1*), and 0.2 mL 40% KOH soln, **967.25B(b)**(*2*). Shake after each addn. To intensify and speed reaction, add few creatine crystals to test medium. Read results 4 hr after adding reagents. Pos. VP test is development of eosin pink color. Salmonellae give neg. test.

(*2*) Incubate remainder of MR-VP medium addnl 48 ± 2 hr at 35°. Perform Me red test by transferring 5 mL culture to test tube and adding 5–6 drops Me red soln, **967.25B(e)**, and read results immediately. Salmonellae give pos. test (red). Neg. test is indicated by yellow color.

(**d**) *Simmons' citrate agar*, **967.25A(j)**.—Inoculate by streaking slant and stabbing butt. Incubate 96 ± 2 hr at 35°. Salmonellae usually give pos. test shown by growth and color change from green to blue (alk.). Color change usually appears first on slant and then spreads thru medium. Neg. test is indicated by no or very little growth and no change in color of medium.

(**e**) *Classification*.—Classify cultures according to results listed in Table **967.27B**. If 1 TSI culture from 25 g sample is classified as *Salmonella* sp., further testing of other TSI cultures from same 25 g sample is unnecessary.

(*1*) *Salmonella* sp.—Cultures that have reaction patterns of Table **967.27B**.

(*2*) *Salmonella arizonae*.—Cultures that have reaction pattern of Table **967.27B**, except footnote reactions [b] and [c].

(*3*) *Non-Salmonella* sp.—Discard, as not *Salmonella*, cultures that give results listed in any 1 subdivision of Table **967.27C**.

### *E. Summary of Classification of Non-Salmonella Cultures*

Classify, by performing addnl tests described in *Edwards and Ewing's Identification of Enterobacteriaceae*, any culture that is not clearly identified as *Salmonella* sp. or *S. arizonae* by classification schemes in Tables **967.27A** and **B** or not eliminated from these groups by test reactions listed in Table **967.27C**.

If neither of 2 TSI cultures carried thru biochem. tests, **967.27C** and **D** and Tables **967.27A–C**, confirms as *Salmonella*, perform biochem. tests, beginning with **967.27C**, on remaining urease-neg. TSI cultures from same 25 g sample.

Refs.: JAOAC **50,** 753(1967); **51,** 870(1968); **52,** 455(1969); **56,** 1027(1973); **59,** 731(1976); **62,** 499(1979); **64,** 893(1981); **64,** 899(1981); **65,** 356(1982).

**Table 967.27C Criteria for Discarding Non-*Salmonella* Cultures**

| Test(s) or Substrate(s) | Results |
|---|---|
| (**a**) Urease test, **967.27B(a)** | Positive (purple-red) |
| (**b**) Indole test, **967.27C(c)**(*3*) | Positive (red/or violet at surface) |
| Polyvalent flagellar test, **967.27B(b)**, **967.27C(d)**, or Spicer-Edwards flagellar (H) test, **967.28D** | Negative (no agglutination) |
| (**c**) Lysine decarboxylase test, **967.27C(a)** | Negative (yellow) |
| KCN broth, **967.27C(c)**(*1*) | Positive (growth) |
| (**d**) Phenol red lactose broth[a], **967.27D(a)** | Positive (yellow and/or gas)[b] |
| (**e**) Phenol red sucrose broth, **967.27D(b)** | Positive (yellow and/or gas)[b] |
| (**f**) KCN broth, **967.27C(c)**(*1*) | Positive (growth) |
| Voges-Proskauer test, **967.27D(c)**(*1*) | Positive (red) |
| Methyl red test, **967.27D(c)**(*2*) | Negative (yellow) |

[a] Malonate broth positive cultures are tested further to det. if they are *Salmonella arizonae*, **967.27D(e)**(*2*)
[b] Do not discard pos. broth cultures if corresponding LIA cultures give typical *Salmonella* reactions; test further to det. if they are *Salmonella* sp. *See* **967.26C(a)**

## 978.24 *Salmonella* sp. in Foods
### Biochemical Identification Kit Method
### Final Action

(Use of com. biochem. kit as alternative to conventional biochem.testing in **967.27B–E** is based upon demonstration in analyst's laboratory of adequate correlation between biochem. kit intended for use and conventional biochem. tests in **967.27B–E**. Com. biochem. kits should not be used as a substitute for serological tests as described in **967.27B–E**, **967.28**.)

### *A. Kits*

(**a**) *API 20E*.—Available from Analytab Products Inc., 200 Express St, Plainview, NY 11803. Kit is series of 20 plastic microtubes contg biochem. test substrate affixed to plastic strip for conducting following 22 tests: urease; oxidase; tryptophan deaminase; *o*-nitrophenyl-β-D-galactosidase (ONPG); lysine and ornithine decarboxylase; arginine dihydrolase; gelatinase; citrate utilization; $H_2S$ production; indole production; acetoin production (Voges-Proskauer or VP test); nitrate reduction; and fermentation of amygdalin, arabinose, glucose, inositol, mannitol, melibiose, rhamnose, sorbitol, and sucrose. Required reagents include Kovacs reagent, **967.25B(a)**; 10% $FeCl_3$ soln (for phenylalanine deaminase test); VP test reagents (5% α-naphthol soln and 40% KOH soln), **967.25B(b)**; nitrate reduction reagents (solns of sulfanilic acid and *N,N*-dimethyl-α-naphthylamine); sterile mineral oil; oxidase test reagents (1% *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.2HCl soln and 0.2% ascorbic acid soln); sterile $H_2O$; and 1.5% $H_2O_2$.

(**b**) *Enterotube II*®.—Available from Roche Diagnostics Systems, Div. of Hoffmann-La Roche, Inc., One Sunset Ave, Montclair, NJ 07042-5188, order No. 43128. Consists of self-contained sterile compartmental plastic tube contg 12 different conventional media and enclosed inoculating needle for conducting following 15 tests: lysine and ornithine decarboxylase; phenylalanine deaminase; urease; Voges-Proskauer (VP); citrate utilization; $H_2S$ production; indole production; and utilization of dulcitol, lactose, adonitol, arabinose, sorbitol, and glucose (acid and gas). Kovacs reagent, **967.25B(a)** (for indole test) and VP reagents, **967.25B(b)**, are also required.

(**c**) *Enterobacteriaceae II Set*.—(Formerly Minitek system.) Available from BBL Microbiological Systems, No. 25147. Consists of system for differentiation of microorganisms by observation of their effect upon chem. substrates impregnated into paper disks for conducting following 25 tests: urease; *o*-nitrophenyl-β-D-galactosidase (ONPG); phenylalanine deaminase; lysine and ornithine decarboxylase; arginine dihydrolase; nitrate reduction; citrate utilization; $H_2S$ production; indole production; malonate utilization; Voges-Proskauer (VP) test; and fermentation of adonitol, arabinose, dulcitol, esculin, glucose, inositol, lactose, mannitol, raffinose, rhamnose, salicin, sorbitol, and sucrose. In addition to inoculum broth, required reagents include Kovacs reagent, **967.25B(a)**, 10% $FeCl_3$ soln (for phenylalanine deaminase test); VP test reagents (5% α-naphthol soln and 40% KOH soln), **967.25B(b)**; nitrate reduction reagents (solns of sulfanilic acid and *N,N*-dimethyl-α-naphthylamine); and sterile mineral oil. Required apparatus includes Minitek pipetter, disposable pipet tips, color comparator cards, disk dispenser, plastic multiwell plates, humidor with sponges for incubation of disks in plates after adding inoculum broth contg test culture, and paper disks impregnated with individual substrates for performing biochem. tests.

Systems (**a**)–(**c**) are also available from Fisher Scientific Co. Systems (**b**) and (**c**) are also available from Scientific Products, Inc., and VWR Scientific, Inc.

### B. Isolation

Prep. samples and isolate presumptive cultures by **967.26**.

### C. Identification

Assemble supplies and prep. reagents required for utilizing kit. Inoculate each unit according to directions supplied by manufacturer, incubating for time and temp. specified. Add reagents, observe, and record results. For presumptive identification, classify cultures according to flow charts and tables supplied by manufacturer as *Salmonella* or non-*Salmonella* sp.

For confirmation of cultures presumptively identified as *Salmonella* sp., perform *Salmonella* serological somatic (O) test, **967.28A**, and *Salmonella* serological flagellar (H) test, **967.28C**, or "Spicer-Edwards" flagellar (H) test, **967.28D**, and classify cultures according to following guidelines:

(**a**) Cultures classified as presumptive *Salmonella* sp. with com. biochem. kits are confirmed as *Salmonella* sp. when culture demonstrates pos. *Salmonella* somatic (O) test and pos. *Salmonella* (H) test.

(**b**) Cultures classified as presumptive non-*Salmonella* sp. with com. biochem. kits are discarded as non-*Salmonella* sp. when the cultures conform to manufacturer's criteria for classifying cultures as non-*Salmonella* sp.

(**c**) Cultures which do not conform to (**a**) or (**b**) should be classified according to addnl tests specified in **967.27B–E**, **967.28** or addnl tests specified in *Edwards and Ewing's Identification of Enterobacteriaceae*, or sent to ref. typing laboratory for definitive serotyping and identification.

Refs.: JAOAC **61**, 1043(1978); **64**, 408(1981).

## 989.12 *Salmonella* sp., *Escherichia coli*, and Other *Enterobacteriaceae* in Foods
### Biochemical Identification Kit Method
### First Action 1989

Use of com. biochem. kit as alternative to conventional biochem. testing in **966.24(a)–(f)** *(E. coli)* and **967.27** *(Salmonella* sp.) is based on demonstration in analyst's laboratory of adequate correlation between biochem. kit intended for use and conventional biochem. tests in **966.24(a)–(f)** and **967.27**. Com. biochem. kit should not be used as substitute for serological tests for *Salmonella* as described in **967.27**, **967.28**. Com. biochem. kit can be used for presumptive identification of other *Enterobacteriaceae* isolated from foods.

### A. Principle

Method uses kit in which inoculum contains preformed enzymes at levels detectable in 4 h by means of sensitive indicator system. Kit contains filter paper discs impregnated with reagents which detect presence of specific enzymes and/or metabolic products produced by certain microorganisms. These reagents include substrate to be acted on by bacterial enzyme, and detection system which reacts with metabolic end product to yield readily identifiable color change. Precise quantities of substrate and/or detection reagents are supplied to each disc so that chem. incompatible materials are sepd until tray is inoculated. Tests included are Voges-Proskauer (VP), nitrate reductase, phenylalanine deaminase, $H_2S$, indole, ornithine decarboxylase, lysine decarboxylase, malonate utilization, urease, esculin hydrolysis, β-galactosidase, and arabinose, adonitol, inositol, and sorbitol fermentations.

### B. Method Performance

| Results | Percent Agreement | 95% Confidence Range (Approx.) |
|---|---|---|
| *Salmonella* sp. | 98.8 | 97.2–100 |
| *Escherichia coli* | 97.7 | 94.6–100 |
| Other enterics[2] | 84.6 | 81.2–88.0 |

[1] Agreement with conventional biochem. tests (AOAC methods).
[2] *Enterobacteriaceae* correctly identified to genera other than *Salmonella* and *E. coli*

### C. Apparatus, Culture Media, and Reagents

Use distd or deionized $H_2O$.

(**a**) *Plate count agar (standard methods agar) slants*.—5.0 g tryptone, 2.5 g yeast ext, 1.0 g dextrose, and 15.0 g agar. Suspend ingredients in 1 L $H_2O$. Heat to boiling to dissolve medium completely. Dispense 8–10 mL portions into 16 × 150 mm tests tubes. Autoclave 15 min at 121°. Before medium solidifies, place tubes in slanted position so that adequate slants are formed.

(**b**) *Physiological saline*.—Dissolve 8.5 g NaCl in 1 L $H_2O$. Final pH *must be* 6.0 ± 0.5. Do not use saline prepns contg preservatives such as Na azide or other bacterial growth inhibitors. Saline does not need to be sterile but should be freshly prepd.

(**c**) *20% KOH soln*.—Slowly add 20 g KOH pellets to 60 mL $H_2O$. Dissolve by stirring. Add sufficient $H_2O$ to prep. 100 mL soln. Keep KOH soln in tightly closed container when not in use. *Caution:* Caustic reagent. Handle with care.

(**d**) *Test tubes*.—16 × 100 mm or larger. One test tube is required for each isolate to be identified.

(**e**) *Pipets*.—1 mL and 5 mL serological, with cotton plug.

(**f**) *Pathotec cytochrome oxidase test*.—No. 34191 (Organon Teknika Corp., 100 Akzo Ave, Durham, NC 27704); or equiv.

(**g**) *MICRO-ID identification kit and manual*.—No. 34146 (Organon Teknika Corp.).

(**h**) *Support rack*.—To hold test kit units (Organon Teknika Corp., No. 34147).

### D. Preparation of Inocula

*(1)* Select isolated colony from agar medium. Transfer colony to plate count agar slant. Incubate 18–24 h at 35°. *Note:* Cultures older than 30 h may give false neg. results.

*(2)* Perform cytochrome oxidase test on portion of growth from slant. Cytochrome oxidase-neg. rods should be further tested.

*(3)* Pipet ca 3.5 mL physiological saline (**b**) into 16 × 100 mm test tube for each isolate to be identified. Transfer growth from each slant into tube of saline until density of suspension of organisms is equiv. to McFarland No. 2.0. *Note:* Sterile test tubes are not required.

### E. General Instructions

Components and procedures of test kit have been stdzd for use in MICRO-ID identification system. Components or procedures other then those supplied by Organon Teknika Corp. may yield unsatisfactory results, and should be pretested.

### F. Inoculation and Reading of Unit

*(1)* Open sealed, moisture-proof, foil package and remove test unit. Do not remove clear plastic tape that covers test wells.

*(2)* Record sample no. and other required information on area provided on right side of cover.

*(3)* Open cover and let unit lie flat on laboratory bench.

*(4)* Pipet ca 0.2 mL of organism suspension into each inoculation well at top of unit.

*(5)* Close cover and stand tray upright in support rack. (Make sure that organism suspension is in contact with all substrate discs. DO NOT moisten detection discs.)

*(6)* Incubate 4 h at 35–37°. DO NOT use $CO_2$ incubator.

*(7)* After 4 h incubation, place each unit flat on bench, open lid, and add 2 drops (ca 0.1 mL) of 20% KOH soln (**c**) to inoculation well of VP test ONLY. Do not add KOH to any other inoculation well. Close lid and hold tray upright. Be certain that KOH flows down into VP test soln.

*(8)* Rotate unit clockwise ca 90° so upper discs in first 5 wells become wet. Hold tray upright and tap gently on bench to dislodge any suspension trapped under upper disc. Be certain that each upper disc in reaction chambers 1–5 is moistened by this procedure.

*(9)* Read all reactions immediately, except VP test, as pos. or neg. according to color changes listed below. Let color develop in VP well for ca 10 min, and then read. Read color of upper disc for first 5 tests; read color of organism suspension for remaining 10 tests. Record result for each biochem. test on encoding forms supplied with system.

| Test | Positive Reaction | Negative Reaction |
|---|---|---|
| Voges-Proskauer | pink to red | light yellow |
| Nitrate reductase | red | colorless to light pink |
| Phenylalanine deaminase | green[1] | light yellow |
| $H_2S$ | brown to black[2] | white |
| Indole | pink to red | light yellow to orange |
| Ornithine decarboxylase | purple to red-purple | amber to yellow |
| Lysine decarboxylase | purple to red-purple | amber to yellow |
| Malonate utilization | green to blue | yellow |
| Urease | orange to red-purple | yellow |
| Esculin hydrolysis | brown to black | no color change or beige |
| β-Galactosidase | light yellow to yellow | colorless |
| Fermentations: | | |
| Arabinose | yellow to amber | red-purple to purple |
| Adonitol | yellow to amber | red-purple to purple |
| Inositol | yellow to amber | red-purple to purple |
| Sorbitol | yellow to amber | red-purple to purple |

[1] In phenylalanine deaminase test, any green color in organism suspension also indicates pos. reaction.

[2] Pos. $H_2S$ reaction might vary from thin, dark line at bottom of detection disc to entire disc turning black. It is often advisable to read this disc before it has been wetted.

*(10)* Use MICRO-ID identification manual (**g**) to det. 5 digit octal no. for each isolate, and record identification of isolate.

### G. Confirmation (Salmonella sp. Only)

For confirmation of cultures presumptively identified as *Salmonella* sp., see **978.24C**.

Ref.: JAOAC **71**, 968(1988).

## 967.28 *Salmonella* in Foods

### Serological Tests

**First Action 1967**
**Final Action 1968**

(Follow manufacturer's instructions for reconstitution, mixing, diln, and operation of *Salmonella* antisera. Dil. and pretest all *Salmonella* serological antisera with known test cultures to ensure reliability of results with unknown cultures. *Caution:* Handle viable cultures carefully to prevent contaminating environment.)

### A. Polyvalent Somatic (O) Slide or Plate Test

Using wax pencil, mark off 2 sections ca 1 × 2 cm on inside of glass or plastic petri dish. Place $^1/_2$ of 3 mm loopful of culture from 24- or 48-hr TSI agar slant on dish in upper part of each marked section. Add 1 drop saline soln, **967.25B(f)**, to lower part of one section only. Add 1 drop *Salmonella* polyvalent somatic (O) antiserum, **967.25B(h)**, to other section only. With clean, sterile transfer loop or needle, emulsify culture in saline soln for one section and repeat for other section contg antiserum. Tilt mixt. in both sections back and forth 1 min and observe against dark background. Any degree of agglutination is pos. reaction.

Classify polyvalent somatic (O) test as:

(**a**) *Positive*.—Agglutination in culture-saline-serum mixt. and no agglutination in culture-saline mixt.

(**b**) *Negative*.—No agglutination in culture-saline-serum mixt. (Polyvalent somatic antisera do not contain agglutinins for antigens of some salmonellae isolated from foods. Neg. somatic reactions occur with *Salmonella* serotypes whose corresponding agglutinins are not contained in the antisera, i.e., *S. cerro*, group K(18); *S. minnesota,* group L(21); *S. alachua,* group O(35).)

(**c**) *Non-specific.*—Both mixts agglutinate. Requires addnl testing as in *Edwards and Ewing's Identification of Enterobacteriaceae.*

### B. Determination of Somatic Grouping (Optional)

Perform serological somatic (O) test on culture as in **967.28A**, using individual group somatic (O) antiserum (including Vi), **967.25B(i)**, instead of *Salmonella* polyvalent somatic (O) antiserum. Repeat test, using each group somatic antiserum or until culture reacts with specific group antiserum.

Suspend cultures pos. with Vi antiserum by emulsifying growth from slant surface in 1 mL physiological saline soln, **967.25B(f)**, to make heavy suspension. Heat in boiling $H_2O$ 20–30 min and let cool. Retest heated suspension, using somatic group D, $C_1$, and Vi antisera. Vi-pos. cultures which react with somatic group D antiserum are probably *S. typhi,* and Vi-pos. cultures which react with somatic group $C_1$ antiserum are probably *S. paratyphi C.* For these cultures to be classified as *Salmonella* sp., they must have characteristics of salmonellae as in Table **967.27A** or **B**. Heated Vi-pos. cultures which do not react with any individual somatic serum but continue to react with Vi antiserum probably belong to *Citrobacter* and are not *Salmonella.* Confirm conclusion by biochem. tests listed in Table **967.27A**.

Cultures that give pos. somatic (O) test with any individual somatic (O) antiserum are recorded as pos. for that somatic (O) group; cultures that do not react with any individual somatic (O) antiserum are recorded as neg. for individual group somatic (O) test.

### C. Polyvalent Flagellar (H) Test Tube Test

Place 0.5 mL appropriately dild *Salmonella* polyvalent flagellar (H) antiserum, **967.25B(j)**, in 10 × 75 or 13 × 100 mm serological test tube and add 0.5 mL antigen to be tested: formalinized brain-heart infusion broth, **967.27B(b)**(*1*), or formalinized trypticase soy-tryptose broth, **967.27B(b)**(*2*) or **967.27C(d)**. If formalinized culture contains granular particles, pellicles, or sediment, also prep. saline control by mixing 0.5 mL formalinized saline soln, **967.25B(g)**, with 0.5 mL formalinized trypticase soy-tryptose or brain-heart infusion broth culture in same size serological test tube. Incubate mixts 1 hr in $H_2O$ bath at 48–50°. Observe preliminary results at 15 min intervals and read final results at 1 hr.

Classify polyvalent flagellar (H) test as:

(**a**) *Positive.*—Agglutination in culture-formalinized saline-serum mixt. and no agglutination in culture-formalinized saline mixt.

(**b**) *Negative.*—No agglutination in culture-formalinized saline-serum mixt. (Polyvalent flagellar antiserum does not contain agglutinins for antigens of some salmonellae isolated from foods. Neg. flagellar reactions occur with *Salmonella* serotypes whose corresponding agglutinins are not contained in the antisera (i.e., *S. simsbury,* $z_{27}$; *S. chittagong,* $z_{35}$)).

(**c**) *Non-specific.*—Both mixts agglutinate. Requires addnl testing as in *Edwards and Ewing's Identification of Enterobacteriaceae.*

Cultures that give typical biochem. results as salmonellae but do not agglutinate in *Salmonella* flagellar (H) antisera must be tested to det. if enough flagellar (H) antigens are present. Test motility of culture as follows:

Inoculate petri dish contg motility test medium, **967.25A(n)**, with 3 mm loopful TSI culture by stabbing medium once, 10 mm from edge of plate to depth of 2–3 mm. (Do not stab to bottom of plate with inoculum.) Do not inoculate any other portion of plate. Incubate 24 hr at 35°. When organism has migrated 40 mm or more toward other side of plate, it is sufficiently motile to retest.

Transfer 3 mm loopful of growth which migrated farthest from inoculation point into tube of trypticase soy-tryptose broth, **967.27C(d)**. Incubate and retest this culture by adding $^1/_2$ vol. formalinized physiological saline soln, **967.25B(g)**, and repeat *Salmonella* serological flagellar (H) test, **967.28C** or **D**.

Incubate cultures that are not motile after first 24 hr incubation for addnl 24 hr at 35°. If still neg., incubate 5 days at 25° before classifying as nonmotile (flagellar (H) antigen not detected).

Cultures that are non-motile or cultures that are *Salmonella* serological flagellar (H) test-neg., when retested, are classified according to results of other tests in *Edwards and Ewing's Identification of Enterobacteriaceae.*

### D. "Spicer-Edwards" Flagellar (H) Test Tube Test

(Alternative to polyvalent flagellar (H) test tube test, **967.28C**, to det. presence or absence of flagellar (H) antigens)

Test each culture, using each of the 7 "Spicer-Edwards" flagellar (H) antisera, **967.25B(k)**. Perform test as in **967.28C**, using 1 of the 7 "Spicer-Edwards" (H) antisera for each test instead of *Salmonella* polyvalent flagellar (H) antiserum. Since there are 7 "Spicer-Edwards" antisera, each culture must be tested 7 times.

Pos. agglutination indicates presence of flagellar (H) antigen. Identify by comparing pattern of agglutination reactions obtained with agglutinins known to be present in each of the 7 "Spicer-Edwards" (H) antisera. Results of these reactions are shown in Table **967.25**.

If culture produces pos. agglutination when tested with each of the 4 "Spicer-Edwards" antisera 1, 2, 3, and 4 (4 plus pattern), then results indicate presence of non-specific antigen other than *Salmonella* antigen or presence of more than single *Salmonella* H antigen which cannot be identified with this antisera until antigens are sepd.

Refs.: JAOAC **50,** 753(1967); **51,** 870(1968); **52,** 455(1969); **56,** 1027(1973); **59,** 731(1976); **62,** 499(1979); **64,** 893(1981); **64,** 899(1981); **65,** 356(1982).

## 975.54 *Salmonella* in Foods

### Fluorescent Antibody (FA) Screening Method

**First Action 1975**
**Final Action 1977**

### A. Precautions

Method is screening test for presence of *Salmonella*; it is not confirmatory test, since conjugate will react with some other members of *Enterobacteriaceae.*

Enrichment broths from samples pos. by FA method must be streaked on selective media as in **967.26B** and typical or suspicious colonies identified as in **967.26C**, **967.27**, **967.28**.

Method must be followed rigorously since errors in prepn of sample, smears, conjugate, and other reagents can lead to invalid results. Microscopic observation of stained smears must be performed with critically aligned and properly functioning equipment.

Visual estimation of degree of fluorescence of stained cells is somewhat subjective and should be conducted by analyst with prior training or experience in both FA methodology and in cultural technic for detection of *Salmonella.*

If sample prepn does not normally include pre-enrichment step (as with meat, poultry, and certain environmental samples), 4 hr post-enrichment incubation period may not be sufficient for development of number of *Salmonella* cells required for detection by FA method. Therefore, include pre-enrichment step or extend post-enrichment incubation time. In some cases when pre-enrichment step is not used, sample is not adequately dild and carryover of debris into post-enrichment broth may interfere with observation of FA stained cells.

### B. Apparatus

(**a**) *Multiwell coated slides.*—Clean thin (1.0–1.2 mm) slides thoroly with detergent and rinse with distd $H_2O$ and alcohol. Apply double row of 4 sep. drops of glycerol (8 drops total) to each of series of slides and spray with fluorocarbon coating material (Fluoroglide, Ace Scientific Co., Inc., 1420 E Linden Ave, Linden, NJ 07036). After few min, rinse off each slide individually under tap and then with distd $H_2O$, and stand on end in rack to dry. (Prepd slides are available from Cell-Line Associates, PO Box 35, Newfield, NJ 08344 and Clinical Sciences, Inc., 30 Troy Rd, Whippany, NJ 07981.)

(**b**) *Fluorescence microscope.*—With exciter filter with wavelength transmission of 330–500 nm and barrier filter with wavelength reception >400 nm.

### C. Reagents

(**a**) *Phosphate-buffered saline (PBS) soln.*—pH 7.5; 0.01*M*; 0.85% NaCl. Dissolve 12.0 g anhyd. $Na_2HPO_4$, 2.2 g $NaH_2PO_4.H_2O$, and 85.0 g NaCl in $H_2O$ and dil. to 1 L. Dil. 100 mL this soln to 1 L with $H_2O$. Adjust pH to 7.5 with 0.1*N* HCl or 0.1*N* NaOH, if necessary.

(**b**) *Carbonate buffer.*—pH 9.0. Mix 4.4 mL 0.5*M* $Na_2CO_3$ (5.3 g in 100 mL $H_2O$) with 100 mL 0.5*M* $NaHCO_3$ (4.2 g in 100 mL $H_2O$). pH should be 9.0; if not, adjust by addn of 0.5*M* $Na_2CO_3$.

(**c**) *Glycerol saline soln.*—pH 9.0. Mix 9 mL glycerol with 1 mL carbonate buffer, (**b**). pH decreases on storage; prep. weekly.

(**d**) *Salmonella polyvalent fluorescent antibody conjugate.*—Fluorescein isothiocyanate-labeled *Salmonella* OH globulin, polyvalent, contg antibodies for all antigens within *Salmonella* O groups A–S, and meeting specifications of Centers for Disease Control, Atlanta, GA 30333 (1975). (Available from Difco Laboratories (FA Salmonella Poly); Clinical Sciences, Inc., 30 Troy Rd, Whippany, NJ 07981). Before use, titer each lot to det. appropriate routine test diln (RTD). Use pure cultures of *Salmonella* representative of several somatic groups. Prep. 5 dilns (1:2, 1:4, 1:8, 1:16, and 1:32) of conjugate in PBS soln, (**a**). Stain duplicate smears from cultures with each diln and det. intensity of fluorescence. RTD is that diln one less than highest diln giving 4+ fluorescence with representative *Salmonella* cultures. Store stock (undild) conjugate of known titer frozen, and dil. when needed. Dild conjugate can be stored at 4° for few weeks as long as control cultures remain pos.

### D. Determination

(**a**) *Pre-enrichment.*—Pre-enrich product in noninhibitory broth to initiate growth of salmonellae. Methods used vary with product as in (*1*)–(*9*). In all cases loosen jar caps 1/4 turn and incubate 24 ± 2 hr at 35°. Except where selenite cystine and tetrathionate broths, **967.25A**(**b**)(*1*) or (*2*) and (**c**), resp., have already been used ((*2*)(b) and (*5*)), transfer 1 mL incubated mixts to selenite cystine broth and tetrathionate broth for selective enrichment as in **967.26B**(**a**). Where these broths have already been used ((*2*)(b) and (*5*)), proceed directly to post-enrichment, (**b**).

(*1*) *Dried yeast (inactive).*—Weigh 25 g into sterile, wide-mouth, screw-cap, 500 mL (pt) jar, add 225 mL sterile trypticase (tryptic) soy broth, **967.25A**(**t**), and mix well to form smooth suspension. Cap jar securely and let stand 60 min at room temp. If pH is <6.6, adjust to 6.8 ± 0.2 with 1*N* NaOH.

(*2*) *Meats, animal substances, glandular products, and fish meal.*—(*a*) *Heated, processed, and dried products.*—Weigh 25 g into sterile blending jar, add 225 mL sterile lactose broth, **940.36A**(**f**), and blend 2 min at 8000 rpm. If product is powd., ground, or comminuted, blending may be omitted. Transfer aseptically to sterile, wide-mouth, screw-cap, 500 mL (pt) jar and adjust pH to 6.8 ± 0.2 with 1*N* NaOH. If product contains large amt of fat, add 2.2 mL of steamed (15 min) Tergitol Anionic 7 (Na heptadecyl sulfate, Union Carbide Corp.).

(*b*) *Raw and highly contaminated products.*— Weigh duplicate 25 g samples into sep. sterile blending jars. Add 225 mL of selenite cystine broth to one jar and 225 mL of tetrathionate broth to other, and blend 2 min. Transfer aseptically to sterile, wide-mouth, screw cap, 500 mL (pt) jars.

(*c*) *Raw frog legs.*—Aseptically place 2 legs into single sterile, wide-mouth, screw cap, 500 mL (pt) jar contg 225 mL sterile lactose broth, **940.36A**(**f**).

(*3*) *Dry whole milk.*—Weigh 25 g into sterile, wide-mouth, screw cap, 500 mL (pt) jar, add 225 mL sterile distd $H_2O$, and mix well. Adjust pH to 6.8 ± 0.2 with 1*N* NaOH, if necessary. Add 0.45 mL 1% aq. brilliant green soln and mix well.

(*4*) *Dried whole eggs, yolks, and whites; pasteurized liquid and frozen eggs; prepared powdered mixes (cake, cookie, donut, biscuit, and bread); and infant formula.*—If product is frozen, thaw rapidly at ≤45° for ≤15 min or overnight at 5–10°. Weigh 25 g into sterile, wide-mouth, screw cap jar. Add 225 mL lactose broth, little at time with mixing, cap jar, and let stand at room temp. 60 min. Mix well and adjust to pH 6.8 ± 0.2 with 1*N* NaOH or HCl.

(*5*) *Nonpasteurized frozen egg products.*—Thaw as in (*4*). Weigh duplicate 25 g samples into sep. sterile, wide-mouth, screw cap, 500 mL (pt) jars. Add 225 mL selenite cystine broth to one jar and 225 mL tetrathionate broth to other, and mix well. Adjust pH to 6.8 ± 0.2 with 1*N* NaOH.

(*6*) *Egg-containing foods (noodles, egg rolls, etc.)*—Proceed as in (*2*)(*a*).

(*7*) *Coconut.*—Proceed as in (*2*)(*a*), using Tergitol Anionic 7, but omitting blending.

(*8*) *Candy and candy coatings.*—Weigh 25 g into sterile blending jar. Add 225 mL sterile reconstituted nonfat dry milk, **967.25A**(**v**), but without brilliant green dye, and blend 2 min. Adjust pH to 6.8 ± 0.2 with 1*N* NaOH, if necessary. Add 0.45 mL 1% aq. brilliant green soln and mix well.

(*9*) *Nonfat dry milk.*—Examine as in **967.26A**(**f**).

(**b**) *Post-enrichment.*—Transfer 1 mL of incubated selenite cystine enrichment broth to 10 mL of sterile selenite cystine broth as post-enrichment. (Other vols may be used if 1:10 diln ratio is maintained.) Take aliquot from upper third of selective enrichment cultures to minimize product carryover. Similarly, transfer 1 mL of incubated tetrathionate enrichment broth to 10 mL of sterile selenite cystine broth. Incubate 4 hr in 35° $H_2O$ bath.

(**c**) *Staining.*—Transfer ca 0.0075 mL of each post-enrichment medium with sterile 2 mm loop into sep. wells of multiwell coated slide, and dry thoroly in air at room temp. Fix by immersion in bath of alcohol-$CHCl_3$-formalin (60 + 30 + 10) 3 min. Rinse 2 or 3 times in alcohol, and air dry at room temp. Change alcohol periodically to prevent cell carryover (250 mL alcohol will rinse 5–10 slides). Slides may also be fixed and rinsed by flooding. Apply solns to one end of slide and allow to flow into wells.

Cover dried smears with titered *Salmonella* polyvalent FA conjugate and let stain in moist chamber 15–30 min. *FA conjugate must not dry on smear.* (Covered plastic petri dish contg piece of filter paper moistened with $H_2O$ is excellent staining chamber.) Drain excess conjugate by standing slide on edge few sec. (Avoid mixing conjugate from one well on slide to another.) Immediately rinse slides in PBS soln, **975.54C(a)**. Then soak slides 10 min in fresh PBS soln and rinse briefly with $H_2O$. Air-dry smears again at room temp. and then mount by placing drop of glycerol saline soln, (**c**), directly onto each smear and covering with No. 1 glass cover slip. Add enough glycerol saline soln to smear to ensure adequate, but not excessive, coverage of all wells after cover slips have been placed. Do not trap air bubbles under cover slip.

(**d**) *Examination*.—Examine smears with fluorescent microscope. Scan entire smear using 40–50× oil immersion objective to locate fluorescent cells. When found, change objective to 100× oil immersion lens for definitive detn of cell morphology and fluorescence. Objectives with iris diaphragm for adjusting numerical aperture are helpful for control of contrast between cells and background. Estimate degree of fluorescence of cells on scale of neg. to 4+ as follows:

4+ = Max. fluorescence; brilliant yellow-green; clearcut cell outline; sharply defined cell center.

3+ = Less brilliant yellow-green fluorescence; clearcut cell outline; sharply defined cell center.

2+ = Definite but dim fluorescence; cell outline less well defined.

1+ = Very subdued fluorescence; cell outline indistinguishable from cell center in most instances.

− = Negligible or complete lack of fluorescence.

Typical pos. smears for salmonellae exhibit ≥2 short to medium rod-shaped cells per field, using 100× objective. Cells should be distributed thruout entire smear. Intensity of fluorescence should be in range of 3+ to 4+. Occasionally cells are observed with proper morphology and cell distribution, but fluorescence is rated 2+. Sometimes 3+ to 4+ fluorescence is observed, but distribution is poor and not all fields contain cells, due to improper processing of slides. Score both cases pos. and subject to confirmatory tests.

Each time samples are tested, carry culture of known *Salmonella* strain thru all cultural, staining, and observation steps as control.

Report: (1) morphological characteristics of fluorescent cells; (2) number of typical cells per field under 100× oil immersion objective; and (3) degree of fluorescence of cells (1+ to 4+).

Ref.: JAOAC **58,** 828(1975).

## 985.42 *Salmonella* in Foods

### Hydrophobic Grid Membrane Filter Screening Method

**First Action 1985**
**Final Action 1986**

(Applicable to detection of *Salmonella* from chocolate, raw poultry meat, pepper, cheese powders, powdered egg, and nonfat dry milk)

### *A. Principle*

Hydrophobic grid membrane filter (HGMF) uses membrane filter imprinted with hydrophobic material in grid pattern. Hydrophobic lines act as barriers to spread of colonies, thereby dividing membrane filter surface into sep. compartments of equal and known size.

### *B. Apparatus, Culture Media, and Reagents*

(**a**) *Hydrophobic grid membrane filter* (*HGMF*)—Membrane filter has pore size of 0.45 μm and is imprinted with nontoxic hydrophobic material in grid pattern. ISO-GRID (available from QA Laboratories Ltd, 135 The West Mall, Toronto, Ontario, Canada, M9C 1C2) or equiv. meets these specifications.

(**b**) *Filtration units for HGMF*.—Equipped with 5 μm mesh prefilter to remove food particles during filtration. One unit is required for each sample. ISO-GRID (available from QA Laboratories Ltd) or equiv. meets these specifications.

(**c**) *Pipets*.—1.0 mL serological with 0.1 mL graduations; 1.1 mL or 2.2 mL milk pipets are satisfactory.

(**d**) *Blender*.—Waring, or equiv. with high-speed operation at 20,000 rpm, and 500 mL glass or metal blender jars with covers. One jar is required for each sample.

(**e**) *Vacuum pump*.—$H_2O$ aspirator vac. source is satisfactory.

(**f**) *Manifold or vacuum flask*.

(**g**) *Peptone diluent*.—Dissolve 1.0 g peptone (Difco 0118) in 1 L $H_2O$. Dispense enough vol. into diln bottles to give 99 ± 1 mL after autoclaving 15 min at 121°.

(**h**) *Lactose broth*.—*See* **940.36A(f)**. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust if necessary to 225 mL. Final pH 6.7 ± 0.2.

(**i**) *Trypticase (tryptic) soy broth*.—Suspend 17.0 g trypticase or tryptose (pancreatic digest of casein), 3.0 g phytone (papaic digest of soya meal), 5.0 g NaCl, 2.5 g $K_2HPO_4$, and 2.5 g glucose in 1 L $H_2O$. Heat gently to dissolve completely. Dispense 225 mL portions into 500 mL flasks. Autoclave 15 min at 121°. Aseptically det. vol. and adjust if necessary to 225 mL. Final pH 7.3 ± 0.2.

(**j**) *Reconstituted nonfat dry milk with brilliant green dye (NFDM-BG)*.—Suspend 100 g dehydrated NFDM in 1 L $H_2O$; mix by swirling until dissolved. Autoclave 15 min at 121°. Add brilliant green dye soln after blending sample/broth mixt. as described below.

(**k**) *Tetrathionate broth (with iodine and brilliant green)*.—Suspend 5.0 g polypeptone, 1.0 g bile salts, 10 g $CaCO_3$, and 30 g $Na_2S_2O_3.5H_2O$ in 1 L $H_2O$, mix thoroly, and heat to bp. (Ppt will not dissolve completely.) Cool to <45° and store at 5–8°. Prep. I-KI soln by dissolving 5 g KI in 5 mL sterile $H_2O$, adding 6 g resublimed I, dissolving, and dilg to 20 mL with sterile $H_2O$. Prep. brilliant green soln by dissolving 0.1 g dye in sterile $H_2O$ and dilg to 100 mL. On day medium is used, add 20 mL I-KI soln and 10 mL brilliant green soln per 1 L basal broth. Resuspend ppt by gentle agitation and aseptically dispense 10 mL portions in 16 × 150 mm sterile tubes. Do not heat medium after addn of I-KI and dye solns. Temper to 25–35° before use.

(**l**) *Selective lysine agar* (*SLA*).—Suspend 5.0 g proteose peptone No. 3, 3.0 g yeast ext, 10.0 g L-lysine.HCl, 3.5 g glucose, 1.5 g bile salts No. 3, 0.001 g crystal violet (1.0 mL of 0.1% (w/v) aq. soln), 0.03 g bromocresol purple, 0.3 g sulfapyridine, and 15.0 g agar in 1 L $H_2O$ and heat to bp with stirring to dissolve completely. Autoclave 15 min at 121°. Cool to 45–50°. Dispense 20 mL vol. in 15 × 100 mm petri dishes. Final pH, 6.8 ± 0.1.

(**m**) *Hektoen enteric agar* (*HE*).—Suspend ingredients (*1*) or (*2*) (varies with manufacturer of formula) in 1 L $H_2O$ and mix thoroly. Heat to boiling with frequent agitation and let boil few moments. Do not overheat. Cool in $H_2O$ bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 7.6 ± 0.2. Do not autoclave.

(*1*) 12.0 g thiotone peptone, 3.0 g yeast ext, 9.0 g bile salts, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.064 g bromothymol blue, 0.1 g acid fuchsin, and 13.5 g agar.

(*2*) 12.0 g proteose peptone, 3.0 g yeast ext, 9.0 g bile salts No. 3, 12.0 g lactose, 12.0 g sucrose, 2.0 g salicin, 5.0 g

NaCl, 5.0 g Na thiosulfate, 1.5 g ferric ammonium citrate, 0.065 g thymol blue, 0.1 g acid fuchsin, and 14.0 g agar.

(**n**) *Triple sugar iron agar (TSI agar)*.—Suspend ingredients (*1*) or (*2*) in 1 L $H_2O$, mix thoroly, and heat with occasional agitation. Boil ca 1 min until ingredients dissolve. Fill 16 × 150 mm tubes $^1/_3$ full and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 3 cm) and adequate slants (ca 5 cm) are formed on solidification.

(*1*) 20 g polypeptone, 5.0 g NaCl, 10 g lactose, 10 g sucrose, 1 g glucose, 0.2 g $Fe(NH_4)_2(SO_4)_2.6H_2O$, 0.2 g $Na_2S_2O_3$, 0.025 g phenol red, and 13 g agar. Final pH, 7.3 ± 0.2.

(*2*) 3.0 g beef ext, 3.0 g yeast ext, 15 g peptone, 5.0 g proteose peptone, 1.0 g glucose, 10 g lactose, 10 g sucrose, 0.2 g $FeSO_4$, 5.0 g NaCl, 0.3 g $Na_2S_2O_3$, 0.024 g phenol red, and 12 g agar. Final pH, 7.4 ± 0.2.

(**o**) *Lysine iron agar (LIA) (Edwards and Fife)*.—Dissolve 5.0 g gelysate or peptone, 3.0 g yeast ext, 1.0 g glucose, 10 g L-lysine, 0.5 g ferric ammonium citrate, 0.04 g anhyd. $Na_2S_2O_3$, 0.02 g bromocresol purple, and 15 g agar in 1 L $H_2O$, heating until dissolved. Dispense 4 mL portions into 13 × 100 mm test tubes and cap or plug so that aerobic conditions are maintained during use. Autoclave 12 min at 121°. Before medium solidifies, place tubes in slanted position so that 4 cm butts and 2.5 cm slants are formed on solidification. Final pH 6.7 ± 0.2.

(**p**) *MacConkey agar (MAC)*.—Suspend 3.0 g proteose peptone or polypeptone, 17 g peptone or gelysate, 10 g lactose, 1.5 g bile salts No. 3 or bile salts mixt., 5.0 g NaCl, 3.0 mL 1% neutral red (30 mg) soln, 1 mL 0.1% crystal violet (1.0 mg) soln, and 13.5 g agar in 1 L $H_2O$ and mix thoroly until homogeneous. Heat, with occasional agitation, and boil 1–2 min until ingredients dissolve. Autoclave 15 min at 121°. Cool to 45–50° and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with plates covered. Do not use wet plates. Final pH 7.1 ± 0.2.

(**q**) *Sodium hydroxide soln*.—1N. Dissolve 42.11 g 95% reagent NaOH in sterile $H_2O$ and dil. to 1 L.

(**r**) *Hydrochloric acid soln*.—1N. Dil. 89 mL to 1 L with sterile $H_2O$.

(**s**) *pH Test paper*.—Min. range 6.0–7.6 with max. gradations of 0.4 pH unit per color change.

(**t**) *Sterile distilled water*.—Dispense 1 L $H_2O$ into 2 L wide-mouth flask or wide-mouth jar; plug or cap loosely. Autoclave 20 min at 121°.

(**u**) *Brilliant green dye soln*.—1%. Dissolve 1 g in sterile $H_2O$ and dil. to 100 mL. (Since some batches of dye are unusually toxic, test all batches of dye before use, and use only those producing satisfactory results when tested with known pos. and neg. test organisms.)

(**v**) *Brilliant green dye water*.—Prep. sterile $H_2O$, (**t**), and add 2 mL of 1% aq. brilliant green dye, (**u**), per L sterile $H_2O$ and mix well.

### C. Preparation of Sample

(**a**) *Powdered egg*.—Aseptically open sample container and aseptically weigh 25 g sample into sterile, empty, wide-mouth, screw-cap pt (500 mL) jar. Add ca 15 mL sterile lactose broth. Stir with sterile glass rod, sterile spoon, or sterile tongue depressor to smooth suspension. Add 3 addnl portions lactose broth, 10, 10, and 190 mL for total of 225 mL. Stir after each addn until sample is suspended without lumps. Cap jar securely and let stand at room temp. 60 min. Mix well by shaking, and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Loosen jar cap ca $^1/_4$ turn and incubate 18–24 h at 35°.

(**b**) *Chocolate*.—Aseptically weigh 25 g sample into sterile blender jar. Add 255 mL sterile reconstituted NFDM-BG. Blend 2 min at high speed and decant blended homogenate into sterile 500 mL jar. Cap jar securely and let stand 60 min at room temp. Mix well by shaking, and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Add 0.45 mL 1% aq. brilliant green dye and mix well. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

(**c**) *Raw meat*.—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth and blend 2 min at high speed. Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Aseptically transfer sample to sterile 500 mL wide-mouth screw-cap jar. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

(**d**) *Cheese powder*.—Aseptically weigh 25 g sample into sterile 500 mL wide-mouth screw-cap jar. Add 225 mL sterile lactose broth and mix well. Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

(**e**) *Pepper*.—Aseptically weigh 25 g sample into sterile 500 mL wide-mouth screw-cap jar. Add 225 mL sterile trypticase soy broth and mix well. Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 with sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

(**f**) *Powdered milk*.—Use sterile funnel to aseptically add 25 g sample slowly and gently to 225 mL sterile brilliant green dye water in 500 mL wide-mouth screw-cap jar. Do not mix. Allow to soak undisturbed 60 min at room temp. Do not mix or adjust pH. Loosen jar cap $^1/_4$ turn and incubate 18–24 h at 35°.

### D. Isolation

(**a**) *Selective enrichment*.—Gently shake incubated sample mixt. and transfer 0.1 mL to 10 mL tempered (25–35°) tetrathionate broth. Mix inoculated broth on vortex mixer or by hand to disperse inoculum. Incubate in $H_2O$ bath 6–8 h at 35 ± 0.5°.

(**b**) *Filtration and selective isolation*.—Mix incubated tetrathionate broth by hand or vortex-mixer to resuspend. For raw meats, prep. $10^{-2}$ diln by transferring 1.0 mL into 99 mL sterile peptone diluent. Mix by shaking. For all other products, use undild tetrathionate.

(*See* Figs **986.32A** and **986.32B**). Turn on vac. source. Place sterile filtration unit on manifold or vac. flask. Open clamp A. Rotate back funnel portion C. Aseptically place sterile HGMF on surface of base D. Rotate funnel forward. Clamp shut by sliding jaws L of stainless steel clamp over entire length of flanges B extending from both sides of funnel C and base D, and rotating moving arm K into horizontal (locked) position.

Aseptically add ca 15–20 mL sterile $H_2O$ to funnel. Pipet 1.0 mL of required tetrathionate diln into funnel. Apply free end of vac. tubing E to suction hole F to draw liq. thru prefilter mesh G. Aseptically add addnl 10–15 mL $H_2O$ to funnel and draw thru mesh as before. Close clamp A to direct vac. to base of filtration unit and draw liq. thru HGMF.

Open clamp A. Rotate moving arm K of stainless steel clamp into unlocked (ca 45° angle) position and slide jaws L off of flanges B. Rotate back funnel C. Aseptically remove HGMF and place on surface of pre-dried SLA. Avoid trapping air bubbles between filter and agar. For nonfat dry milk samples, insert second sterile HGMF into same filtration unit, repeat filtering procedure and place second HGMF on surface of pre-dried HE. Incubate SLA 24 ± 2 h at 43 ± 0.5°, and HE 24 ± 2 h at 35°. If HGMFs do not have typical or suspicious colonies or do not contain growth, record as neg. test result.

**(c)** *Appearance of typical Salmonella colonies.*—*(1) On SLA.*—Blue-green, blue, or purple colonies (lysine-pos. reaction). Typically, *Salmonella* produces relatively flat colonies which are neither watery nor mucoid. Lysine-neg. colonies are typically yellow or yellow-green. However, this can be masked if large no. of lysine-pos. colonies are present on HGMF.

*(2) On HE.*—Black, or green with black centers. Some *Salmonella* will produce yellow colonies with black centers or green colonies with no blackening. $H_2S$ reaction can be partially suppressed if very heavy growth is present on HGMF.

### E. Treatment of Typical or Suspicious Colonies

**(a)** *Inoculation of TSI, LIA, and MAC or HE.*—*(1) Raw meats.*—Select 5 typical or suspicious colonies from each HGMF.

*(2) All other products.*—Select 3 typical or suspicious colonies from each HGMF.

Using sep. sterile, completely cooled needle for each colony, pick each selected colony and inoculate TSI slant with portion of colony by stabbing butt and streaking slant. Without heating needle or obtaining more inoculum, inoculate LIA with portion of colony by stabbing butt in 2 places and streaking slant. Without heating needle or obtaining more inoculum, streak remainder of inoculum to MAC or HE. Incubate TSI, LIA, and MAC or HE 24 ± 2 h at 35°. Cap tubes loosely to maintain aerobic conditions while incubating slants to prevent excessive $H_2S$ production.

**(b)** *Presumptive positive reactions.*—*Salmonella* cultures typically have alk. (red) slant and acid (yellow) butt, with or without $H_2S$ (blackening of agar) in TSI agar. In LIA, *Salmonella* cultures typically have alk. (purple) reaction in butt. Consider only distinct yellow coloration in butt of LIA tube as acidic (neg.) reaction. Do not eliminate cultures that produce discoloration in butt solely on this basis. Most *Salmonella* cultures produce $H_2S$ in LIA. Retain all presumptive pos. *Salmonella* cultures on TSI agar (alk. slant and acid butt) for biochem. and serological tests whether or not corresponding LIA reaction is pos. (alk butt) or neg. (acid butt). Do not exclude TSI culture that appears to be non-*Salmonella* if reaction in LIA is typical (alk. butt) for *Salmonella*. Treat these cultures as presumptive pos. and submit them to further examination. LIA is useful in detection of *S arizonae* and atypical *Salmonella* strains that utilize lactose and/or sucrose. Discard only apparent non-*Salmonella* TSI agar cultures (acid slant and acid butt) if corresponding LIA reactions are not typical (acid butt) for *Salmonella*.

### F. Purification and Identification

**(a)** *Appearance of Salmonella colonies.*—*(1) On MAC.*—Typical colonies appear transparent and colorless, sometimes with dark centers. *Salmonella* will clear areas of pptd bile caused by other organisms sometimes present in medium.

*(2) On HE.*—Blue-green to blue colonies with or without black centers. Many *Salmonella* cultures may have large glossy black centers or may appear as almost completely black colonies.

**(b)** *Purification of mixed cultures.*—Examine MAC or HE. If pure, proceed with identification. If mixed culture, pick with needle ≥2 well isolated typical or suspicious colonies and inoculate TSI, LIA, and MAC or HE as described above. Incubate and examine for presumptive pos. reactions.

**(c)** *Identification.*—Carry out biochem. and serological identification procedures on 3 presumptive pos. TSI cultures from each HGMF as described in **967.27B–E**, **967.28**. As alternative to conventional tube system for *Salmonella*, any one of 4 commercial biochem. systems (API, Enterotube, Minitek, or Micro-ID) may be used for presumptive generic identification of foodborne *Salmonella*. *See* **978.24**.

Ref.: JAOAC **68**, 555(1985).

## 986.35 *Salmonella* in Foods

### Colorimetric Monoclonal Enzyme Immunoassay Screening Method

**First Action 1986**
**Final Action 1988**

Method is screening procedure for presence of *Salmonella* in all foods; it is not a confirmatory test because monoclonal antibodies used in test may cross-react with small percentage of non-*Salmonella*.

Enrichment broths and M-broth from samples pos. by enzyme immunoassay (EIA) method must be streaked on selective media as in **967.26B** and typical or suspicious colonies must be identified as in **967.26C**, **967.27**, **967.28**.

Detn of pos. result is objective and must be performed using filter photometer having 405–420 nm filter. Pos. result is valid only when neg. and pos. controls possess acceptable optical density readings.

### A. Principle

Detection of *Salmonella* antigens is based on solid phase immunoassay and uses mag. force to transfer solid phase from one reaction mixt. to another. Monoclonal antibodies to *Salmonella* antigen are bound to surface of beads made of ferrous metal. Beads are placed in sample to be assayed. If *Salmonella* antigens are present in sample, they will attach to specific antibody on beads. Beads are washed and then released into reaction mixt. contg peroxidase-conjugated anti-*Salmonella* immunoglobulins. Conjugate will bind to *Salmonella* antigens if they are attached to antibody molecules on surface of beads. Beads are washed to remove unbound conjugate and then placed in substrate soln. Appearance of color indicates presence of *Salmonella* antigen in sample. Fig. **986.35A** shows schematic representation.

### B. Method Performance

For all foods:

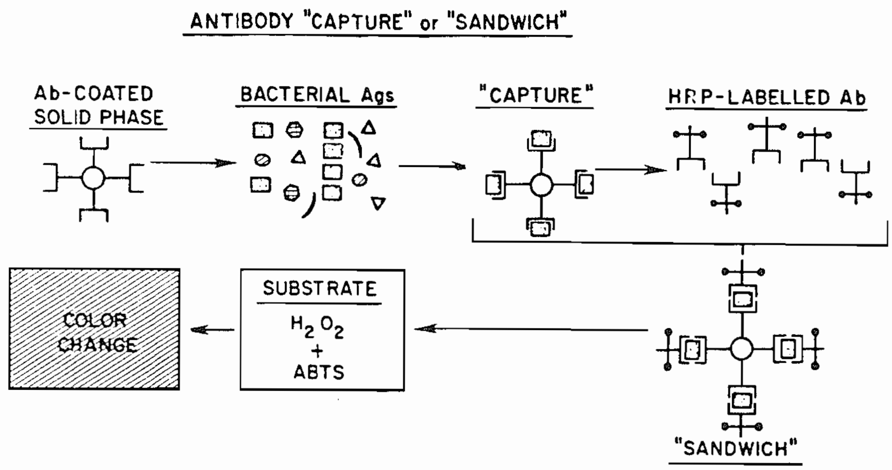
| Results | Percent | 95% Confidence Range (Approx.) |
|---|---|---|
| Agreement[1] | 96.9 | 95.7–98.1 |
| False neg. (BAM)[2] | 1.5 | 0.4–2.5 |
| False neg. (EIA)[3] | 3.4 | 1.8–5.0 |

[1] This rate reflects no. of samples read identically between AOAC/BAM (Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA) culture method and EIA.

[2] This rate reflects no. of samples found to be pos. by EIA but detd to be neg. by AOAC/BAM culture method.

[3] This rate reflects no. of samples found to be pos. by AOAC/BAM culture method but detd to be neg. by EIA.

Of 21 laboratories, 10 (48%) had complete agreement between culture method and EIA (153/153); 17 laboratories (81%)




Ab = antibody
Ag = antigen
HRP = horseradish peroxidase
$H_2O_2$ = hydrogen peroxide
ABTS = 2,2′-azino-di[3-ethylbenzthiazoline-sulfonate(6)]

**FIG. 986.35A—Antibody "capture" or "sandwich"**

showed agreement on ≥96% of samples; 20 (95%) showed agreement on ≥93% of samples.

### C. Reagents

Items (**a**)–(**m**) are available as *Salmonella* Bio-EnzaBead Screen Kit (Organon Teknika Corp., 100 Akzo Ave, Durham, NC 27704).

(**a**) *Antibody-coated beads.*—Monoclonal antibodies to *Salmonella*, 2 vials (48 beads/vial). Store bead vials tightly capped at 2–8°. Beads are stable 14 days after opening.

(**b**) *Control antigens.*—Pos. control (heat-treated *S. javiana*) which reacts with antibodies to *Salmonella*, 1 vial; neg. control which is nonreactive with antibodies to *Salmonella*, 1 vial. Reconstituted control antigens are stable 28 days when stored at 2–8°.

(**c**) *Conjugate diluent.*—1 vial (24 mL/vial). Contains 1% bovine serum in phosphate-buffered saline contg 0.05% Tween 20 and 0.01% thimerosal as preservative.

(**d**) *Reagent water.*—1 bottle (125 mL/bottle). Store at room temp. or warm to room temp. before use.

(**e**) *Phosphate-buffered saline.*—PBS, pH = 7.5 ± 0.2; 1 bottle (125 mL/bottle). Contains 1.2 g $Na_2HPO_4$, 0.22 g $NaH_2PO_4.H_2O$, and 8.5 g NaCl/L $H_2O$.

(**f**) *Peroxidase-conjugated antibodies to Salmonella.*—1 vial (lyophilized). When reconstituted, conjugate is stable 28 days when stored at 2–8°.

(**g**) *Wash solution* (*50×*).—1 vial (2.5 mL). Contains 2.5% surfactant.

(**h**) *ABTS substrate.*—2 vials (lyophilized). After reconstitution, each vial contains 0.03% 2,2′-azino-di(3-ethyl-benzthiazoline-sulfonate). Reconstituted substrate is stable 14 days when stored tightly capped at 2–8°. Let reconstituted substrate warm to room temp. before dispensing.

(**i**) *Substrate diluent for ABTS.*—2 vials (30 mL/vial). Contains $H_2O_2$.

(**j**) *"Stop" soln.*—1 vial (5 mL/vial). Contains 1.25% NaF. *Caution:* Avoid contact with skin. If contact occurs, wash area with $H_2O$.

(**k**) *Microtitration plates.*—Plate ($3^5/_{16}$ × 5 in.) possessing 96 wells, each having capability of holding >0.3 mL fluid. These must be designed in 8 × 12 format which will fit into mag. transfer device. Spaces between wells should be hollowed out, and not filled in with plastic coming to top of well. Available as "Accessory Package" (Organon Teknika Corp.), or equiv. may be used. *Note:* Not all microtitrn plates meet these criteria.

(**l**) *Package insert.*

(**m**) *Data record sheets.*

(**n**) *M-broth.*—5.0 g yeast ext, 12.5 g tryptone, 2.0 g D-mannose, 5.0 g Na citrate, 5.0 g NaCl, 5.0 g $K_2HPO_4$, 0.14 g $MnCl_2$, 0.8 g $MgSO_4$, 0.04 g $FeSO_4$, 0.75 g Tween 80. Suspend ingredients in 1 L $H_2O$, and heat to boiling for 1–2 min. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Cap tubes loosely and autoclave 15 min at 121°. Tighten caps securely for storage. Final pH should be 7.0 ± 0.2.

(**o**) *Diagnostic reagents.*—Necessary for cultural confirmation of presumptive pos. EIA tests; *see* **967.25B**.

### D. Apparatus

Items (**a**)–(**e**) are available from Organon Teknika Corp.

(**a**) *Magnetic transfer device.*—Mag. app. which houses microtitrn plates and is used to transfer metal beads from one reagent to another as well as to wash metal beads.

(**b**) *Incubator.*—37° with 100 rpm agitator.

(**c**) *Enzyme immunoassay reader.*—Photometer with 405–420 nm screening filter which will read thru microtitrn plates. Must be able to be set to zero while reading thru unreactive substrate well (blank). Reader should be equipped with printer so that records of analysis can be kept. Semiautomated Organon Teknika 30 or equiv. meets these specifications.

(**d**) *Bead dispenser.*—Either single bead dispenser or 96-well bead dispenser, or suitable alternative. Places beads into wells of microtitrn plate.

(**e**) *Micropipet.*—Capable of delivering accurate amts in range 50–300 μL. Micropipets capable of delivering these vols to multiple wells simultaneously (multichannel) or individually (single channel) are needed.

(**f**) *Centrifuge.*—Having min. capacity to spin centrf. tubes (≤20 mm diam.) at 1500 × *g* in swinging bucket rotor or 3000

$\times$ $g$ in fixed angle rotor for 20 min. IEC Centra 7 or Centra 8 tabletop centrf. with IEC 216 horizontal rotor (available from International Equipment Co., or equiv., meets these criteria.

**(g)** *Boiling water bath.*—Able to attain and maintain 100°. Must be able to hold centrf. tubes upright. Microwave or autoclave set at 100° is acceptable alternative, as are generators of flowing stream. *Caution:* $H_2O$ baths which do not maintain boiling conditions are unacceptable.

**(h)** *Vortex mixer.*—Capable of vigorous agitation of centrf. tube, such that pellet at bottom of conical tube can be resuspended. S/P mixer (available from Scientific Products, Inc.) or equiv. meets these criteria.

### E. General Instructions

Include pos. control antigen and duplicate neg. controls with each group of test samples. All controls must function properly for test to be valid. One addnl well per group of test samples should be left empty initially. This well, filled with substrate, will be used to "blank" assay reader. See sample data record sheet (Fig. **986.35B**).

Use data record sheets to identify location of each test sample.

Do not use mag. transfer device without top and bottom plate in position. Always insert plates in device with notched side facing operator.

*Do not reuse* wells of a plate or the beads.

Use sep. pipets for each sample and kit reagent to avoid cross-contamination. Take care not to contaminate substrate with conjugate.

The components and procedures of this test kit have been standardized for use in Bio-EnzaBead procedure. Use of components or procedures other than those supplied by Organon Teknika Corp. may yield unsatisfactory results.

### F. Preparation of Sample

**(a)** *Pre-enrichment.*—Pre-enrich product in non-inhibitory broth to initiate growth of salmonellae. Methods used may vary with product, and should be performed as indicated in **967.26A**, or in Bacteriological Analytical Manual, 6th ed., AOAC, Arlington, VA, Chap. 7, section C, with the following exception:

*Raw or highly contaminated products.*—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (ca 20 000 rpm). Cap jars securely and let stand at room temp. 60 min. Mix well by shaking and det. pH with test paper. Adjust pH to 6.8 $\pm$ 0.2, if necessary, using sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Aseptically transfer contents of each jar to sterile wide-mouth, screw-cap 500 mL jar. Loosen jar caps $^1/_4$ turn and incubate 24 $\pm$ 2 h at 35°.

**(b)** *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment mixts to selenite cystine broth and 1 mL into tetrathionate broth as in **967.26B(a)**. (For dried active yeast, substitute lauryl sulfate tryptose broth **967.25A(u)** for selenite cystine broth.) Incubate 18–24 h at 35°.

**(c)** *Post-enrichment.*—Remove selective broths from incubation and mix by hand or by vortex mixer. Remove 0.5 mL from tetrathionate tube and transfer to 10 mL tube of sterile M-broth which has been warmed to 35°. Also remove 0.5 mL from selenite cystine tube and transfer to same tube of M-broth and vortex-mix well. Incubate M-broth tube 6 h at 35°. Return tetrathionate and selenite cystine tubes to 35° incubator for addnl 6 h.

**(d)** *Centrifugation and preparation of sample for EIA analysis.*—Remove M-broth from incubation and mix tube by hand or vortex mixer. Pipet 10 mL into centrf. tube ($\leq$20 mm diam.) and label tube. Refrigerate (2–8°) remaining M-broth and tetrathionate and selenite cystine tubes from **(c)** above for cultural confirmation of any enzyme immunoassay pos. samples. These broths may be refrigerated, if necessary, for $\leq$18 h at 2–8°. Centrf. M-broth at *min.* speed of 1500 $\times$ $g$ (swinging bucket rotor) or 3000 $\times$ $g$ (fixed angle rotor) for 20 min. Suction off supernate from tube, using trap flask filled with disinfectant. Resuspend pellet with 1 mL PBS. Vortex-mix tube to mix well. Heat resuspended pellet in boiling $H_2O$ bath or

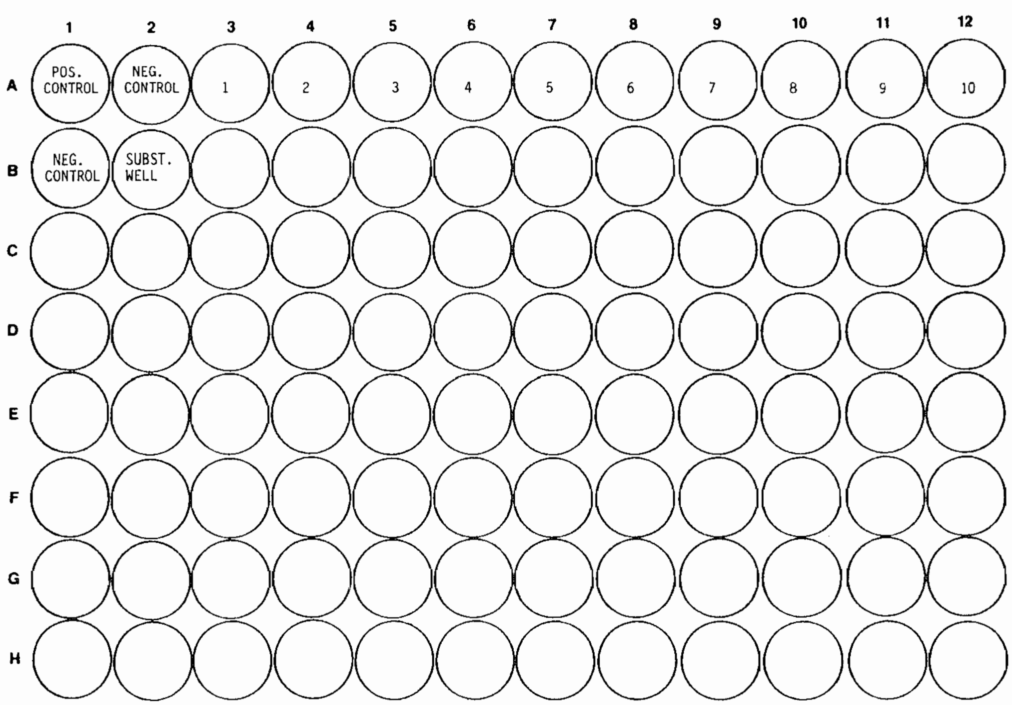



**FIG. 986.35B—Data record sheet for identifying location of test samples**

in flowing steam 20 min. Cool heated exts to 25–37° prior to analysis by EIA. *Note:* Heated exts which are not cooled to this temp. can destroy monoclonal antibodies on metal beads.

### G. Enzyme Immunoassay

*(1)* Reconstitute control antigens by adding 2.5 mL PBS to each vial. Swirl gently to dissolve. Following record sheet, add 0.2 mL aliquot of neg. and pos. control antigens and 0.2 mL aliquot of test samples into designated wells of 96-well plate. *Note:* Use 2 neg. controls and one pos. control for each group of samples. Label plate "antigen."

*(2)* Using bead dispenser or forceps, place antibody-coated beads in wells of one of empty plates according to record sheet. If using forceps, remove beads from vials by rolling beads into cap or onto gauze pad. Do *not* put bead in substrate blanking well.

*(3)* To start assay, simultaneously transfer beads to "antigen" plate by using mag. transfer device as follows:

(a) Invert 96-well plate and insert it with notched end facing operator into top slot until it snaps into position. Magnet should be in UP position.

(b) Slide plate contg beads (with notched end facing operator) into lower slot until it snaps into position, centering it under inverted top plate.

(c) To remove beads from bottom plate, lower magnet to full DOWN position (all beads should now be in inverted plate). Without disturbing top plate, remove lower plate (save for later step) and slide plate contg samples under inverted plate.

(d) Raise magnet to allow all beads to drop into wells of "antigen" plate.

*(4)* Remove bottom plate contg samples and beads. Incubate plate with agitation (10–100 rpm) for 20 min at 37°. During incubation period, proceed to steps *5* and *6*.

*(5)* If entire plate is being used, prep. 1× wash soln by adding 1.5 mL 50× wash soln to 75 mL reagent water in clean glass or plastic screw-cap bottle. Mix by inverting bottle several times. Add 0.3 mL 1× wash soln into appropriate wells of 96-well plate previously saved. Label plate "wash 1." Similarly, fill second plate with 1× wash soln and label "wash 2." If entire plate is not being used, calc. amt of wash soln required by multiplying number of tests by 0.6 and prep. amt of 1× wash soln required based on 0.5 mL 50× wash soln to 25 mL reagent water.

*(6)* Prep. conjugate soln by adding 24 mL 1× conjugate diluent to lyophilized material in vial. Mix gently by inverting bottle several times. Date vial. Add 0.2 mL conjugate into appropriate wells of sep. plate. Label plate "conjugate."

*(7)* Following 20 min incubation (above), wash beads as follows:

(a) Assure that 96-well plate (from step *3*a) is inverted in top slot of mag. transfer device and that magnet is in UP position.

(b) Slide "antigen" plate contg beads under top plate and lower magnet to DOWN position (all beads should now be in inverted plate).

(c) Remove bottom "antigen" plate without disturbing top plate and place it in container for proper disposal.

(d) Slide "wash 1" under top plate and wash beads 12 times by raising and lowering magnet to extreme UP and full DOWN positions (count UP and DOWN as 1 wash).

(e) With magnet in DOWN position (beads in top inverted plate), remove wash plate without disturbing top plate and save for step *11*c. *Note:* Proceed immediately with next step. Do not let beads dry in top plate.

*(8)* Slide "conjugate" plate under top plate and raise magnet to let beads drop into wells.

*(9)* Remove bottom plate contg conjugate and beads and incubate with agitation (10–100 rpm) for 20 min at 37°.

*(10)* While beads are incubating, reconstitute ABTS substrate by adding contents of substrate diluent vial to lyophilized substrate. Mix gently by inverting bottle several times. Date substrate. Add 0.2 mL room temp. substrate into each appropriate well of unused plate. Also, put 0.2 mL into extra well which will be used to "blank" EIA reader.

*(11)* Following conjugate incubation period, wash beads as follows:

(a) Assure that 96-well plate (from step *3*a) is still inverted in top slot of mag. transfer device and that magnet is in UP position.

(b) Slide "conjugate" plate contg beads under top plate and lower magnet to DOWN position (all beads should now be in inverted plate).

(c) Remove bottom plate and slide "wash 1" under top plate. Raise magnet to extreme UP position, allowing beads to fall into "wash 1."

(d) Remove top plate and *replace with unused top plate*. Lower magnet to remove beads from "wash 1." Wash beads twice in "wash 1" by raising and lowering magnet to its extreme UP and full DOWN positions (count UP and DOWN as 1 wash).

(e) With magnet in DOWN position (beads in top plate), remove "wash 1" and insert "wash 2."

(f) Wash beads 9 times by raising and lowering magnet to extreme UP and full DOWN positions.

(g) With magnet in UP position (beads in "wash 2"), *replace top inverted plate with unused plate*. *Note:* This change must be made to avoid contamination of substrate with conjugate.

(h) Lower magnet and remove bottom "wash 2" without disturbing top plate.

*(12)* Immediately slide "substrate" plate under top plate and raise magnet to let all beads drop into the wells.

*(13)* Remove bottom plate contg substrate and beads and incubate uncovered at room temp. (20–25°) for 10 min. *Do not agitate plate.*

*(14)* After 10 min, add 0.025 mL (25 μL) "stop" soln to each well including substrate blanking well. Gently swirl beads in plate to disperse colored reaction product forming at surface of beads. Remove beads (be sure that 96-well plate is inverted in top slot of mag. transfer device) by placing "substrate" plate in mag. transfer device and lowering magnet.

*(15)* Remove "substrate" plate and release beads into used plate by sliding plate into mag. transfer device and raising magnet. Mix contents of substrate blanking well with pipet tip.

*(16)* Read results on EIA reader.

*(17)* Sterilize all used plates, tubes, etc., prior to disposal. Tightly close and return unused reagents to 2–8° storage.

### H. Reading

Insert 405 nm filter and bring reader to zero (blank reader) on well contg only substrate and "stop" soln. Then read each individual control and sample well. Average optical density readings of the 2 neg. control wells. For test to be valid, pos. control should read ≥0.200 and av. of neg. controls should read ≤0.120. Record optical density (OD) of each well on data sheet. Samples reading ≥0.200 should be considered pos. Samples reading <0.200 should be considered neg.

### I. Confirmation of Positive EIA Samples

Pos. EIA reading indicates that *Salmonella* may be present. However, since antibodies may cross-react with a few other organisms, cultural confirmations should be performed by streaking HE, XLD, and BS plates from tetrathionate broth, selenite cystine broth, and M-broth tubes as described in **967.26B**, and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **69,** 786(1986).

## 987.11 *Salmonella* in Low-Moisture Foods

### Colorimetric Monoclonal Enzyme Immunoassay Screening Method

### First Action 1987

Method is screening procedure for presence of *Salmonella* in low-moisture foods.

Enrichment broths and M-broths from samples pos. by enzyme immunoassay (EIA) method must be streaked on selective media as in **967.26B** and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Detn of pos. result is objective and must be performed using filter photometer having 405 nm filter. Pos. result is valid only when neg. and pos. controls exhibit acceptable optical density readings.

#### *A. Principle*

*See* **986.35A**.

#### *B. Method Performance*

| Results | Percent | 95% Confidence Range (Approx.) |
|---|---|---|
| Agreement[1] | 97.0 | 95.8–98.2 |
| False neg. rate[2] | 3.3 | 1.7–4.9 |

[1] This rate reflects no. of samples read identically between 302AOAC/BAM (Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA) culture method and EIA.
[2] This rate reflects no. of samples found to be pos. by AOAC/BAM culture method but detd to be neg. by EIA.

Of 15 laboratories, 7 (47%) had complete agreement between culture method and EIA (156/156); 14 laboratories (93%) showed agreement on ≥97% of samples.

#### *C. Reagents*

*See* **986.35C**.

#### *D. Apparatus*

*See* **986.35D**.

#### *E. General Instructions*

*See* **986.35E**.

#### *F. Preparation of Sample*

**(a)** *Pre-enrichment.*—Pre-enrich low-moisture food product in non-inhibitory broth to initiate growth of salmonellae. Methods used may vary with product, and should be performed as indicated in **967.26A**, or in Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA, Chap. 7, section C, except incubation time is 18–24 h.

**(b)** *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment mixt. to selenite cystine broth and 1 mL to tetrathionate broth as in **967.26B(a)**. Pre-warm both broths to 35° before inoculation. (For dried active yeast, substitute lauryl sulfate tryptose broth, **967.25A(u)**, for selenite cystine broth.) Incubate 6–8 h in 35° *water bath.*

**(c)** *Post-enrichment.*—Remove selective broths from incubation and mix by hand or by vortex mixer. Remove 1.0 mL from tetrathionate tube and transfer to 10 mL tube of sterile M-broth. Also remove 1.0 mL from selenite cystine tube and transfer to another 10 mL tube of sterile M-broth. Incubate both M-broths and remaining tetrathionate and selenite cystine broths for 14–18 h at 35°.

**(d)** *Preparation of sample for EIA analysis.*—Remove the 2 tubes of M-broth (M-broth-Tet and M-broth-SC) from incubation and mix tubes by hand or by vortex mixer. Remove 0.5 mL from M-broth-Tet tube and transfer to glass screw-cap test tube. Also remove 0.5 mL from M-broth-SC tube and transfer to same screw-cap test tube. Refrigerate (2–8°) remaining M-broths and tetrathionate and selenite cystine broths from **(c)** for cultural confirmation of EIA-pos. samples. Heat combined M-broths in boiling $H_2O$ bath or in flowing steam 20 min. Cool heated exts to 25–37° prior to analysis by EIA. *Note:* Heated exts which are not cooled to this temp. can destroy monoclonal antibodies on metal beads.

#### *G. Enzyme Immunoassay*

*See* **986.35G**.

#### *H. Reading*

*See* **986.35H**.

#### *I. Confirmation of Positive EIA Samples*

Pos. EIA reading indicates that *Salmonella* may be present. However, since antibodies may cross-react with a few other organisms, culture confirmations should be performed by streaking HE, XLD, and BS plates from tetrathionate broth, selenite cystine broth, and the associated M-broth tubes as described in **967.26B**, and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **70,** 530(1987).

## 989.14 Salmonella in Foods

### Colorimetric Polyclonal Enzyme Immunoassay Screening Method

### First Action 1989

Method is screening procedure for presence of *Salmonella* in all foods; it is not a confirmatory test because polyclonal antibodies used in test may cross-react with small percentage of non-*Salmonella*.

Enrichment broths and M-broths from samples pos. by enzyme immunoassay (EIA) method must be streaked on selective media as in **967.26B** and typical or suspicious colonies must be identified as in **967.26C**, **967.27**, **967.28**.

Detn of pos. result may be performed *(1)* visually by aid of color comparator card where pos. result is valid when neg. and pos. controls match those described on card or *(2)* instrumentally using filter photometer having 414 nm filter where pos. result is valid only when neg. and pos. controls possess acceptable optical density readings.

#### *A. Principle*

Detection of *Salmonella* antigens is based on enzyme immunoassay using highly purified antibodies prepd from antigens unique to *Salmonella*. Polyclonal antibodies to *Salmonella* antigen are adsorbed onto internal surface of 96-well microtiter tray. Sample to be assayed is placed into well of tray. If *Salmonella* antigens are present in sample, they will attach to specific antibody adsorbed on well. All other material in samples is washed away. Conjugate is added and will bind to *Salmonella* antigens if they are attached to adsorbed antibody on surface of well. Wells are washed to remove unbound conjugate, and enzyme substrate is added. Dark blue-green color indicates presence of *Salmonella* antigen in sample.

### B. Method Performance

For all foods:

| Results | Percent | 95% Confidence Range (Approx.) |
|---|---|---|
| Agreement[1] | 96.8 | 95.4–98.2 |
| False neg. (BAM)[2] | 1.6 | 0.5–2.7 |
| False neg. (EIA)[3] | 1.4 | 0.4–2.4 |

[1]This rate reflects no. of samples read identically between AOAC/BAM (Bacteriological Analytical Manual, 1984, 6th ed., AOAC, Arlington, VA) culture method and EIA.
[2]This rate reflects no. of samples found to be pos. by EIA but neg. by AOAC/BAM culture method.
[3]This rate reflects no. of samples found to be pos. by AOAC/BAM culture method but neg. by EIA.

Of 14 laboratories, 3 had complete agreement between culture method and EIA method. Excluding 1 food group, turkey, 13 of the 14 laboratories had perfect agreement between BAM/AOAC and EIA methods. Laboratory that did not have perfect agreement had difference in each of pepper, nonfat dry milk, and chocolate food groups.

### C. Reagents

Items **(a)**–**(m)** are available as TECRA *Salmonella* Visual Immunoassay (Bioenterprises Pty Ltd, 28 Barcoo St, Roseville, NSW 2069, Australia). Substitutions must be pretested for equivalency.

**(a)** *Antibody adsorbed strips.*—Removawell® (Dynatech Laboratories, Inc.) strips. Polyclonal antobodies to *Salmonella,* 96 wells. Store wells at 2–8° when not in use.

**(b)** *Tray.*—Sufficient to secure individual wells or strips.

**(c)** *Control antigens.*—Pos. control (lyophilized). Purified *Salmonella* antigen, which reacts with antibodies to *Salmonella,* 1 vial. Neg. control (lyophilized lactose), which is nonreactive with antibodies to *Salmonella,* 1 vial. Reconstituted control antigens are stable 28 days when stored at 2–8°.

**(d)** *Controls diluent.*—1 vial (5 mL/vial). Contains 0.006 g Tris [tris(hydroxymethyl)aminomethane], 0.044 g NaCl, 0.0025 g Tween 20 (polyoxyethylene 20 sorbitan monolaurate), and 0.005 g thimerosal in $H_2O$.

**(e)** *Conjugate.*—1 vial (lyophilized). Contains 147 ng anti-*Salmonella* antibodies (from sheep) conjugated to horseradish peroxidase, 0.00686 g $Na_2B_4O_7$, 0.12 g Dextran T10, 0.06 g hydrolyzed gelatin, 0.0024 g $CaCl_2$, and 120 ng thimerosal. Reconstituted conjugate is stable 28 days when stored at 2–8°.

**(f)** *Conjugate diluent.*—1 vial (22 mL/vial). Contains 0.42 g $Na_2B_4O_7$, 0.193 g NaCl, 0.22 g hydrolyzed gelatin, and 0.0022 g thimerosal in $H_2O$.

**(g)** *Substrate.*—1 vial (lyophilized). Contains 0.011 g 2,2′-azino-di(3-ethylbenzthiazoline sulfonate) and 0.123 g $NaH_2PO_4$. Reconstituted substrate is stable 28 days when stored at 2–8°.

**(h)** *Substrate diluent.*—1 vial (22 mL/vial). Contains 0.116 g citric acid, 0.0011 g $H_2O_2$, and 0.0185 g NaOH in $H_2O$.

**(i)** *"Stop" soln.*—1 vial (6 mL/vial). Vial contains 0.15 g NaF in $H_2O$. *Caution:* Avoid contact with skin. If contact occurs, wash area with $H_2O$.

**(j)** *Wash soln concentrate.*—1 vial (25 mL/vial). Contains 1.45 g Tris, 7.03 g NaCl, 0.5 g Tween 20, and 0.0025 g thimerosal in $H_2O$.

**(k)** *Package insert.*

**(l)** *Data record sheet.*

**(m)** *Color comparator card.*—For visual interpretation of pos. and neg. tests.

**(n)** *M-broth.*—5.0 g yeast ext, 12.5 g tryptone, 2.0 g D-mannose, 5.0 g Na citrate, 5.0 g NaCl, 5.0 g $K_2HPO_4$, 0.14 g $MnCl_2$, 0.8 g $MgSO_4$, 0.04 g $FeSO_4$, 0.75 g Tween 80. Suspend ingredients in 1 L $H_2O$ and heat to boiling for 1–2 min. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Cap tubes loosely and autoclave 15 min at 121°. Tighten caps securely for storage. Final pH should be 7.0 ± 0.2.

**(o)** *Diagnostic reagents.*—Necessary for culture confirmation of presumptive pos. EIA tests; see **967.25B**.

### D. Apparatus

**(a)** *Incubator.*—35–37°.

**(b)** *Multipipets.*—Capable of delivering accurate amts in ranges 50–250 μL and 5–50 μL.

**(c)** *Water bath.*—Capable of maintaining 100°. Autoclave set at 100° is acceptable alternative, as are generators of flowing steam.

**(d)** *Plastic squeeze bottle.*—500 mL, for dispensing wash soln. Automatic washer may be used.

**(e)** *Plastic film wrap or sealable plastic container.*—To cover wells during incubation.

**(f)** *Enzyme immunoassay reader.*—Optional. Photometer with 414 ± 10 nm screening filter which will read thru microtiter plates.

### E. General Instructions

Components of kit must be refrigerated when not in use. Kit is intended for 1-time use only; do not reuse wells containing sample, reagents, or wash solution.

Include duplicate pos. and neg. control antigens with each group of test samples. All controls must function properly for test to be valid.

Use data record sheet to identify location of each test sample.

Use sep. pipets for each sample and kit reagent to avoid cross-contamination. If plastic troughs are used to dispense conjugate and substrate, ensure that they are always kept separate.

Components in kit are intended for use as integral unit. Do not mix components of different batch numbers.

### F. Preparation of Sample

**(a)** *Pre-enrichment.*—Pre-enrich product in noninhibitory broth to initiate growth of salmonellae. Methods used may vary with product and should be performed as indicated in **967.26A**, or in Bacteriological Analytical Manual, 6th ed., AOAC, Arlington, VA, Chap. 7, section C, with following exception:

*Raw or highly contaminated products.*—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (ca 20 000 rpm). Cap jars securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH to 6.8 ± 0.2, if necessary, using sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Aseptically transfer contents of each jar to sterile wide-mouth, screw-cap 500 mL jar. Loosen jar caps 1/4 turn and incubate 24 ± 2 h at 35°.

**(b)** *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment mixts to selenite cystine broth and 1 mL to tetrathionate broth as in **967.26B(a)**. For all foods other than raw or highly contaminated products, incubate 6–8 h at 35°. Selective enrichments of raw or highly contaminated product must be incubated 16–20 h at 35°.

**(c)** *Post-enrichment.*—Remove selective broths from incubation and mix by hand or by vortex mixer. Remove 1 mL from tetrathionate tube and transfer to 10 mL tube of sterile M-broth which has been warmed to 35°. Also remove 1 mL from selenite cystine tube and transfer to sep. tube of M-broth. For all foods other than raw or highly contaminated products, incubate M-broth tubes 14–18 h and return selective enrichment broth tubes to 35° incubator and incubate for addnl 16–18 h. For raw or highly contaminated products, incubate M-broth tubes 6 h at 35° and return selective enrichment broth tubes to 35° and incubate for addnl 6 h at 35°.

(**d**) *Preparation of sample for EIA analysis.*—Remove M-broth tubes from incubation and mix tubes by hand or vortex mixer. Combine 1.0 mL from each M-broth tube in clean screw-cap tube and heat in boiling $H_2O$ bath or in flowing steam 15 min. Refrigerate (2–8°) remaining M-broth and tetrathionate and selenite cystine tubes from (**c**) for culture confirmation of any EIA pos. samples. Cool heated M-broths to 25–37° prior to analysis by EIA.

### G. Enzyme Immunoassay

*(1)* Following reagents must be prepd prior to commencing assay:

(a) Prep. working strength wash soln by dilg contents of 1 vial of wash soln conc. to 1 L with distd or deionized $H_2O$ into plastic reagent bottle. Plastic squeeze bottle is ideal for washing trays manually.

(b) Prep. reconstituted neg. control by transferring 2 mL controls diluent to vial of lyophilized neg. control antigen; mix thoroly. Similarly prep. reconstituted pos. control by transferring 2 mL controls diluent to vial of lyophilized pos. control antigen; mix thoroly.

(c) Prep. reconstituted conjugate by adding 5 mL conjugate diluent to vial of lyophilized conjugate. Let conjugate rehydrate at room temp., mix, and then pour contents of vial into conjugate diluent vial. Finally, gently mix reconstituted conjugate.

(d) Prep. reconstituted substrate by adding vial of substrate diluent to lyophilized substrate. Be sure substrate has dissolved and mixt. is room temp. prior to use. Reconstituted substrate will appear pale green.

(e) Use stop soln as received. No reconstitution is required.

*(2)* Secure desired no. of test (Removawell) strips in tray, allowing 1 well per food sample plus 4 wells for controls. PRESS WELLS FIRMLY INTO PLACE. Remove sealing film from top of wells to be used. Transfer 0.2 mL of each heated M-broth sample to single well. Transfer 0.2 mL aliquots of reconstituted neg. control into 2 wells and 0.2 mL aliquots of reconstituted pos. control into 2 wells. Record sample position on sample record sheet provided. *Note:* Be sure numbered tag at end of each test strip has been removed.

*(3)* Cover tray and incubate 30 min at 35–37° in std laboratory incubator. Tray must be covered to prevent evapn. Plastic film or sealed plastic container may be used.

*(4)* After incubation, wash plate by hand using plastic squeeze bottle contg working strength wash soln or use automatic washer charged with working strength wash soln as follows:

(a) Quickly invert tray, emptying its contents into container.

(b) Remove any residual liquid by FIRMLY tapping tray face-down on paper towel several times.

(c) Completely fill each well with working strength wash soln.

(d) Repeat (a)–(c) **2** more times.

*(5)* Empty tray according to *4*(a) and (b); then add 0.2 mL reconstituted conjugate to each well. Cover tray and incubate 30 min at 35–37°.

*(6)* Empty contents of tray and wash it thoroly **4** times according to *4*(a)–(c); then empty tray according to *4*(a) and (b).

*(7)* Add 0.2 mL reconstituted substrate to each well. Incubate at room temp. (20–25°) until pos. control has reached color equiv. to pos. control on color comparator card or to $A \geq 1.0$. Because color development tends to conc. around edges of wells, it is important to tap sides of plate *gently* to mix contents prior to reading result. In this way, accurate readings will be obtained.

*(8)* Add 0.02 mL stop soln to each well. Incubation time should be ca 10–20 min. If >25 min has elapsed and $A$ of 1.0 has not been attained, test is invalid.

### H. Reading

Results of tests can be detd *(1)* visually or *(2)* with microtiter tray reader.

*(1)* Place tray on white background, and then compare individual test wells with color comparator. Pos. control should give strong blue-green color indicating that all reagents are functional. If pos. control is lighter than "Positive Control" on color comparator card, test is invalid; refer to "Troubleshooting Guide" in package insert (**k**). If neg. control is darker than "Negative" on color comparator card, it is probable that tray was inadequately washed, and assay must be repeated. Duplicate control antigens should appear equiv. by eye.

*(2)* $A$ max. of blue-green end product occurs at 414 nm; therefore, tray can be read at 414 ± 10 nm. For single and dual wavelength readers, set reader to zero (blank) on air. For dual wavelength readers, set second ref. wavelength at 490 ± 10 nm. $A > 0.3$ indicates pos. result. $A > 0.25$ for neg. control indicates insufficient washing of tray. Pos. control should give $A \geq 1.0$.

### I. Confirmation of Positive EIA Samples

Pos. EIA reading indicates that *Salmonella* may be present. However, since antibodies may cross-react with a few other organisms, culture confirmations must be performed by streaking HE, XLD, and BS plates from tetrathionate broth, selenite cystine broth, and M-broth tubes as described in **967.26B**, and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **71**, 973(1988).

## 989.15 *Salmonella* in Foods
### Fluorogenic Monoclonal Enzyme Immunoassay Screening Method
### First Action 1989

Method is screening procedure for presence of *Salmonella* in all foods; it is not a confirmatory test because monoclonal antibodies used in test may cross-react with small percentage of non-*Salmonella*.

Enrichment broths and M-broths from samples pos. by enzyme immunoassay (EIA) method must be streaked on selective media as in **967.26B** and typical or suspicious colonies must be identified as in **967.26C**, **967.27**, **967.28**.

### A. Principle

Detection of *Salmonella* antigens is based on enzyme immunoassay which measures *Salmonella* antigen in foods and feeds. Monoclonal antibodies to *Salmonella* antigen are coated on internal surface of plastic microtiter strip wells, and sample to be assayed is added to strip well. If *Salmonella* antigens are present in sample, they will be bound to antibody adsorbed onto surface of well. All other material in sample is washed away.

*Salmonella* antibody conjugated to alk. phosphatase is added and will bind to *Salmonella* antigens if they are attached to adsorbed antibody on surface of well. This forms antibody-antigen-antibody complex. Unbound conjugate is removed by washing and fluorescent substrate is added. Samples with fluorescent value greater than or equal to recommended cutoff value are considered pos. for *Salmonella* antigens.

### *B. Method Performance*

For all foods:

| Results | Percent | 95% Confidence Range (Approx.) |
|---|---|---|
| Agreement[1] | 98.5 | 97.9–99.1 |
| False neg. (BAM)[2] | 1.1 | 0.8–1.4 |
| False neg. (EIA)[3] | 0.8 | 0.0–2.7 |

[1] This rate reflects no. of samples read identically between AOAC/BAM (Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA) culture method and EIA.
[2] This rate reflects no. of samples found to be pos. by EIA but neg. by AOAC/BAM culture method.
[3] This rate reflects no. of samples found to be pos. by AOAC/BAM culture method but neg. by EIA.

Of 11 laboratories submitting usable data, 6 (55%) had complete agreement between culture method and EIA; 10 laboratories (91%) showed agreement on ≥96% of samples; all laboratories showed agreement on ≥93% of samples.

### *C. Reagents*

Items (**a**)–(**j**) are available as Q-TROL *Salmonella* Detection Kit (Dynatech Laboratories, Inc., 14340 Sullyfield Circle, Chantilly, VA 22021).

(**a**) *Antibody coated microtiter wells.*—Monoclonal antibody to *Salmonella*, eight 12-well strips. Stable 28 days, after opening, when stored at 2–8°.

(**b**) *Microtiter strip well holder.*—Sufficient for securing individual wells or strips.

(**c**) *Control antigens.*—Pos. control (lyophilized boiled suspension of *S. typhimurium*) purified *Salmonella* antigen, which reacts with antibodies to *Salmonella*, 1 vial; neg. control (lyophilized boiled suspension of *Proteus mirabilis*), which is nonreactive with antibodies to *Salmonella*, 1 vial. Reconstituted control antigens are stable 28 days when stored at 2–8°.

(**d**) *Tween 20.*—1 vial. 25% Tween 20 (polyoxyethylene (20) sorbitan monolaurate) in $H_2O$. After opening, soln is stable 28 days when stored at 2–8°.

(**e**) *Phosphate buffer-saline tablets.*—For prepn of PBS-Tween soln. Dissolve 1 tablet in 100 mL distd or deionized $H_2O$ to prep. 0.01M phosphate-buffered 0.85% saline. Add 8 drops Tween 20. PBS-Tween soln is used to rehydrate pos. and neg. control antigens and for wash steps, and is stable 7 days when stored at 2–8°.

(**f**) *Enzyme conjugate.*—1 vial contg antibody of *Salmonella* conjugated to alk. phosphatase (lyophilized). Reconstituted conjugate is stable 28 days when stored at 2–8°.

(**g**) *Conjugate diluent.*—1 vial (10 mL/vial). Contains 0.05M tris buffer (pH 8), 0.02% $NaN_3$, 1 mM $MgCl_2$, and 1% bovine serum albumin.

(**h**) *Substrate tablets.*—0.13 mg 4-methylumbelliferyl phosphate (4-MUP) per tablet. Reconstituted substrate must be used within 2 h.

(**i**) *Substrate diluent.*—1 vial (31.5 mL/bottle). Aq. soln of 10% diethanolamine with 0.02% $NaN_3$ as preservative.

(**j**) *Stop soln.*—1 vial (5.5 mL/vial). Aq. soln of 2% $Na_3PO_4$.

(**k**) *M-broth.*—5.0 g yeast ext, 12.5 g tryptone, 2.0 g D-mannose, 5.0 g Na citrate, 5.0 g NaCl, 5.0 g $K_2HPO_4$, 0.14 g $MnCl_2$, 0.8 g $MgSO_4$, 0.04 g $FeSO_4$, 0.75 g Tween 80. Suspend ingredients in 1 L $H_2O$ and heat to boiling for 1–2 min. Dispense 10 mL portions into 16 × 125 mm screw-cap test tubes. Cap tubes loosely and autoclave 15 min at 121°. Tighten caps securely for storage. Final pH should be 7.0 ± 0.2.

(**l**) *Diagnostic reagents.*—Necessary for cultural confirmation of presumptive pos. EIA tests; see **967.25B**.

### *D. Apparatus*

(**a**) *Fluorometer.*—To measure relative fluorescence of contents of microtiter well (Micro FLUOR® Reader, Dynatech Laboratories, Inc.; or equiv.).

(**b**) *Microtiter strip well-washer/aspirator.*—With 12 channels to wash entire strip.

(**c**) *Pipets.*—Capable of delivering 50–200 μL.

(**d**) *Water bath.*—Capable of maintaining 100°. Autoclave set at 100° is acceptable alternative, as are generators of flowing steam.

### *E. General Instructions*

Components of kit must be refrigerated when not in use. Kit is intended for 1-time use only; do not reuse wells contg sample, reagents, or wash soln.

Include 3 neg. and 1 pos. control antigens with each group of test samples. All controls must function properly for test to be valid.

*Caution:* Diluents for conjugate and substrate contain $NaN_3$ as preservative. Flush drains with $H_2O$ if any solns contg $NaN_3$ are discarded in sink. Flushing will prevent formation of lead or copper azide in plumbing, which may explode upon percussion (such as hammering). 4-MUP diluent and stop soln are basic and may cause skin irritation. If contact with skin occurs, flush area with $H_2O$.

Use data record sheet to identify location of each test sample.

Use sep. pipets for each sample and kit reagent to avoid cross-contamination. If plastic troughs are used to dispense conjugate and substrate, ensure that they are always kept separate.

Components in kit are intended for use as integral unit. Do not mix components of different batch numbers.

### *F. Preparation of Sample*

(**a**) *Pre-enrichment.*—Pre-enrich product in noninhibitory broth to initiate growth of salmonellae. Methods used may vary with product and should be performed as indicated in **967.26A**, or in Bacteriological Analytical Manual, 6th ed., AOAC, Arlington, VA, Chap. 7, sec. C, with following exception:

*Raw or highly contaminated products.*—Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (ca 20 000 rpm). Cap jars securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH to 6.8 ± 0.2, if necessary, using sterile 1N NaOH or HCl, capping jar securely and mixing well before detg final pH. Aseptically transfer contents of each jar to sterile wide-mouth, screw-cap 500 mL jar. Loosen jar caps 1/4 turn and incubate 24 ± 2 h at 35°.

(**b**) *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment mixts to selenite cystine broth and 1 mL into tetrathionate broth as in **967.26B(a)**. For all foods, incubate 6–8 h in 35° water bath.

(**c**) *Post-enrichment.*—Remove selective broths from incubation and mix by hand or by vortex mixer. Remove 1 mL from tetrathionate tube and transfer to 10 mL tube of sterile M-broth which has been warmed to 35°. Also remove 1 mL from selenite cystine tube and transfer to a separate tube of M-broth. For all foods, incubate M-broth tubes for 14–18 h and return selective enrichment broth tubes to 35° incubator and incubate for addnl 16–18 h.

(**d**) *Preparation of sample for EIA analysis.*—Remove M-broth tubes from incubation and mix tubes by hand or vortex mixer. Combine 0.5 mL from each M-broth tube in clean screw-cap tube and heat 20 min in boiling $H_2O$ bath or flowing steam. Refrigerate (2–8°) remaining M-broth and tetrathionate and selenite cystine tubes from (**c**) for cultural confirmation of any

EIA pos. samples. Cool heated M-broths to 20–30° prior to analysis by EIA.

### G. Enzyme Immunoassay

*(1)* Following reagents must be prepd prior to commencing assay:

(a) *PBS-Tween soln.*—For every two 12-well strips to be used, dissolve 1 PBS tablet in $H_2O$ and prep. soln as in (**e**).

(b) *Reconstituted control antigens.*—Transfer 3 mL PBS-Tween soln to neg. control vial and mix contents thoroughly. Transfer 3 mL PBS-Tween soln to pos. control vial and mix contents thoroughly. These solns are reconstituted neg. and pos. controls, resp.

(c) *Reconstituted enzyme conjugate.*—Add 10 mL (1 vial) conjugate diluent to conjugate vial. Mix and let contents of vial rehydrate at room temp.

(d) *Stop soln.*—No reconstitution is required. Warm soln at 35° if crystals are present.

*(2)* Turn on power to reader and printer. Allow at least 2 h warm-up.

*(3)* Remove necessary number of microtiter strips from Al foil pouch, allowing 1 well per food sample plus 4 wells for controls. Secure strips in strip well holder. Transfer 100 μL neg. control antigen into each of wells A-1, A-2, and A-3. Transfer 100 μL pos. control antigen into well designated A-4. Transfer 100 μL each heated M-broth sample to single well. Record sample position on sample record sheet provided.

*(4)* Incubate tray for 60 min at 20–25°.

*(5)* After incubation, aspirate samples from wells and add 300 μL PBS-Tween soln to each well by use of washer/aspirator.

(a) Repeat this step 4 more times.

(b) Aspirate last wash. Invert tray and firmly tap it on absorbent paper several times to remove last traces of fluid.

*(6)* Add 100 μL reconstituted enzyme conjugate to bottom of each well and incubate 40 min at 20–25°.

*(7)* During this incubation period, prep. substrate by adding one 4-MUP substrate tablet to 5.2 mL substrate diluent. Dissolve 1 substrate tablet for every 2 microtiter strips to be used. Swirl soln occasionally to dissolve tablet(s).

*(8)* Repeat steps *5*(a) and (b).

*(9)* Add 200 μL 4-MUP substrate to bottom of each well. Incubate 20 min at 20–25°.

*(10)* Add 50 μL stop soln to each well.

### H. Reading

Place tray in reader. Read relative fluorescent units (RFU) of each control and sample well. Calc. av. RFU of the 3 neg. control wells. Individual neg. control values should be ≥0.85 av. RFU and ≤1.15 av. RFU. If 1 value is outside this range, discard that value and recalc. mean. If 2 values are outside range, test is invalid and must be repeated. Multiply av. valid neg. controls by 2.3 to det. cutoff value. Any sample with value at or above cutoff value is considered reactive.

If av. of neg. control values exceeds 1600 RFU, cutoff will exceed dynamic range of reader and test is invalid. Poor washing and deterioration of substrate may result in high readings of neg. control.

### I. Confirmation of Positive EIA Samples

Pos. EIA reading indicates that *Salmonella* may be present. However, since antibodies may cross-react with a few other organisms, culture confirmations should be performed by streaking HE, XLD, and BS plates from tetrathionate broth, selenite cystine broth, and M-broth tubes as described in **967.26B**, and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **72**, 318(1989).

## 987.10 *Salmonella* in Foods
### DNA Hybridization Screening Method
**First Action 1987**
**Final Action 1989**

Method is test procedure for presence of *Salmonella* in all foods. Because a certain percentage of false pos. reactions are expected, all pos. assays should be confirmed by standard culture methods. Enrichment broths and GN broths from samples pos. by DNA hybridization method should be streaked to selective media as in **967.26B** and typical or suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

### A. Principle

Detection of *Salmonella* DNA in cultured food samples uses specific DNA probes. Following pre-enrichment, selective enrichment, and post-enrichment of test samples, bacteria are collected on membrane filters by vac. filtration. Bacteria are lysed, DNA is denatured, and resultant single-stranded DNA is fixed to membrane filters. Filters are then incubated in hybridization soln contg $^{32}P$-labeled *Salmonella*-specific DNA molecules. If *Salmonella* target DNA is present in test sample, radiolabeled DNA probes will hybridize to target DNA sequences. Unbound probe is washed away and radioactivity on filters is measured. Radioactivity on filter above threshold value indicates presence of *Salmonella* in test sample.

### B. Method Performance

For all foods:

| Results | Percent | 95% Confidence Range |
|---|---|---|
| Agreement[1] | 95.6 | 94.0–97.2 |
| False neg. (BAM)[2] | 5.9 | 3.7–8.1 |
| False neg. (DNAH)[3] | 0.2 | 0.0–0.6 |

[1] Rate reflects no. of samples read identically between AOAC/BAM (Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA) culture method and DNA hybridization (DNAH) method.
[2] Rate reflects no. of samples found to be pos. by DNAH method but detected as neg. by AOAC/BAM culture method.
[3] Rate reflects no. of samples found to be pos. by AOAC/BAM culture method but detected as neg. by DNAH method.

Of 11 laboratories, 2 had complete agreement between culture and DNAH methods; 4 showed agreement on ≥97% of samples; 7 showed agreement on ≥95% of samples; 10 showed agreement on ≥93% of samples.

### C. Reagents

Items (**a**)–(**e**) are available as GENE-TRAK® DNA Hybridization Test for Detection of *Salmonella* (GENE-TRAK Systems, Inc., 31 New York Ave, Framingham, MA 01701).

(**a**) *Filter cups.*—Filter cup assemblies contg 25 mm diam. membrane filters (112 assemblies/box). Sufficient for 96 tests plus controls.

(**b**) *Soln set.*—Contains *(1)* 1 bottle (240 mL) denaturation soln (0.2N NaOH and 0.6M NaCl). (*Caution:* Contains NaOH. If contact with skin occurs, wash skin thoroly with $H_2O$.) *(2)* 1 bottle (240 mL) neutralization soln (1.0M Tris, pH 7.0, and 0.6M NaCl). *(3)* 1 bottle (240 mL) fixation soln (95% EtOH). (*Caution:* Flammable, contains alcohol.) *(4)* 1 bottle (120 mL) pre-hybridization soln (0.9M NaCl, 0.09M Na citrate, 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinyl pyrrolidone, 1mM $Na_2$ ethylenediamine tetraacetate, and 0.5% Na dodecyl sulfate (SDS), pH 6.5–7.5). *(5)* 1 bottle (60 mL) hybridization soln (same as soln *4*, with 10% dextran sulfate). *(6)* 3 bottles (240 mL each) wash soln (0.03M NaCl, 0.003M Na citrate, and 0.5% SDS, pH 6.5–7.5).

Sufficient for 96 tests plus controls. All solns are stable 6 months from date of manufacture when stored at 15–24°.

**(c)** *Salmonella probe and controls set.*—Contains *(1)* 1 bottle (20 mL) pos. control soln (heat-killed *S. typhimurium*). *(2)* 1 bottle (60 mL) neg. control soln (heat-killed *Escherichia coli*). *(3)* 1 vial (0.75 mL, 75 μCi $^{32}P$) $^{32}P$-labeled *Salmonella* DNA probe soln.

Sufficient for 96 tests plus controls. Shipped frozen on dry ice; thaw at room temp. (15–24°) before use. Solns are stable 8 days from receipt when stored at 2–8°.

**(d)** *Instruction manual.*

**(e)** *Data sheets.*

**(f)** *Gram negative (GN) broth.*—20.0 g tryptose, 1.0 g dextrose, 2.0 g D-mannitol, 5.0 g Na citrate, 0.5 g Na desoxycholate, 4.0 g $K_2HPO_4$, 1.5 g $KH_2PO_4$, 5.0 g NaCl. Dissolve ingredients in 1 L $H_2O$. Dispense 10 mL portions into 16 × 125 mm test tubes (or equiv.). Cap tubes loosely and autoclave 15 min at 121°. Final pH should be 7.0 ± 0.2 at 25°.

**(g)** *Diagnostic reagents.*—Necessary for cultural confirmation of pos. DNA hybridization tests; see **967.25B**.

### D. Apparatus

Items **(a)**–**(e)** are available from GENE-TRAK Systems, Inc.

**(a)** *Manifold kit.*—Vac. filtration manifold device consisting of base with vac. control valve and top to house filter cup assemblies.

**(b)** *Bottle holder.*—Plastic rack to hold soln bottles in water bath.

**(c)** *Shield.*—½ in. thick Lucite used to protect worker from beta particle emissions from $^{32}P$ decay when radiolabeled DNA probe soln is used.

**(d)** *Vacuum pump.*—Adjustable between 8 and 15 in. Hg. Connected to manifold base thru trap consisting of 1 or 2 L vac. filtration flask and requisite tubing.

**(e)** *Beta detector.*—Any instrument capable of measuring radioactive decay of $^{32}P$ on dry, 25 mm membrane filters with efficiency of 0.4–0.5, e.g., scintillation counter. Use of scintillation counter requires appropriate carrier vials and insert minivials to hold filters upright.

**(f)** *Heating water bath.*—Capable of maintaining 65 ± 1.0°. Able to accommodate bottle holder 12 × 6 × 4½ in. and $H_2O$ level of 4½ in.

**(g)** *Micropipet.*—Capable of delivering accurate amts in range continually adjustable in 1 μL increments between 100 and 200 μL.

**(h)** *Vortex mixer.*—For mixing broth in culture tubes.

**(i)** *Polypropylene screw-cap centrifuge tubes.*—50 mL, conical bottom.

### E. General Instructions

Test uses radioactive compd. Personnel must receive appropriate training in use of radioactive materials and have proper facilities available for use of this substance. Facility must possess current, appropriate radioactive materials license issued by U.S. Nuclear Regulatory Commission or other agency with regulatory control.

Disposal of radioactive waste must be in accordance with radioactive materials license of facility.

Treat all materials in contact with bacterial cultures or culture filtrates as biohazardous material and decontaminate by appropriate methods.

Do not touch membrane filters. Handle with forceps only.

Return pos. and neg. controls and DNA probe soln to 2–8° storage immediately after use.

Include 1 pos. control and 3 neg. controls with each group of test samples.

Components and procedures of this test kit have been standardized for use in GENE-TRAK assay. Use of components or procedures other than those supplied or recommended by GENE-TRAK Systems, Inc., may yield unsatisfactory results.

### F. Sample Preparation

**(a)** *Pre-enrichment.*—Pre-enrich product in non-selective medium to initiate growth of salmonellae. Procedure will vary with product type and should be performed as indicated in **967.26A** or in Bacteriological Analytical Manual (1984) 6th ed., AOAC, Arlington, VA, Chap. 7, section C, with the following exception:

*Raw meats and raw milk products:* Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL sterile lactose broth. Blend 2 min at high speed (ca 20 000 rpm). Cap jar securely and let stand 60 min at room temp. Mix well by shaking and det. pH with test paper. Adjust pH, if necessary, to 6.8 ± 0.2 using sterile 1N NaOH or HCl; cap jar securely and mix well before detg final pH. Aseptically transfer contents to sterile wide-mouth, screw-cap 500 mL jar. Loosen jar cap ¼ turn and incubate 24 ± 2 h at 35°.

**(b)** *Selective enrichment.*—Transfer 1 mL incubated pre-enrichment culture to tube contg 10 mL selenite cystine broth and 1 mL to tube contg 10 mL tetrathionate broth (pre-warmed to 35°) as in **967.26B(a)**. Incubate 6 h at 35° with the following exception:

*Raw meats and raw milk products:* Incubate selenite cystine and tetrathionate broth 18 ± 2 h at 35°.

**(c)** *Post-enrichment.*—Remove selective enrichment cultures from incubation and mix by hand or with vortex mixer. Transfer 1 mL tetrathionate culture to tube contg 10 mL GN broth (prewarmed to 35°). Transfer 1 mL selenite cystine culture to sep. tube contg 10 mL GN broth. Incubate GN broths 12–18 h at 35° with the exception of raw meats and raw milk products (see below). Return tetrathionate and selenite cystine tubes to 35° for incubation up to total of 24 ± 2 h.

*Raw meats and raw milk products:* Incubate GN broths 6 h at 35°. Return tetrathionate and selenite cystine tubes to 35° for incubation up to total of 24 ± 2 h.

### G. Filtration

*(1)* Connect manifold base to vac. pump thru trap. Add disinfectant soln to filtration flask. Two manifolds may be connected in parallel to single vac. source. This configuration will accommodate up to 24 food samples in 1 run plus requisite pos. and triplicate neg. controls.

*(2)* Place manifold top(s) on manifold base(s). Fit manifold top(s) with filter cup assemblies; 1 pos. control filter cup, 3 neg. control filter cups, and up to 24 pre-numbered sample filter cups for each set of assays. Fit unused manifold positions with No. 3 rubber stoppers (provided).

*(3)* Remove sample GN broths from 35° incubation. Vortex-mix or otherwise mix each culture. For each sample, pipet 1 mL from each of the 2 GN broths (one derived from tetrathionate, one from selenite cystine) into single filter cup. Record sample numbers and filter numbers on data sheet.

*(4)* Mix pos. and neg. control solns. Pipet 2 mL pos. control soln into pos. control filter cup. Pipet 2 mL neg. control soln into each of the 3 neg. control filter cups.

Note: Pos. and neg. controls are shipped frozen on dry ice. Thaw at room temp. (15–24°) before first use. Store controls at 2–8° between uses.

*(5)* When all filter cups have been loaded with samples, open vac. control valve on manifold base and turn on vac. pump. Apply vac. (8–10 in. Hg) until all samples are filtered. Avoid vac. of excessive pressure or prolonged duration. Close valve on manifold base and turn off pump. Be sure vac. on manifold base is released before proceeding.

*(6)* Add (squirt in) ca 1.5–2.0 mL denaturation soln (*Soln 1*) into each filter cup, completely covering surface of filters. Wait 2 min, then apply vac. as before (see step *5*) until soln has filtered thru all of the cups. Turn off vac.

*(7)* Add (squirt in) ca 1.5–2.0 mL neutralization soln (*Soln 2*) into each filter cup. Wait 2 min, then apply vac. Turn off vac. after soln has filtered thru all of the cups.

*(8)* Add (squirt in) ca 1.5–2.0 mL fixation soln (*Soln 3*) into each filter cup. Wait 2 min, then apply vac. Turn off vac. after soln has filtered thru all of the cups.

*(9)* Snap off top part of each cup assembly. Be careful not to discard membrane filters. With forceps, remove membrane filters from cup assembly bases and place on sheet of absorbent paper to dry. All other parts of cup assembly should be treated as biohazardous waste and discarded. Manifold bases and tops should be treated with disinfectant soln (do not autoclave).

### *H. DNA Hybridization Assay*

*(1)* Using forceps, place filters (up to 24 sample filters plus 1 pos. and 3 neg. control filters) into 50 mL polypropylene centrf. tube, (**i**).

*(2)* Mix bottle of pre-hybridization soln (*Soln 4*) that has been equilibrated to 65°. Pour *Soln 4* into conical tube contg filters to 25 mL mark. Store remaining *Soln 4* at room temp. With back-and-forth motion, gently shake tube until all filters are completely immersed in soln and none are stuck to sides of tube. Filters should stack in vertical array at bottom of tube. Incubate tube for 30 min in 65° $H_2O$ bath.

*(3)* Carefully drain pre-hybridization soln from tube and discard soln. Use of funnel will prevent losing stack of filters into collection vessel. Immediately mix bottle of hybridization soln (*Soln 5*) that has been equilibrated to 65° and add 12 mL to conical tube (measure accurately with pipet). Return remaining *Soln 5* to room temp. storage.

*(4)* Working behind Lucite shield, remove plastic vial contg thawed $^{32}$P-labeled *Salmonella* probe soln from Lucite container. Mix probe soln by tapping on lower portion of plastic vial. Using precision micropipet, add probe soln to conical tube according to following schedule:

| Age of Probe, Days | Calendar Day | μL of Probe Soln |
|---|---|---|
| 0[1] | Mon. | — |
| 1 | Tue. | 127 |
| 2 | Wed. | 133 |
| 3 | Thur. | 140 |
| 4 | Fri. | 147 |
| 5 | Sat. | 154 |
| 6 | Sun. | 162 |
| 7 | Mon. | 170 |
| 8 | Tue. | 179 |

[1] Day 0 is indicated by date on vial. Day 0 is normal shipping day; Day 1 is normal day of receipt, but this may vary. User should always refer to age and calendar day for detn of probe soln vol. to use.

Dispose of pipet tip in container reserved for solid radioactive waste. Return remaining probe soln to 2–8° storage in its Lucite container.

*(5)* Tighten cap on conical tube. With back-and-forth motion, gently shake tube until all filters are completely immersed in soln and none are stuck to sides of tube. Again, filters should stack in vertical array at bottom of tube. Incubate tube for 2 h in 65° $H_2O$ bath.

*(6)* Working behind Lucite shield, carefully drain soln from conical tube into container reserved for liq. radioactive waste, using funnel to protect against losing stack of filters into waste vessel. Drain off as much soln as possible. (Radiochemical concn of waste is ca 1.0 μCi $^{32}$P/mL in molecular form of DNA.)

*(7)* Immediately add 25 mL wash soln (*Soln 6*) that has been equilibrated to 65° by pouring soln into conical tube to 25 mL mark. Return bottle of *Soln 6* to 65° $H_2O$ bath. Gently shake tube for at least 10 s, or until all filters are free from sides of tube and are stacked in vertical array at bottom of tube. Incubate conical tube for 5 min in 65° $H_2O$ bath.

*(8)* Remove tube from $H_2O$ bath and gently shake for at least 10 s, then carefully drain soln into liq. radioactive waste container. Drain off as much soln as possible.

*(9)* Repeat steps *7* and *8* five addnl times for total of 6 washes. Store *Soln 6* at room temp. after use.

*(10)* Using forceps, remove filters from conical tube and place them on sheet of absorbent paper. Discard conical tube into solid radioactive waste container. Using forceps, sep. filters and let dry briefly (5–10 min).

*(11)* Turn on power to beta detector, (**e**), and let instrument warm up ca 10 min. Counting time switch should be set to 0.5 min position.

To count each filter, center filter, using forceps, on lower window of beta detector. Detector windows are delicate; be careful not to damage them with forceps. Close detection unit and press start switch. Radioactivity on filter will be counted for 30 s, and result will be displayed as counts per minute (cpm). Record result for each filter in cpm on data sheet. Filters should be saved until data analysis is complete. Afterwards, they should be discarded as solid radioactive waste.

Alternatively, filters can be counted using std scintillation counter. Appropriate carrier vials and insert minivials are required so that filters can be positioned upright. Count each filter for 30 s using settings capable of counting $^{32}$P on dry filters (do *not* use scintillation cocktail) with efficiency of 0.4–0.5. Record data as cpm.

### *I. Data Analysis*

*(1)* Det. av. of 3 neg. control filters. If this av. is >500 cpm, *all* filters must be re-washed (repeat steps *7* and *8* under *DNA Hybridization Assay*) and re-counted. Accept data as final after addnl wash, even if av. of 3 neg. control filters is still >500 cpm.

*(2)* CPM of pos. control filter should be at least 5 times av. of neg. control filters; otherwise assay is invalid.

*(3)* Add 500 cpm to av. of the 3 neg. control filters. This sum is CUTOFF value.

*Neg. criterion:* Test sample is considered to be neg. (nonreactive for presence of *Salmonella*) if its cpm is ≤CUTOFF value.

*Pos. criterion:* Test sample is considered to be pos. (reactive for presence of *Salmonella*) if its cpm is >CUTOFF value.

### *J. Confirmation of Positive DNA Hybridization Assays*

Because a certain percentage of false pos. DNA hybridization assays can be expected (3–4%), all samples found pos. by DNA hybridization assay must be confirmed by culture methods. HE, XLD, and BS plates should be streaked from tetrathionate broth, selenite cystine broth, and GN broth tubes as described in **967.26B**, and typical and suspicious colonies should be identified as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **70**, 527(1987).

## 989.13 Motile *Salmonella* in Foods

**Immunodiffusion Screening Method**

**First Action 1989**

Method is screening procedure for presence of motile *Salmonella* in all foods. It is not a confirmatory test because polyvalent H (flagellar) antibodies used in test may cross-react with small percentage of non-*Salmonella*. *Note:* Method does not detect nonmotile salmonellae.

If test is pos., enrichment broth from inoculation chamber of test unit must be streaked onto selective/differential agar media as in **967.26B**, or, if test is performed on raw or highly contaminated product, enrichment broth must be transferred to tetrathionate broth and incubated 18–24 h in 43 ± 0.5° $H_2O$ bath, and then streaked onto selective/differential agar media. Typical or suspicious colonies must be identified as in **967.26C**, **967.27**, **967.28**.

### *A. Principle*

Detection of *Salmonella* is based on presence and observation of *Salmonella* immobilized in motility medium by polyvalent H (flagellar) antibodies. Immobilization of motile *Salmonella* results in development of well defined band of cells (immuno-band). Fig. **989.13** shows small disposable plastic device (1–2 TEST unit) which has 2 chambers. Smaller inoculation chamber contains selective tetrathionate broth supplemented with brilliant green and L-serine. Enriched sample is inoculated into this chamber. Central motility chamber of unit contains peptone-based, nonselective motility medium. Motility chamber is sealed with gel-former plug. Tip of this plug forms void in motility medium for addn of flagellar antibody prepn. For shipping, opening between 2 chambers is sealed with polyethylene chamber plug, which is removed and discarded prior to addn of inoculum. *Salmonella* inoculated into tetrathionate-brilliant green-serine broth move from this medium into motility medium to react with flagellar antibodies.

### *B. Method Performance*

For all foods:

| Results | Percent | 95% Confidence Range (Approx.) |
|---|---|---|
| Agreement[1] | 96.1 | 94.5–97.7 |
| False neg. (BAM/AOAC)[2] | 1.7 | 0.5–3.0 |
| False neg. (immunodiffusion)[3] | 3.6 | 1.8–5.5 |

[1] Rate reflects no. of samples read identically between BAM/AOAC (Bacteriological Analytical Manual (1984) 6th ed. AOAC, Arlington, VA) culture method and immunodiffusion method.
[2] Rate reflects no. of samples found to be pos. by immunodiffusion method but detd as neg. by BAM/AOAC culture method.
[3] Rate reflects no. of samples found to be pos. by BAM/AOAC culture method but detd as neg. by immunodiffusion method.

Of 17 laboratories, 8 had complete agreement between culture and immunodiffusion methods; 11 showed agreement on ≥97% of samples; 14 showed agreement on ≥95%; 16 showed agreement on ≥93%.

### *C. Reagents*

Items (**a**)–(**c**) are available as BioControl 1–2 TEST (BioControl Systems, Inc., 19805 North Creek Pkwy, Bothell, WA 98011). Store at refrigerator temp. (4–8°C; 39–46°F). Items are stable 3 months (90 days) from date of receipt.

(**a**) *1–2 TEST unit*.—See Fig. **989.13**.
(**b**) *Iodine-iodide soln*.—1 vial for 12 units.
(**c**) *Antibody*.—1 vial for 12 units.
(**d**) *Diagnostic reagents*.—Necessary for cultural confirmation of presumptive pos. 1–2 TESTS. See **967.25B**.

### *D. Preparation of Sample*

Most foods require only pre-enrichment of product in noninhibitory broth to initiate growth of salmonellae before inoculation into unit. Exceptions are given below. Methods used for sample prepn may vary with product and should be performed as in **967.26A** and **975.54D**.

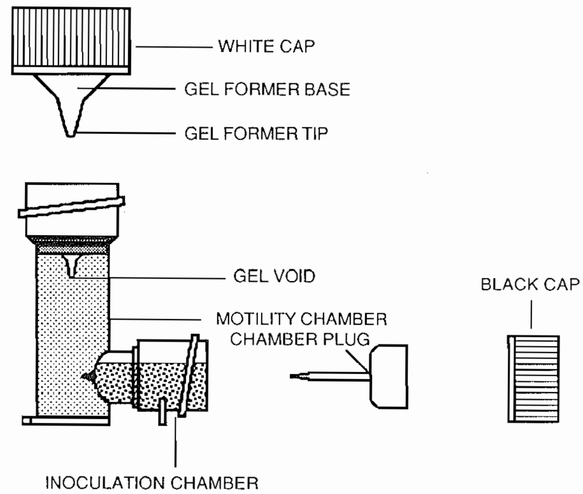



**FIG. 989.13—Test unit for immunodiffusion screening method for motile *Salmonella***

*Raw flesh foods or highly contaminated products*.—No pre-enrichment is required. Aseptically weigh 25 g sample into sterile blender jar. Add 225 mL tetrationate broth without brilliant green dye. Blend 2 min. Securely cap jars and let stand 60 min at room temp. Mix well by shaking. Add 2.25 mL 0.1% soln of brilliant green dye. Aseptically transfer contents of each jar to sterile 500 mL wide-mouth, screw-cap jar. Loosen jar caps $^1/_4$ turn and incubate 24 ± 2 h at 35°.

*Flour-containing products (soy flour, wheat flour, dough, pasta, cake mix, and processed animal by-products)*.—Transfer 1 mL incubated pre-enrichment mixt. to tetrathionate broth as in **967.26B(a)**. Incubate 24 ± 2 h at 35°.

### *E. General Instructions*

Components and procedures of test kit have been stdzd for use in 1–2 TEST procedures. Components or procedures other than those supplied by BioControl Systems, Inc., may yield unsatisfactory results, and should be pretested.

### *F. Immunodiffusion Detection*

(**a**) *Test unit preparation*.—Each test unit has 2 chambers: inoculation chamber and motility chamber (Fig. **989.13**). Each step of prepn sequence can be performed on individual unit or multiple units as needed. Sample nos. can be recorded on lower portion of motility chamber but must NOT interfere with reading of results. Alternatively, sample nos. may be recorded on flat surface of white cap. When cap is replaced, it must be screwed on tightly.

*(1)* Position unit with black cap UP, and remove black cap. Add 1 drop of iodine-iodide soln to inoculation chamber, and replace black cap. Gently shake unit to mix and resuspend enrichment ingredients.

*(2)* Position unit with white cap UP, and remove white cap. Snip off tip of gel-former plug with scissors and discard tip. Cut should be made at point where tip meets base of plug. If tip of gel-former plug is not removed, antibody soln will be displaced from gel void when white cap is replaced.

*(3)* Add 1 drop of antibody prepn to gel void in motility chamber. Replace white cap. Antibody prepn should fill ca $^2/_3$ of gel void. This can be detd by observing blue antibody soln in gel void.

*(4)* Position unit with black cap UP, and remove black cap. Remove chamber plug from inoculation chamber with sterile forceps and discard plug. Do not replace black cap until unit is inoculated. If chamber plug is not removed, bacteria will be unable to move from inoculation chamber to motility chamber.

(**b**) *Inoculation.*—Prior to inoculation, be sure that enrichment broth contg sample is well mixed. Use pipet to transfer 0.1 mL enriched sample into inoculation chamber. Replace black cap.

(**c**) *Incubation.*—Place inoculated unit in incubator with white cap UP. Incubate unit in shipper/incubation tray at 35° for min. of 8 h.

(**d**) *Reading positive results.*—After 8 h incubation, unit may be inspected for pos. results: With white cap UP, hold unit next to strong light. Desktop fluorescent light is recommended for reading test results. Carefully observe motility chamber gel by rotating unit back and forth thru various angles in front of light source.

Pos. test is indicated by presence of white band that is U-shaped or meniscus-shaped. Band, which forms as motile *Salmonella* are immobilized by antibodies that have diffused into gel, is seen in upper half of motility chamber gel.

Pos. test indicates that sample contains *Salmonella*. Pos. test results should be confirmed by std culture methods outlined in *Confirmation of Positive Samples*.

Pos. unit can be stored up to 1 week at refrigerator temp. (4–8°).

(**e**) *Reading negative results.*—If no band is seen after initial 8 h incubation, reincubate units for min. of 6 h but not more than 12 h. After this incubation period, read units as described in (**d**), *Reading positive results*. Units that show no band after this second incubation indicate neg. test results. Neg. units that were incubated at least 14 h require no addnl incubation. Neg. test results indicate that sample does not contain levels of motile *Salmonella* detectable by immunodiffusion test.

Neg. units show uniform turbidity thruout motility chamber as result of movement of motile bacteria in gel. However, after initial 8 h incubation, movement of bacteria thru gel may not be complete.

### *G. Confirmation of Positive Samples*

Presence of band of cells indicates that *Salmonella* may be present in sample. Perform cultural confirmation by using 3 mm loop to obtain inoculum from tetrathionate-brilliant green-serine broth in inoculation chamber and streaking HE, XLD, and BS plates.

For raw or highly contaminated products, transfer 0.1 mL of the tetrathionate-brilliant green-serine broth to test tube contg 10 mL tetrathionate-brilliant green broth. Incubate 18–24 h in 43 ± 0.5° $H_2O$ bath, and then streak into HE, XLD, and BS plates. Identify typical or suspicious colonies from selective plates as in **967.26C**, **967.27**, **967.28**.

Ref.: JAOAC **72,** 303(1989).

## VIBRIO

## 988.20 *Vibrio cholerae* in Oysters
### Elevated Temperature Enrichment Method
### First Action 1988

### *A. Principle*

Recovery of *V. cholerae* is based on selection of typical colonies on isolation agar. Although *V. cholerae* grows well at 35°, many other species of competing bacteria also proliferate in enrichment broth when incubated at 35°. Some species of competing microflora mimic colonial appearance of *V. cholerae* on isolation medium. Subsequent selection of these mimicking colonies decreases probability of recovering any *V. cholerae* colonies that may be present, and increases labor and materials needed for analysis. Ability of almost all strains of *V. cholerae* to grow at 42° distinguishes them from many other bacterial species associated with oysters and results in higher confirmation rate of suspects as *V. cholerae*.

### *B. Culture Media and Reagents*

(**a**) *AP broth.*—Suspend 10.0 g peptone and 10.0 g NaCl in 1 L $H_2O$ and mix thoroly. Adjust pH so that value after sterilization is 8.5 ± 0.2. Dispense portions into 500 mL flasks so that final vol. after autoclaving 10 min at 121° is 225 mL.

(**b**) *TCBS agar.*—Suspend 5.0 g yeast extract, 10.0 g proteose peptone No. 3, 10.0 g Na citrate, 10.0 g $Na_2S_2O_3$, 8.0 g oxgall, 20.0 g sucrose, 10.0 g NaCl, 1.0 g ferric citrate, 0.04 g bromthymol blue, 0.04 g thymol blue, and 15 g agar in 1 L $H_2O$ and mix thoroly. Heat with frequent agitation until medium just boils, 1–2 min. Cool in $H_2O$ bath and pour 20 mL portions into 15 × 100 mm petri dishes. Let dry ca 2 h with covers partially removed; then close plates. Final pH, 8.6 ± 0.2. Do not autoclave. Do not use wet plates.

(**c**) *$T_1N_1$ agar.*—Suspend 10.0 g tryptone or typticase, 10.0 g NaCl, and 20.0 g agar in 1 L $H_2O$ and mix thoroly. Heat with frequent agitation until medium boils. Dispense into 16 × 125 mm screw-cap tubes (if tubed medium is required). Autoclave 15 min at 121°. Slant tubes until cool or let medium cool to 50° and pour into 15 × 100 mm petri dishes. Let dry 2 h with plates covered. Do not use wet plates. Final pH, 7.2 ± 0.2.

(**d**) *Tryptone broth.*—Suspend 10.0 g tryptone or trypticase in 1 L $H_2O$ and mix thoroly. Dispense 5 mL portions into 16 × 125 mm or 16 × 150 mm test tubes. Autoclave 15 min at 121°. Final pH, 6.9 ± 0.2.

(**e**) *Kligler iron agar (KIA).*—Suspend 3.0 g beef extract, 3.0 g yeast extract, 15.0 g peptone 5.0 g proteose peptone, 10.0 g lactose, 1.0 g dextrose, 0.2 g $FeSO_4$, 5.0 g NaCl, 0.3 g $Na_2S_2O_3$, 12.0 g agar, and 0.024 g phenol red in 1 L $H_2O$, mix thoroly, and heat with occasional agitation. Boil ca 1 min until ingredients dissolve. Fill 13 × 100 mm screw-cap tubes $^1/_3$ full and cap to maintain aerobic conditions during use. Autoclave 15 min at 121°. Before medium solidifies, place tubes in slanted position so that deep butts (ca 3 cm) and adequate slants (ca 5 cm) are formed on solidification. Final pH, 7.4 ± 0.2.

(**f**) *Hugh-Leifson glucose broth (HLGB).*—Suspend 2.0 g peptone, 0.5 g yeast extract, 30.0 g NaCl, 10.0 g dextrose, 0.015 g bromcresol purple, and 3.0 g agar in 1 L $H_2O$, mix thoroly, and heat with agitation. Boil ca 1 min until ingredients are dissolved. Final pH, 7.4 ± 0.2. Fill 13 × 100 mm screw-cap test tubes $^1/_3$ full and cap. Autoclave 15 min at 121°. After inoculation cover with ca 1 mL sterile mineral oil to test for fermentation of dextrose.

(**g**) *Purple carbohydrate broth.*—Suspend 10.0 g proteose peptone No. 3, 1.0 g beef extract, 5.0 g NaCl, and 0.015 g bromcresol purple in 1 L $H_2O$ and heat with gentle agitation until dissolved. Dissolve 10.0 g inositol or 10.0 g mannitol in basal broth. Dispense 2.5 mL portions into 13 × 100 mm test tubes. Autoclave 10 min at 121°. Final pH, 6.8 ± 0.2.

(**h**) *Decarboxylase test media (Moeller).*—Suspend 5.0 g peptone, 5.0 g beef extract, 0.5 g dextrose, 0.01 g bromcresol purple, 0.005 g cresol red, and 0.005 g pyridoxal in 1 L $H_2O$ and heat with gentle agitation until dissolved. Dissolve 10.0 g L-lysine·2HCl, 10.0 g L-arginine·HCl, or 10.0 g L-ornithine·2HCl in basal broth. Use 1 portion of basal medium, without adding any amino acid, as control. Dispense 3–4 mL portions into 13 × 100 mm screw-cap tubes. Cap loosely and autoclave 10 min at 121°. Screw caps on tightly for storage. After inoculation cover with ca 1 mL sterile mineral oil. Final pH, 6.0 ± 0.2.

### C. Diagnostic Reagents

(**a**) *Oxidase test soln*.—Dissolve 1.0 g *N,N,N',N'*-tetramethyl-*p*-phenylenediamine·2HCl in 100 mL $H_2O$. Store ≤7 days in dark glass bottle in refrigerator. Do not autoclave.

(**b**) *String test soln*.—Dissolve 0.5 g Na desoxycholate in 100 mL $H_2O$. Store tightly capped in refrigerator. Do not autoclave.

(**c**) *V. cholerae polyvalent (O) antiserum*.—Contains agglutinins for Inaba and Ogawa (O) antigens (Difco, or equiv.). Rehydrate with 5.0 mL sterile physiological saline soln (**e**). Store refrigerated.

(**d**) *V. cholerae individual somatic (O) antisera*.—For Inaba and Ogawa (O) groups (Difco, or equiv.). Rehydrate and store as described in (**c**).

(**e**) *Sterile physiological saline soln*.—Dissolve 8.5 g NaCl in 1 L $H_2O$ and autoclave 15 min at 121°.

(**f**) *NaOH soln, 1N*.—Dissolve 42.11 g 95% reagent grade NaOH in sterile $H_2O$ and dil. to 1 L.

(**g**) *HCl soln, 1N*.—Dil. 89 mL HCl to 1 L with sterile $H_2O$.

(**h**) *Sterile mineral oil*.—Autoclave 500 mL mineral oil in 1 L flask for 30 min at 121°.

(**i**) *Bromcresol purple soln, 0.2%*.—Dissolve 0.2 g bromcresol purple in sterile $H_2O$ and dil. to 100 mL.

### D. Apparatus

(**a**) *Incubator*.—Air, 35 ± 2°.

(**b**) *$H_2O$ bath*.—Covered, 42 ± 0.2°.

(**c**) *High-speed blender*.—2 speed, with high-speed operation at 18 000–21 000 rpm, and 1 L glass or metal blender jars with covers. Use 1 jar for each test sample.

(**d**) *Sterile equipment*.—*(1)* Flasks or jars, 500 mL capacity. *(2)* Knives and spoons for opening and manipulating oysters. *(3)* Petri dishes, 15 × 100 mm. *(4)* Pipets, 1.0 and 10.0 mL with 0.1 mL graduations. *(5)* Inoculating needles and loops, ca 3 mm. *(6)* Culture tubes, 13 × 100 mm, 16 × 125 mm, and tube racks. *(7)* Wooden applicator sticks.

(**e**) *Balance*.—2000 ± 0.1 g capacity.

## *V. cholerae* Recovery

### E. Preparation of Test Sample

Aseptically remove oyster meats and liquor from ca 12 shell stock oysters or 12 shucked oysters from container. Aseptically weigh ca 200 g oyster meat and liquor into sterile empty blender jar. Blend at high speed 1 min. Aseptically weigh 25 g portions into 500 mL flasks contg 225 mL AP broth. Cover flask with sterile Al foil. Swirl mixt. 25 times clockwise and 25 times counterclockwise to suspend oyster homogenate. Incubate 6–8 h at 42 ± 0.2° in $H_2O$ bath.

### F. Isolation

Gently remove flasks from $H_2O$ bath. Streak 3 mm loopful of surface or pellicle growth from incubated AP broth on TCBS agar plate. Incubate plates 18–24 h at 35°. Typical *V. cholerae* colonies on TCBS agar appear large, smooth, yellow, and slightly flattened with opaque centers and translucent peripheries. Colonies of *V. mimicus*, which is closely related to *V. cholerae*, appear as smooth, green, slightly flattened colonies.

### G. Treatment of Typical or Suspicious Colonies

*Inoculation of $T_1N_1$ agar*.—Pick with needle 2–5 suspicious colonies from TCBS agar plate. Streak to $T_1N_1$ agar and incubate 18–24 h at 35°.

*Initial screening reactions*.—Scrape agar surface with sterile wooden applicator stick and touch to filter paper impregnated with oxidase reagent.

*Oxidase test*.—*V. cholerae* cultures are oxidase pos. and should produce dark purple spot within 1 min.

*String test*.—Emulsify oxidase pos. cultures in drop of 0.5% Na desoxycholate by stirring with same wooden applicator stick used previously. Within 1 min, *V cholerae* cultures form mucoid mass, which strings (string test) when stick is lifted 2–3 cm from slide. Treat oxidase and string test pos. cultures as presumptive *V. cholerae* and submit them to further examination.

*Inoculation of Kligler iron agar (KIA) and tryptone broth*.—Inoculate KIA slant with each suspect colony by streaking slant and stabbing butt with inoculating needle. After inoculating KIA with needle, do not obtain more inoculum from colony and do not heat needle, but directly inoculate tryptone broth. Incubate KIA and tryptone broth overnight at 35°. Cap tubes lightly to maintain aerobic conditions while incubating slants to prevent excessive $H_2S$ production.

*KIA*.—*V. cholerae* cultures typically have alk. (red) slant and acid (yellow) butt, without $H_2S$ (blackening of agar) or gas (cracking or lifting of agar). Do not eliminate KIA culture as *V. cholerae* solely on basis of acid slant.

*Tryptone broth*.—*V. cholerae* cultures typically produce growth in tryptone broth without added NaCl. Discard only apparent non-*V. cholerae* cultures. Test retained presumptive pos. KIA and tryptone cultures to det. if they are *V. cholerae*. Biochem. reactions characteristic of *V. cholerae* are summarized in Table **988.20**.

## *V. cholerae* Identification

### H. Identification Tests

Pure 18–24 h $T_1N_1$ agar cultures are required for inoculation of biochem. media. Select isolated colony and transfer with needle to each biochem. medium without obtaining more inoculum or heating needle.

*Dextrose fermentation*.—After inoculation, cover with ca 1 mL sterile mineral oil and incubate overnight at 35°. *V. cholerae* gives pos. test, shown by acid reaction (yellow). Discard all cultures that give neg. test.

*Acid production from mannitol and inositol*.—Incubate at 35° and read daily up to 4 days. Pos. tests are shown by acid production (yellow). *V. cholerae* gives pos. mannitol and neg. inositol test. Do not eliminate culture as *V. cholerae* solely on neg. mannitol test.

*Decarboxylase broth*.—After inoculation cover with ca 1 mL sterile mineral oil and incubate at 35°. Read daily up to 4 days. Pos. test is shown by purple alk. reaction thruout broth (final color is slightly darker than original purple of medium). Sometimes tubes that become yellow after 8–12 h incubation change to purple later. Neg. test is permanently yellow thruout broth and is seen with decarboxylase control tube without added

**Table 988.20 Biochemical Reactions of *V. cholerae***

| Test or substrate | Positive ( + ) | Negative ( − ) | *V. cholerae* reaction |
|---|---|---|---|
| $H_2S$ (KIA) | blackening | no blackening | − |
| Gas (KIA) | lifting or cracking | no lifting or cracking | − |
| Tryptone | visible growth | no visible growth | + |
| HLGB | yellow | purple | + |
| Mannitol | yellow | purple | + |
| Inositol | yellow | purple | − |
| Decarboxylase broth: | | | |
| Lysine | purple | yellow | + |
| Arginine | purple | yellow | − |
| Ornithine | purple | yellow | + |

amino acid. If medium appears to be discolored (neither purple nor yellow), add several drops of 0.2% bromcresol dye. *V. cholerae* gives pos. (purple) reaction in lysine and ornithine and neg. (yellow) reaction in arginine.

### *Serological Tests for V. cholerae*

Reconstitute antisera with 5.0 mL sterile 0.85% saline and refrigerate. Pretest all antisera with known test cultures to ensure reliability of results with unknown cultures. *Caution:* Handle viable cultures carefully to prevent contaminating environment. Use pure 18–24 h $T_1N_1$ cultures for all serological tests. Perform serological test only on cultures that give biochem. reactions typical of *V. cholerae*.

#### *I. Polyvalent Somatic O Group 1 Slide or Plate Test*

Use wax pencil to mark off 2 sections ca 1 × 2 cm on inside of glass or plastic petri dish. Place 1 drop of 0.85% saline soln to one section and 1 drop of *V. cholerae* polyvalent somatic (O) antisera to other section. With sterile wooden applicator stick or inoculating loop or needle, emulsify culture in saline soln for one section and repeat for other section contg antiserum. Tilt mixt. in both sections back and forth 1 min and observe against dark background. Any degree of agglutination is pos. reaction.

Classify polyvalent somatic (O) group 1 test as:

*Positive.*—Agglutination in culture-saline-serum mixt.

*Negative.*—No agglutination in culture-saline-serum mixt.

*Nonspecific.*—Both mixts agglutinate.

#### *J. Determination of Individual Somatic O Group 1 Serotypes*

Test only somatic O group 1 pos. cultures in individual O group 1 antisera. Perform serological somatic O group 1 test on culture as above, by using Inaba and Ogawa antiserum instead of *V. cholerae* polyvalent somatic O group 1 antiserum.

Classify individual somatic O group 1 test as:

*Inaba positive.*—Agglutination in culture-saline-Inaba antiserum mixt. and no agglutination in culture-saline or in culture-saline-Ogawa antiserum mixt.

*Ogawa positive.*—Agglutination in culture-saline-Ogawa antiserum mixt. and no agglutination in culture-saline or in culture-saline-Inaba antiserum mixt.

*Hikojima positive.*—Agglutination in both culture-saline-Inaba antiserum mixt. and culture-saline-Ogawa antiserum mixt. but no agglutination in culture-saline mixt.

*Negative.*—No agglutination in culture-saline-Inaba antiserum mixt., culture-saline-Ogawa antiserum mixt., or in culture-saline mixt. This pattern indicates faulty individual somatic O group 1 antisera or presence of non-O group 1 antisera in polyvalent somatic O group 1 antiserum.

*Nonspecific.*—All mixts agglutinate.

Ref.: JAOAC **71,** 584(1988).

## MISCELLANEOUS

## 975.56 Virus in Beef (Ground)

### Microbiological Method

**First Action 1975**
**Final Action 1989**

#### *A. Media and Reagents*

(**a**) *Diethylaminoethyl (DEAE) dextran sulfate soln.*—Add 1 g DEAE dextran sulfate, 2 × $10^6$ MW (Pharmacia Fine Chemicals, Inc., 800 Centennial Ave, Piscataway, NJ 08854), to $H_2O$, dil. to 100 mL, mix on mag. stirrer, and filter thru 0.22 μm filter.

(**b**) *Magnesium chloride soln.*—Add 50.75 g $MgCl_2.6H_2O$ to $H_2O$, dil. to 100 mL, mix on mag. stirrer, and filter thru 0.22 μm filter.

(**c**) *Neutral red soln.*—Add 1 g neutral red to 1 L $H_2O$, mix overnight on mag. stirrer, autoclave 15 min at 121°, and dispense into 100 mL bottles for storage at 10°.

(**d**) *Sodium bicarbonate soln.*—pH 8.0. Add 75 g $NaHCO_3$ to $H_2O$, dil. to 1 L, and filter thru 0.22 μm filter.

(**e**) *Tissue culture.*—Propagate Vero monkey kidney cell cultures (ATCC CCL 81) in 6 oz (45 sq cm) prescription bottles contg growth medium, (**f**). After cell sheets are confluent, ca 7 days, decant medium, add 10 mL 0.02% $Na_4EDTA$ in phosphate buffered saline soln, **975.54C(a)**, and shake. When cells resuspend, ca 20 min, pour suspension into centrf. tube, centrf. 15 min at 700 rpm in International PR-2, rotor 259, and decant supernate. Add 146 mL growth medium to cell pellet, mix, and distribute into 8 prescription bottles. Repeat propagation until enough cultures are prepd to perform analysis.

(**f**) *Growth medium.*—To Leibovitz medium (L-15) (Grand Island Biological Co., 3175 Staley Rd, Grand Island, NY 14072) add equal vol. Eagle's minimum essential medium (MEM) with Hank's salt (Grand Island Biological Co.). Add 10% fetal bovine serum (flow Laboratories). To final mixt. add 10 mL of 7.5% $NaHCO_3$ soln, (**d**). Medium will maintain cells 15 days without having to be changed.

(**g**) *Virus.*—Poliovirus 1, Chat, attenuated (ATCC VR-192). Passage in Vero cell cultures. Prep. virus pool and titer. Dil. pool to provide 10–50 plaque forming units (pfu)/g.

(**h**) *High antibiotic minimum essential medium (HAMEM).*—Prep. MEM with nonessential amino acids in Hank's salt soln contg in each L: 20 mL fetal bovine serum, 5.0 mL $MgCl_2$ soln, (**b**), 10 mL DEAE dextran sulfate soln, (**a**), 4.643 g K penicillin G, 5.0 g streptomycin sulfate, 0.25 g tetracycline.HCl, and 5.0 mg amphotericin B. Adjust to pH 8.5 with 1*N* NaOH for elution of virus and to prevent coagulation of sample slurry.

(**i**) *Agar medium.*—Mix 400 mL "2×" MEM (filtered thru 0.22 μm filter), 20 mL fetal bovine serum, 30 mL $NaHCO_3$ soln, (**d**), 15 mL neutral red soln, (**c**), 10 mL DEAE dextran sulfate soln, (**a**), 10 mL $MgCl_2$ soln, (**b**), 2 mL amphotericin B soln (10 μg/mL), 2 mL tetracycline.HCl soln (50 μg/mL), 5 mL K penicillin G soln (1435 units/mL), and 6 mL streptomycin sulfate soln (1 mg/mL).

(**j**) *Agar overlay medium.*—Add 9.5 g Oxoid Ion agar No. 2 or 12 g Difco purified agar to $H_2O$, dil. to 490 mL, mix on mag. stirrer, autoclave 15 min at 121°, and temper in 47° $H_2O$ bath. Add tempered agar to 500 mL agar medium, (**i**), and temper in 43° $H_2O$ bath. Add 10 mL canned sterile milk (Realfresh, Inc., PO Box 1551, Visalia, CA 93277) just before use.

#### *B. Preparation of Sample*

Place 100 g sample in plastic Whirl-Pac bag (Fisher Scientific Co.) and add 200 mL HAMEM, (**h**). Shake vigorously by hand, adjust pH of slurry to 8.5, and let stand 1 hr at room temp, shaking vigorously 1 min every 20 min. Readjust pH to 8.5 and pour thru funnel contg 5 g Pyrex glass wool pretreated with HAMEM. Let filter 1 hr (ca 180 mL filtrate is obtained) and compress glass wool and slurry with wooden tongue depressor to express remaining liq.

#### *C. Assay*

Inoculate 1 mL filtrate into each of 10 bottles of Vero cell monolayers, (**e**), rotating bottles to obtain even distribution of inoculum. Incubate 1 hr at 36°. Return bottles to room temp.

Dispense 18 mL agar overlay medium, (**j**), into each bottle against inside surface away from cell sheet. Cap bottles and turn so overlay gently floods cell surface. Let solidify at room temp. 30 min with bottles covered to exclude light. Turn bottles so that overlay side is up, and incubate in dark at 36°. Remove bottles daily from incubator, and count and mark plaques until no new plaques appear in 48 hr. Discard after 14 days.

Plaque forming units (pfu)/100 g sample = (Av. plaque count/bottle) × (total vol. filtrate/mL filtrate inoculated per bottle).

Ref.: JAOAC **58,** 576(1975).

## 985.43 Poliovirus 1 in Oysters

**Microbiological Method**
**First Action 1985**
**Final Action 1989**

### *A. Apparatus*

(**a**) *Tissue culture flasks.*—Polystyrene, 150 sq. cm (Corning Glass Works, or equiv.).

(**b**) *Specimen containers.*—220 mL, polyethylene, disposable (Becton-Dickinson Labware, 2 Ridgewater Ln, Lincoln Park, NJ 07035, or equiv.).

(**c**) *Blender.*—Waring, or equiv.

(**d**) *Funnel.*—PF 100 polypropylene (No. 4252-0100, Nalge Co, 75 Panorama Creek Dr, PO Box 20365, Rochester, NY 14602, or equiv.).

(**e**) *Centrifuge bottle.*—250 mL, linear, polyethylene (Nalge No. 3121-0250, or equiv.).

(**f**) *Refrigerated centrifuge.*—Sorvall RC 5B (Ivan Sorvall, Inc., Norwalk, CT 06852, or equiv.).

(**g**) *Shaker.*—Wrist action (Burrell Corp., or equiv.).

### *B. Media*

(Use double-distd $H_2O$ for prepn of media and reagents.)

(**a**) *MEMH.*—Eagle's min. essential medium with Hanks' balanced salts (Gibco Laboratories, Life Technologies, Inc., 3175 Staley Rd, PO Box 68, Grand Island, NY 14072-0122, or equiv.).

(**b**) *Plaque assay agar.*—Add 12 g purified agar (Difco, or equiv.) to 500 mL $H_2O$ autoclave 15 min at 121°, and temper fluid 30 min at 47°.

(**c**) *Plaque assay medium.*—Add 10 mL 50% $MgCl_2.6H_2O$, 10 mL 1% DEAE, 15 mL 7.5% $NaHCO_3$, 15 mL 0.1% neut. red, 30 mL 10% (w/v) nonfat dry milk autoclaved 10 min, and 1 mL (50 mg) gentamicin sulfate to 420 mL double strength MEMH. (All concns w/v with $H_2O$). Bring to 36° in $H_2O$ bath and place in 36° incubator until use.

### *C. Reagents*

(**a**) *Freon TF.*—(DuPont, Inc., or equiv.).

(**b**) *Antifoam.*—Antifoam C (Dow Corning, or equiv.)

(**c**) *Nonfat dry milk.*—Quality equiv. to Carnation Co., Los Angeles, CA, product.

(**d**) *Cat Floc.*—10% (w/v) (Calgon Corp., PO Box 1346, Ellwood, PA 15230, or equiv.).

(**e**) *DEAE.*—1% (w/v) diethylaminoethyl dextran, MW = $5 \times 10^5$ (Pharmacia Fine Chemicals, Inc., 800 Centennial Ave, Piscataway, NJ 08854 or equiv.).

### *D. Preparation of Cells*

*Buffalo African Green Monkey cell line (BGM).*—(BGM cell line is available from several commercial sources.). Incubate cells at 36° in planting medium composed of equal vols of Leibovitz L-15 medium and MEMH supplemented with 10% fetal bovine serum plus 100 U penicillin G, 100 μg streptomycin sulfate, and 50 μg gentamicin/mL. Treat confluent cultures with 0.02% tetrasodium ethylenediamine tetraacetate in phosphate-buffered saline, split 1 to 10, and passage weekly in tissue culture flasks.

### *E. Preparation of Virus*

*Poliovirus 1.*—Chat strain (ATTCC VR-192). Propagate in monolayers of BGM cells and harvest after observing 4 + cytopathic effect. Filter culture fluid contg virus thru 0.22 μm porosity membrane. Shake filtrate vigorously with equal vol. Freon TF, and centrf. 30 min at 4000 × *g* at 10°. Refilter supernate.

### *F. Sample Preparation*

For each 100 g sample, select ca 10 std size oysters, shuck, and pour liquor and meat into specimen container. Adjust liquor and meat in container to 100 g.

Pour 100 g oyster sample into 1 L blender, and pipet 1 mL antifoam onto oysters. Rinse specimen container with 150 mL 37° $H_2O$, and pour rinse into blender. Pipet 2.5 mL 1N HCl into mixt. and blend contents 20 s at 18 500 rpm. Adjust homogenate, using 1 s blending mixes, to pH 4.8 ± 0.1 by adding 1 N HCl or 1 N NaOH as needed. Pour homogenate thru polypropylene funnel into 250 mL linear polyethylene centrif. bottle. Cap centrif. bottles tightly to prevent leakage during various assay procedures. Centrf. 10 min at 5000 × *g* without refrigeration in GSA rotor, using refrigerated centrif. Pour off supernate and discard.

### *G. Elution of Virus from Oyster Tissue*

Add following cold reagents (5°) to pellet (ca 40 g): 50 mL MEMH without phenol red or $NaHCO_3$, 10 mL 10% w/v nonfat dry milk, 2 mL 50% $MgCl_2.6H_2O$, 50 mL Freon TF, and 3 mL 1N NaOH. Vigorously shake mixt. horizontally 5 min on wrist-action shaker, adjust pH to 9.1 ± 0.1, and centrif. mixt. 20 min at 5000 × *g* at 10°.

### *H. Concentration of Virus by Precipitation*

Pipet supernate (ca 80 mL) into 250 mL centrf. bottle, taking care not to harvest Freon TF, which settles below supernate. Discard sediment and Freon TF. Add enough 37° $H_2O$ to supernate to bring liq. level to shoulder of top of bottle. Form floc (15 min) by adjusting pH of liq. to 4.8 ± 0.1 with 1N HCl (ca 1 mL). Centrif. sample 10 min at 1500 × *g* (3000 rpm) without refrigeration. Pour off and discard supernate.

### *I. Elution of Viruses from Precipitate*

Add 4 mL 0.2M $Na_2HPO_4$ (ca 23°) and 1 mL freshly prepd 10% Cat floc to pellet (10 g) and suspend pellet by vortex-mixing 30 s. Centrf. suspension (pH 7.5 ± 0.2) 20 min at 10 000 × *g* (8000 rpm) at 10°.

### *J. Plaque Assay of Eluate*

Pipet supernate (ca 10 mL) onto two 150 sq. cm BGM cell monolayers previously rinsed with 50 mL MEMH adjusted with 7.5% $NaHCO_3$ to pH 7.0. Discard pellet. Incubate inoculated cell monolayers 2 h at 36°. Tilt culture flasks at least twice during incubation to redistribute eluate over cell monolayers; then overlay monolayers as described below.

### *K. Overlay Procedure*

Pour tempered plaque agar into 36° medium, and mix by inverting flask few times. Pour mixt. (60 mL) into cell monolayer flask onto side opposite cell sheet, and rotate flask so that agar flows over monolayer once and then covers cells. Let overlay agar solidify at room temp. (ca 23°). Cover flasks with cloth to exclude light during solidification (ca 15 min). Incubate 7 days at 36°, agar side up, in dark incubator. Mark plaques and count as they appear.

Ref.: JAOAC **68,** 884(1985).

## SOMATIC CELLS

### 973.68★ Somatic Cells in Milk

**Optical Somatic Cell Counting (OSCC) Method I**

**First Action 1973**
**Final Action 1980**
**Surplus 1989**

*See* **46.152–46.160**, 14th ed.

### 978.25★ Somatic Cells in Milk

**Optical Somatic Cell Counting (OSCC) Method II**

**First Action 1978**
**Final Action 1980**
**Surplus 1989**

*See* **46.161–46.170**, 14th ed.

### 978.26 Somatic Cells in Milk

**Optical Somatic Cell Counting (OSCC) Method III**

**First Action 1978**
**Final Action 1979**

#### *A. Principle*

Fresh or preserved milk samples are automatically sampled at 40°, mixed with buffer and dye, and stirred. Portion of mixt. is transferred to rotating disk which serves as object plane for microscope. Xe arc lamp excites somatic cell nuclei-dye complex to emit fluorescent light, and energy emitted by each nucleus is measured as elec. pulse.

#### *B. Apparatus*

*Optical somatic cell counter.*—Fossomatic (manufactured by Foss Food Technology Corp.), consisting of heating coils, rotating table, stirrer, syringes for delivering buffer and dye, rotating disk, microscope equipped to detect fluorescence, and totalizing circuit and printer (*see* Fig. **978.26**).

#### *C. Reagents*

(**a**) *Ethidium bromide dye soln.*—(*1*) *Stock soln.*—0.1%. Dissolve 1.00 g ethidium bromide (Aldrich Chemical Co., Inc., or equiv.) in 1 L $H_2O$ by heating to 40–50° and mixing thoroly. Stock soln is stable 60 days in light-proof, air-tight bottle. (*2*) *Working soln.*—0.002%. Dil. 20 mL dye stock soln to 1 L with KH phthalate buffer soln, (**c**), and mix thoroly.

(**b**) *Rinsing liquid.*—(*1*) *Stock soln.*—1% Triton X-100 (Rohm & Haas Co., or equiv.). Dissolve 10 mL Triton X-100 in 1 L $H_2O$. Stock soln is stable 25 days in air-tight container. (*2*) *Working soln.*—Add 10 mL stock soln to 25 mL $NH_4OH$ (1 + 3), dil. to 10 L with $H_2O$, and mix thoroly.

(**c**) *Potassium hydrogen phthalate buffer soln.*—0.025*M*. Dissolve 51.0 g KH phthalate and 13.75 g KOH in 10 L $H_2O$ by heating to 50° and mixing thoroly. Add 10 mL 1% Triton X-100, (**b**)(*1*), and again mix thoroly. Store ≤7 days in air-tight container.

#### *D. Analytical System*

Two fl oz (60 mL) milk sample is heated to 40°, placed on self-feeding rack, and stirred to ensure even cell distribution just before 200 μL milk is withdrawn. Sample is combined with 1.800 mL 60° buffer soln and 2.000 mL 60° dye soln. Final mixt. is stirred continuously until 20 μL is spread 10 μm deep on edge of rotating disk, 0.5 mm wide along effective length of 3500 mm. Field is viewed with 15× microscope objective. Cell-dye complex is excited by filtered blue light (400–570 nm) from Xe lamp to emit red fluorescence, and filtered

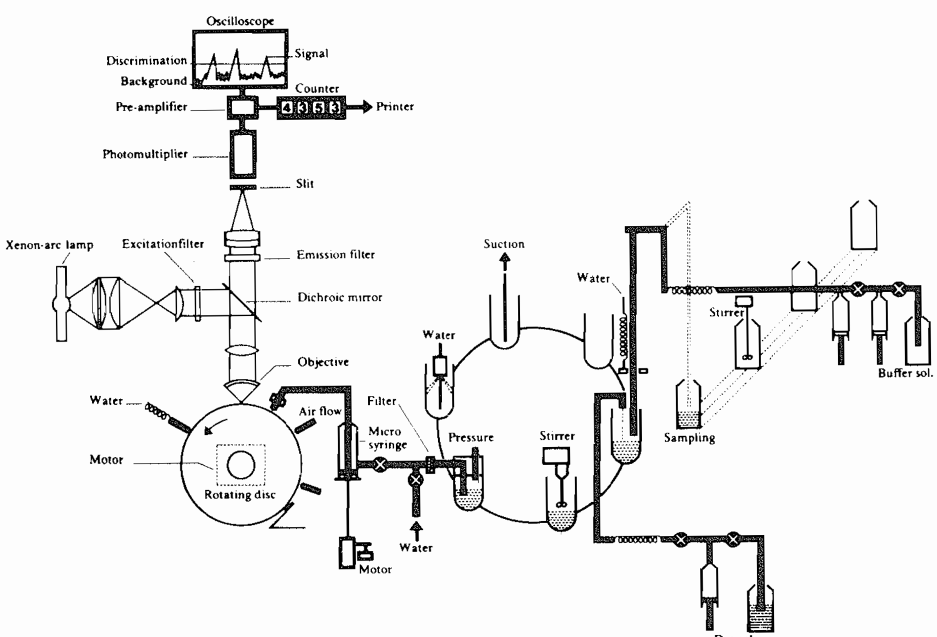



**FIG. 978.26—Optical somatic cell counter flow diagram (Fossomatic) (Method III)**

fluorescence (590–700 nm) over background (removed by discriminator) is sensed by photomultiplier. Each pulse is transformed and reading of total cells/20 μL is digitized on display as well as on printer. Rinsing liq. is used to flush system between milk samples to ensure no carryover effect of sample.

Somatic cells/mL milk = No. pulses × 1000

### *E. Standardization*

Perform direct somatic cell counts (DMSCC) on 3 std milk samples within range 300,000–2,000,000 cells/mL as in **973.68** or **978.25**. (Before analysis, obtain 3 subsamples of each std to avoid excessive reheating.) To arrive at optimum discriminator setting, compare stds over ≥5 discriminator settings having increments of 0.25–0.5 between settings. Choose initial setting near previous operating point and additional settings to provide ≥1 set of readings above and ≥1 below apparent optimum. Optimum is setting at which deviations of Fossomatic readings from those of stds are minimal, with 1 of opposite sign from rest. Check instrument every 700–800 samples or after each 4 hr of operation against std milk samples preserved with 0.05% $K_2Cr_2O_7$.

Ref.: JAOAC **61**, 779(1978).

## 980.33★ Somatic Cells in Milk

**Membrane Filter-Deoxyribonucleic Acid (MF-DNA) Method**

**First Action 1980**
**Final Action 1981**
**Surplus 1989**

*See* **46.176–46.180**, 14th ed.

## SPECIAL REFERENCE

FDA Bacteriological Analytical Manual (BAM) 6th edition (1984) AOAC, Suite 400, 2200 Wilson Blvd, Arlington, VA 22201-3301.

# 18. Drugs: Part I

**Martin Finkelson, *Associate Chapter Editor***
*Food and Drug Administration*

## 960.53 Drugs

**General Directions**

***A. Extraction with Lighter-Than-Water Solvents***

Perform preliminary steps directed in method prior to extn. Ext. aq. soln in separator with specified vols of solv. (ether, pet ether, etc.) by shaking ≥1 min. Let sep. completely, swirl to remove $H_2O$ droplets, transfer lower aq. layer to second separator, and decant solv. layer thru pledget of solv.-washed cotton or glass wool in short-stem funnel inserted in neck of third separator. Wash mouth of separator with fine stream of solv. Repeatedly shake aq. soln with addnl portions of solvs until substance sought is extd, using second and first separators alternately for shaking, collecting solvs by filtering into third. If aq. soln is to be further examined, dry cotton pledget in funnel by drawing air thru stem, and wash with 5 mL $H_2O$ into main aq. ext.

***B. Purified Diatomaceous (Siliceous) Earth for Partition Chromatography***

Celite 545, acid-washed (Manville Filtration and Minerals), or equiv., is usually suitable for column chromatgy. When interfering materials are present, purify as follows: Place pad of glass wool in base of chromatgc tube ≥100 mm diam. and add siliceous earth to ht ca 5 times diam. Add vol. HCl equal to ca $^1/_3$ vol. of earth, and let percolate. Wash with MeOH, using small vols at first to rinse walls of tube, and then until washings are neut. to moistened indicator paper. Extrude into shallow dishes, heat on steam bath to remove MeOH, and dry at 105° until material is powdery and MeOH-free. Store in tightly closed containers.

## 927.09 Sampling of Drugs

**Final Action**

Methods require analysis of sample portions stated. Use individual dosage form or fraction of composite, depending on information needed. Sampling procedure used may affect interpretation of results. Typical procedures for obtaining random samples are described below.

***A. Tablets and Pills***

(**a**) *Bulk lots.*—Remove approx. equal numbers of units from ≥10 locations spaced thruout mixed lot, collecting ≥100 units. Calc. av. wt/unit, and reduce to fine powder that would completely pass 60 mesh seive. Mix thoroly.

(**b**) *Containers.*—Remove equal numbers of units from one or more containers representing same lot to achieve ≥60 units. Calc. av. wt/unit, and reduce to fine powder that would completely pass 60 mesh seive. Mix thoroly.

***B. Capsules***

Follow sampling plan in **927.09A**(**a**) or (**b**). Weigh counted capsules and det. gross wt/capsule. Open capsules and quant. remove contents. If dry contents, reduce to fine powder that would completely pass 60 mesh seive. Mix thoroly. As necessary, clean capsules (cutting in 2 if necessary) and wash by agitating with alternate portions of alcohol and ether, with final rinse of ether. (Few drops of HOAc mixed with alcohol aids cleaning.) Remove ether, deduct wt cleaned, empty capsules from gross wt, and calc av. net contents.

***C. Ampuls***

Before opening ampuls, dislodge any liq. in neck. Open sufficient number of ampuls to provide required sample vol., and transfer contents of each with dry hypodermic syringe to dry stdzd cylinder graduated to contain. Alternatively, contents of syringe may be discharged into dry, tared weighing vessel, and vol. calcd as wt, in g, of fluid delivered divided by density of injection soln.

***D. Vials***

(**a**) *Solns.*—With successive use of clean, dry hypodermic syringes of appropriate size, remove indicated number of doses from vial, delivering each into same stdzd cylinder graduated to contain. Alternatively, use gravimetric procedure described in **927.09C** (ampuls).

(**b**) *Suspensions.*—Follow procedure under (**a**), except shake vial vigorously until product is homogeneous (but ≥15 s), then remove sample immediately.

***E. Ointments***

Remove entire contents of sufficient number of weighed tubes to tared, covered weighing vessel contg stirring rod. Mix pooled contents to obtain composite, cover vessel, and det. wt of composite (C). Weigh empty tubes and calc. total wt of ointment removed (O). Use ratio O/C to correct wt of anal. samples subsequently taken from composite.

Refs.: Banes, "Principles of Regulatory Drug Analysis," AOAC, 2200 Wilson Blvd, Arlington, VA 22201 (1966).
Banes, "A Chemist's Guide to Regulatory Drug Analysis," AOAC, 2200 Wilson Blvd, Arlington, VA 22201 (1974).
JAOAC **10,** 99(1927); **56,** 508(1973); **67,** 357(1984).

## SOLVENTS

## 942.28★ Acetone and Alcohols in Drugs

**Qualitative Tests**

**Final Action**
**Surplus 1972**

*See* **36.006–36.010**, 12th ed.

## 973.69 Acetone and Alcohols in Drugs

**Gas Chromatographic Method**

**First Action 1973**
**Final Action 1975**

(Applicable to liq. prepns contg ethanol with isopropanol or acetone or individual compds)

### A. Reagents and Apparatus

(**a**) *Ethanol std stock soln.*—(*1*) *2% (v/v).*—Dil. 5.0 mL absolute alcohol to 250 mL with $H_2O$. (*2*) *0.2% (v/v).*—Dil. 10.0 mL soln (*1*) to 100 mL with $H_2O$.

(**b**) *Isopropanol std stock soln.*—2% (v/v). Dil. 5.0 mL isopropanol to 250 mL with $H_2O$.

(**c**) *Acetone std stock soln.*—2% (v/v). Dil. 5.0 mL acetone to 250 mL with $H_2O$.

(**d**) *Acetonitrile internal std stock soln.*—2% (v/v). Dil. 5.0 mL $CH_3CN$ to 250 mL with $H_2O$.

(**e**) *Gas chromatograph.*—With 1.8 m (6′) × 4 mm id glass column, packed with 80–100 mesh Porapak Q (Waters Associates, Inc.) and H flame ionization detector. Approx. operating conditions: temps (°)— column 135, detector 155, injection port 165; N carrier gas flow rate 120 mL/min. $CH_3CN$ peak should elute in 5 min. Adjust H and air flow rates and electrometer sensitivity so that 5 μL 0.2% EtOH std soln gives 50–70% scale deflection.

### B. Preparation of GC Column

Carefully plug column exit with small pad of glass wool. Apply vac. to exit and slowly add packing material thru inlet, tapping very gently to pack firmly. Pack to within 1 cm of area heated by injection port. Plug with glass wool and condition overnight at 235° with slow N stream.

### C. Preparation of Sample

(**a**) *Ethanol.*—Prep. soln contg ca 2% (v/v) EtOH by stepwise diln with $H_2O$. Proceed as in **973.69D**.

(**b**) *Isopropanol.*—Prep. soln contg ca 2% (v/v) isopropanol by stepwise diln with $H_2O$. Proceed as in **973.69D**.

(**c**) *Acetone.*—Prep. soln contg ca 2% (v/v) acetone by stepwise diln with $H_2O$. Proceed as in **973.69D**.

If acetone concn is unknown, prep. 50% diln of product with $H_2O$, prep. acetone std soln, and inject sample and std as in **973.69D**. To det. amt acetone, adjust product and std dilns to give comparable peak hts; % internal std added to the 2 solns should be equal to % acetone present in std soln.

### D. Determination

Pipet 10 mL sample soln into 100 mL vol. flask. Pipet 10 mL each std stock soln needed into sep. 100 mL vol. flask. Pipet 10 mL internal std stock soln into each flask and dil. to vol. with $H_2O$.

Inject 5 μL sample and std solns, each in duplicate, using 10 μL syringe. Approx. retention times of peaks relative to $CH_3CN$ internal std peak are as follows: EtOH, 0.76; acetone, 1.32; isopropanol, 1.40.

Calc. % EtOH, acetone, or isopropanol in sample as

$$\% \, C = C' \times (H/H') \times (I'/I) \times f$$

where $C$ and $C'$ = % component in sample and std, resp., $H$ = av. sample peak ht or area in sample chromatogram, $H'$ = av. std peak ht or area in std chromatogram, $I$ and $I'$ = resp. values for internal std, and $f$ = sample diln factor.

Ref.: JAOAC **56**, 684(1973).

CAS-67-64-1 (acetone)
CAS-64-17-5 (ethyl alcohol)
CAS-67-63-0 (isopropyl alcohol)

## 928.10★ Ether in Drugs

**Dichromate Oxidation Method**

**Final Action**
**Surplus 1965**

(Not applicable in presence of essential oils)

*See* **32.370–32.374**, 10th ed.

## 953.18★ Propylene Glycol in Drugs

**Periodate Oxidation Method**

**Final Action**
**Surplus 1975**

*See* **36.016–36.017**, 12th ed.

## 929.10★ Chloroform or Carbon Tetrachloride in Drugs

**Volhard Titrimetric Method**

**Final Action**
**Surplus 1970**

*See* **36.355–36.357**, 11th ed.

## 934.08★ Tetrachloroethylene in Drugs

**Total Chloride Method**

**Final Action**
**Surplus 1970**

*See* **36.434–36.435**, 11th ed.

## 939.15★ Trichloroethylene in Drugs

**Total Chloride Method**

**Final Action 1965**
**Surplus 1970**

*See* **36.437–36.439**, 11th ed.

## 963.30★ Chlorinated Hydrocarbons in Drugs

**Infrared Spectrophotometric Method**

**First Action 1963**
**Surplus 1982**

*See* **36.018–36.022**, 14th ed.

# HALOGENATED DRUGS

## 936.17★ Chlorobutanol in Drugs

**Gravimetric Method**

**Final Action**
**Surplus 1972**

*See* **36.023–36.024**, 12th ed.

## 967.29 Chlorobutanol in Drugs

**Gas Chromatographic and Infrared Methods**

**First Action 1967**
**Final Action 1968**

(*Caution:* See safety notes on flammable solvents, toxic solvents, carbon disulfide, and carbon tetrachloride.)

### A. Reagents

(**a**) *Diatomaceous earth.*—*See* **960.53B**.

(**b**) *Chlorobutanol.*—USP. Store over soln satd with both sugar and salt; product contains 1/2 mole $H_2O$ of hydration.

(**c**) *Glass wool.*—Fine; washed with $CS_2$ and dried.

(**d**) *Dichlorodimethylsilane*.—Dissolve 5 mL in 100 mL toluene. (*Caution:* Dichlorodimethylsilane is toxic. Avoid contact with skin or eyes. Use effective fume removal device.)

### B. Apparatus

(**a**) *Chromatographic tube*.—23 × 400 mm with drip tip small enough to fit into 10 mL vol. flask, and with close-fitting tamping rod.

(**b**) *Infrared spectrophotometer*.—With matched 1 mm path length, liq.-filled NaCl cells.

(**c**) *Gas chromatograph*.—With 1.8 m (6') × 4 mm glass column, packed with Carbowax 6000 on 100–110 mesh Anakrom ABS, H flame ionization detector, and strip chart recorder.

### C. Preparation of GC Column

Carefully wash inside of column and small amt of glass wool with dichlorodimethylsilane soln, rinse with MeOH, and dry. Slowly sprinkle ca 25 g Anakrom ABS into 400 mL beaker almost filled with $CCl_4$. Remove fine particles remaining at surface with vac. line and trap. Decant solv., oven-dry support, and transfer 20.0 g to 500 mL filter flask fitted with trap and stopper. Dissolve 5.0 g polyethylene glycol (Carbowax 6000) in 100 mL toluene, warming if necessary. Add Carbowax soln to flask and apply vac. 5 min, swirling occasionally. Return to atm. pressure and let stand 5 min. Transfer slurry with rapid swirling to buchner fitted with coarse paper. Maintain reduced pressure on funnel 5 min; then dry coat support by spreading on smooth surface. Air dry 1 hr. Oven-dry addnl hr at 90°.

Carefully plug column exit with small pad fine glass wool and thru-hole septum. Apply vac. to exit port and slowly add coated support thru injection port, tapping very gently to pack firmly. Pack to within 1 cm of area heated by flash heater. Plug with fine glass wool and condition ca 3 days at 200° with slow N stream.

### D. Preparation of Standard Solutions

Dissolve ca 0.5 g chlorobutanol.$^1/_2H_2O$, accurately weighed, in 1 mL alcohol and transfer to 100 mL vol. flask with 8 mL alcohol. Dil. to vol. with $H_2O$. Using 5 mL aliquots, prep. duplicate diat. earth columns with trap layers as in **967.29E**. Prep. and elute columns individually. Calc. mg anhyd. chlorobutanol/mL $CS_2$ = $C_{hyd} \times 0.9517$. (Chlorobutanol is appreciably volatile at room temp.; expose to atm. as little as possible.)

### E. GC Determination

Weigh 3 g diat. earth, add 2 mL 1*N* HCl, and mix until uniform. Transfer to chromatgc tube plugged with small pad glass wool and tamp moderately tight.

Calc. vol. sample contg ca 25 mg chlorobutanol. Weigh diat. earth equal to 1 g/mL sample. Pipet sample into diat. earth, and mix (ca 1 min) until uniform. Transfer quant. to same column, dry washing with small amt of dry diat. earth, and tamp firmly. Pack as few portions as possible, each portion ≤5 g diat. earth. Rinse beaker with small portions $CS_2$ and transfer to column until sample portion is wet with $CS_2$. Let each portion sink into column before adding next. Add 20 mL $CS_2$ to column and collect eluate in 10 mL vol. flask. Rinse column tip with few drops $CS_2$ (pipet) when 8–9 mL collects. Continue to collect eluate to vol., stopper flask, and mix. This should yield proper concn for either GC or IR detns. (Tightly stoppered solns of chlorobutanol in $CS_2$ may be stored overnight.)

Inject, as below, 6 µL sample soln and est. concn from std curve.

### F. GC Standard Curve

Operating conditions: temps (°)—column 135, detector 215, flash heater 230; N flow rate, ca 35 mL/min to elute chlorobutanol in ca 6 min; and H flow rate, 30 mL/min.

Adjust electrometer sensitivity so that 12 µg chlorobutanol gives ca 50% deflection. Inject 4, 5, 6, 7, 8, and 9 µL of each std eluate from 10 µL syringe. Read vol. in syringe before and after injection; take difference as vol. injected. Plot net (Δ) % deflection against µg anhyd. chlorobutanol injected.

### G. Preparation of Infrared Standard

Record spectrum of each std eluate from 9 to 15 µm, using quant. instrument settings and $CS_2$ in ref. beam. Det. av. *A* of max. at 12.5–12.6 µm, using baseline technics with min. ca 9.5 and ca 14.2 µm as base. ($\Delta A$ is linear from 0.5 to 4.5 mg chlorobutanol/mL $CS_2$.)

### H. Infrared Determination

Det. *A* of sample soln at 12.5–12.6 µm, as above.

$$\text{mg Chlorobutanol in sample aliquot} = (\Delta A \text{ sample eluate}/\Delta A \text{ std eluate}) \times \text{mg chlorobutanol in 10 mL } CS_2 \text{ std}$$

Ref.: JAOAC **50**, 669(1967).

CAS-57-15-8 (chlorobutanol)

## 931.11 Iodoform Drug Substance
**Gravimetric Method**
**Final Action**

### A. Reagents

(**a**) *Ammonium thiocyanate std soln*.—0.05*N*. Stdze against 0.1*N* $AgNO_3$, using equal vol. alcohol and 3 mL $FeNH_4(SO_4)_2$ soln as indicator.

(**b**) *Ferric ammonium sulfate indicator*.—Dissolve 8 g $FeNH_4(SO_4)_2.12H_2O$ in 100 mL $H_2O$.

### B. Determination

Accurately weigh ca 0.25 g $CHI_3$ and transfer quant. to 200 mL erlenmeyer. Add 40 mL alcohol, swirl gently until $CHI_3$ dissolves, filter if necessary, and immediately add 40 mL 0.1*N* $AgNO_3$ and 10 mL $HNO_3$. Swirl gently ca 5 min, let stand at room temp. 2–3 hr, and then swirl occasionally as aid in flocculating the AgI. Titr. excess $AgNO_3$ with 0.05*N* $NH_4SCN$, using 3 mL of the $FeNH_4(SO_4)_2$ indicator. 1 mL 0.1*N* $AgNO_3$ = 0.01312 g $CHI_3$. Or: Proceed as in **932.17**, last par. beginning "Collect AgI on weighed gooch, . . ."

Ref.: JAOAC **14**, 370(1931).

CAS-75-47-8 (iodoform)

## 932.17 Iodoform in Ointments
**Gravimetric Method**
**Final Action**

Transfer ca 2.5 g sample to tared 50 mL beaker and weigh. Add 5 mL $CHCl_3$, stir gently with glass rod, and transfer bulk of undissolved ointment and $CHCl_3$ soln to 250 mL g-s flask. Add 5 mL $CHCl_3$ to ointment remaining in beaker and stir until dissolved. Add soln to flask and finally wash beaker 3 times, using ≤5 mL $CHCl_3$ each time, and add washings to flask. Or: weigh sample in small, tared glass capsule, drop capsule with contents into 250 mL g-s flask, and add ≤20 mL $CHCl_3$. (Use glass capsule only in volumetric detn.) Swirl gently until all ointment dissolves. Add 40 mL 0.1*N alc. $AgNO_3$* and swirl to wash down any $CHI_3$ that adheres to sides of flask. Slowly add 10 mL $HNO_3$ and let stand at room temp. ca 18 hr. Titr. excess of 0.1*N* alc. $AgNO_3$ with 0.05*N* $NH_4SCN$, **931.11A(a)**, using 3 mL $FeNH_4(SO_4)_2$ indicator, **931.11A(b)**, vigorously shaking mixt. near end of titrn. 1 mL 0.1*N* $AgNO_3$ = 0.01312 g $CHI_3$.

For gravimetric detn use ordinary erlenmeyer instead of g-s flask. Weigh ointment base into 100 mL beaker and add $CHCl_3$. After ointment base dissolves, filter thru gooch, using suction. Wash beaker and crucible once with alcohol. Wash crucible several times with $CHCl_3$ without suction. Collect filtrate in erlenmeyer and add 40 mL 0.1*N* $AgNO_3$ and 10 mL $HNO_3$ in small portions. Let mixt. stand 18 hr. Collect AgI on weighed gooch, using suction. Wash with $H_2O$ and then with alcohol. Finally, wash repeatedly with $CHCl_3$ without suction. Dry gooch and contents at ca 125° to const wt. 1 g AgI = 0.5590 g $CHI_3$.

Ref.: JAOAC **15,** 434(1932).

CAS-75-47-8 (iodoform)

## 932.18 Iodoform on Gauze

**Final Action**

Weigh, in tared g-s weighing bottle, sample of $CHI_3$ gauze contg ca 1 g $CHI_3$. ($CHI_3$ gauze is usually moist and loses wt rapidly when exposed to air.) Transfer to 150 mL beaker, add ca 75 mL alcohol, and stir until $CHI_3$ dissolves. Filter into 200 mL vol. flask, draining alc. soln by pressing on gauze. Wash with four or five 25 mL portions alcohol, filter washings, and finally dil. to vol. with alcohol. Pipet 40 mL aliquot into 200 mL erlenmeyer and immediately add 40 mL 0.1*N* $AgNO_3$ and 10 mL $HNO_3$. Proceed as in **932.17**, first par., beginning ". . . let stand at room temp. ca 18 hr."

Ref.: JAOAC **15,** 441(1932).

CAS-75-47-8 (iodoform)

# INORGANIC DRUGS

## 927.10★ Arsenic in Iron-Arsenic Tablets

**Distillation Method**

**Final Action**
**Surplus 1980**

*See* **36.034–36.036**, 13th ed.

## 928.11★ Arsenic in Iron Methylarsenate

**Distillation Method**

**Final Action**
**Surplus 1980**

*See* **36.037**, 13th ed.

## 977.28 Arsenic in Cacodylate Injections

**Differential Pulse Polarographic Method**

**First Action 1977**
**Final Action 1979**

### *A. Apparatus*

(**a**) *Polarograph*.—Capable of effectively scanning −0.15 to −0.9 v in differential pulse mode. Typical instrument settings: scan rate, 2 mv/sec; scan direction, "−"; range, 1.5 v; initial potential, −0.15 v; modulation amplitude, 50 mv; operation mode, differential pulse; current range, 0.05 ma, or as needed; output offset, as required; display direction, "+"; drop time, 1 sec; low pass filter, off; selector, off; pushbutton, initial; recorder: X-axis, 0.1 v/in., Y-axis, 1 v/in.

(**b**) *Cells*.—Std cell bottom with satd calomel ref. electrode (SCE), C rod counter electrode, and dropping Hg indicating electrode.

(**c**) *Pipet*.—100 μL Eppendorf pipet, or equiv.

### *B. Reagents*

(**a**) *Supporting electrolyte*.—1*M* HCl. Add 82 mL HCl to ca 500 mL $H_2O$ in 1 L vol. flask, dil. to vol. with $H_2O$, and mix.

(**b**) *Arsenic std soln*.—2.000 mg As/mL. Dissolve 0.2640 g $As_2O_3$, dried 1 hr at 105°, in ca 25 mL 1*N* NaOH in 100 mL vol. flask, acidify to litmus with 1*M* HCl, and dil. to vol. with $H_2O$.

### *C. Digestion*

Transfer to 100 mL borosilicate beaker accurately measured vol. of injection, dild if necessary, and contg ca 29 mg Na cacodylate (ca 10 mg As), and add 1.0 g $Mg(NO_3)_2.6H_2O$ and 1 mL $HNO_3$. Heat on hot plate at low heat until $H_2O$ is evapd; then at high heat to dry residue. Complete digestion by placing beaker in furnace at 450° until no brown fumes are evolved (30 min). Remove from furnace and let cool to room temp. Perform blank similarly.

### *D. Reduction of Arsenic*

Add to residue 20 mL 5*M* HCl (2 + 3) and swirl to dissolve, warming on steam bath, if necessary; then add 5 mL 40% HBr and 0.3 g $H_2NNH_2.H_2SO_4$. Cover beaker with watch glass and place on steam bath 30 min. Cool to room temp., transfer with $H_2O$ to 100 mL vol. flask, dil. to vol. with $H_2O$, and mix.

### *E. Determination*

Add to polarographic cell 20 mL supporting electrolyte and bubble N thru soln 5 min; then direct gas to sweep over soln. Switch selector to "Cell", and allow sufficient time for recorder pen to come to rest. Depress "Scan" pushbutton and record polarogram from −0.15 to −0.9 v. Pipet 2 mL of sample soln into cell, bubble N thru soln 1 min, direct gas to sweep over soln, and record polarogram as before, using same instrumental settings. Repeat operations on 2 addnl 100 μL aliquots of std soln. Polarograph blank similarly. Plot std addn curve as follows: mg std added, 0, 0.2, and 0.4, on X-axis and first As peak ht which appears at ca −0.37 v against SCE on Y-axis. Extrapolate linear plot to X-axis to obtain mg As in aliquot. Correct for blank, if necessary. Stds need not be polarographed beyond ca −0.60 v, since anal. peak is at ca −0.37 v against SCE.

Ref.: JAOAC **60,** 1015(1977).

CAS-7440-38-2 (arsenic)

## 926.16★ Arsenic in Sodium Cacodylate

**Titrimetric Method**

**Final Action**
**Surplus 1977**

*See* **36.044**, 12th ed.

## 932.19 Bismuth Compounds in Drugs
### Gravimetric Method
### Final Action 1965

(Applicable in absence of Pb. *Caution:* See safety notes on distillation, nitric acid, and hydrogen sulfide.)

Thoroly mix sample and weigh 0.5 g into 500 mL Kjeldahl flask. Ignite gently over small flame, using wire gauze under flask, and increase heat towards end. Let cool, add 15–20 mL $HNO_3$, evap. to dryness, and ignite as before until yellow or orange $Bi_2O_3$ is formed. Cool residue and dissolve in 10–15 mL warm $HNO_3$, using few mL 3% $H_2O_2$ if residue does not dissolve readily. Boil off excess $H_2O_2$ and wash into 400 mL beaker with $H_2O$, rinsing flask well. Dil. to ca 200 mL, make just neut. to litmus with $NH_4OH$, and add 5 mL HCl. Ppt with $H_2S$ completely.

Transfer ppt to filter paper and wash once with HCl (5 + 200) and then several times with $H_2O$. Dissolve ppt of $Bi_2S_3$ on filter with hot $HNO_3$ (1 + 2). Small residue of S (and HgS if Hg salts are present) usually remains. Neutze filtrate with $NH_4OH$ (2 + 3) and ppt with 25 mL 20% $(NH_4)_2CO_3$ soln. Conc. to ca 150 mL (by boiling, if desired) and let stand on steam bath 1–2 hr. Collect ppt in previously ignited, weighed gooch, wash with small amt of $H_2O$, dry, ignite in furnace at ca 550°, and weigh as $Bi_2O_3$.

Ref.: JAOAC **15,** 422(1932).

## 972.46 Bismuth Compounds in Drugs
### Polarographic Method
### First Action 1972
### Final Action 1974

#### *A. Apparatus*

(**a**) *Polarograph.*—Any voltammetric or polarographic instrument with necessary accessories (cells, electrodes, Hg, capillaries) capable of scanning 0 to −1.0 v and measuring 0.040 μa/mm.

(**b**) *Cells.*—Microcell with satd calomel or Hg pool ref. electrode.

#### *B. Reagents*

(**a**) *Supporting electrolyte.*—1*M* HCl. Add 171 mL HCl to ca 1 L $H_2O$ in 2 L vol. flask and mix. Cool to room temp., dil. to vol. with $H_2O$, and mix.

(**b**) *Gelatin maximum suppressor.*—1 mg/mL. Accurately weigh 100 mg gelatin into small beaker and dissolve in small amt of $H_2O$ on steam bath. Transfer quant. to 100 mL vol. flask and dil. to vol. with $H_2O$. Prep. fresh daily.

(**c**) *Bismuth std solns.*—(*1*) *Stock soln.*—1 mg/mL. Transfer 122.2 mg Bi subcarbonate, equiv. to 100 mg Bi (mg Bi subcarbonate×0.8182 (factor derived from primary std) = mg Bi), to 100 mL vol. flask with supporting electrolyte, (**a**). Dil. to vol. with same soln. (*2*) *Working soln.*—0.2 mg/mL. Pipet 20.0 mL stock soln into 100 mL vol. flask, add 1.0 mL gelatin max. suppressor, (**b**), and dil. to vol. with supporting electrolyte, (**a**). Mix thoroly.

#### *C. Preparation of Sample*

(**a**) *Tablets.*—Det. av. wt/tablet. Grind to pass No. 60 sieve. Quant. transfer amt tablet material contg 10 mg Bi, accurately weighed, to 50 mL vol. flask with aid of 1*M* HCl, (**a**). Add 0.5 mL gelatin max. suppressor, (**b**), and dil. to vol. with 1*M* HCl. Shake thoroly or use sonic vibrator. Filter thru rapid paper just before polarographic detn.

(**b**) *Magma, emulsions, and injectables.*—Mix *thoroly* to disperse suspension. Immediately transfer aliquot contg ca 100 mg Bi to 100 mL vol. flask. Rinse inside of pipet with 1*M* HCl, (**a**), and dil. to vol. with same soln. Pipet 10 mL into 50 mL vol. flask, add 0.5 mL gelatin max. suppressor, (**b**), and dil. to vol. with 1*M* HCl. Mix thoroly.

(**c**) *Powder.*—Transfer entire contents of vial, or amt material equiv. to 200 mg Bi for bulk powders or capsules, to 200 mL vol. flask. Wash vial into flask and dil. sample to vol. with 1*M* HCl, (**a**). Proceed as for magma, beginning "Pipet 10 mL . . ."

#### *D. Determination*

Transfer soln to cell and bubble N thru soln 10 min. Record polarogram from 0 to −1.0 v against satd calomel ref. electrode. Measure ht of diffusion current ($I_d$) at half-wave potential ($E^1/_2$), and det. Bi concn by comparing wave hts of sample soln with those of std soln polarographed immediately before and after samples. Do all detns at same current sensitivity and consecutively.

$E^1/_2$ value is qual. identification of Bi.

Calc. concn of Bi as follows:

(**a**) *Tablets.*—mg Bi/tablet = $50 \times (I/I') \times C \times (W/W')$;

(**b**) *Magma, emulsions, and injectables.*—mg Bi/mL = $500 \times (I/I') \times (C/V)$;

(**c**) *Powder.*—mg Bi/mg sample = $1000 \times (I/I') \times (C/W')$;

where $I$ and $I'$ = diffusion currents of sample and std, resp.; $C$ = mg Bi/mL working soln, (**c**)(*2*); $W$ and $W'$ = av. tablet wt and wt sample taken (mg), resp.; and $V$ = mL liq. prepn taken.

#### *E. Measurement of Diffusion Current*

Measure diffusion current ($I_d$ at $E^1/_2$). Draw lines tangent to tops of residual and limiting currents. Draw third line tangent to vertical slope. Measure its length, mark off half-way point at $I_d/2$; then drop perpendicular thru this point and thru abscissa (applied voltage). Diffusion current is perpendicular portion of this line cutting thru limiting current and residual current tangent lines.

Ref.: JAOAC **55,** 155(1972).

## 967.30 Calcium and Magnesium in Drugs
### Titrimetric Method
### First Action 1967
### Final Action 1968

(Applicable to pharmaceuticals and vitamin-mineral prepns)

#### *A. Reagents*

Use $H_2O$ redistd from glass (preferable) or deionized $H_2O$.

(**a**) *Calcium carbonate.*—Primary std grade, dried 2 hr at 285°.

(**b**) *Hydroxy naphthol blue.*—Ca indicator (Mallinckrodt Chemical Works No. 5630 in dispenser bottle ready for use, or equiv.). Store in dark and replace after 1 year.

(**c**) *Calmagite.*—Ca + Mg indicator (Mallinckrodt No. 4283 in dispenser bottle ready for use, or equiv.). Store in dark and replace after 1 year.

(**d**) *Disodium dihydrogen ethylenediamine tetraacetate (EDTA) std soln.*—0.01*M*. Dissolve 3.72 g $Na_2H_2EDTA.2H_2O$ (99+ % purity) in $H_2O$ in 1 L vol. flask, dil. to vol., and mix. Accurately weigh enough $CaCO_3$ (ca 40 mg) to give ca 40 mL titrn with 0.01*M* EDTA and transfer to 400 mL beaker. Add 50 mL $H_2O$ and enough HCl (1 + 3) to dissolve the $CaCO_3$. Dil. to ca 150 mL with $H_2O$ and add 15 mL 1*N* NaOH, disregarding any ppt or turbidity. Add ca 200 mg hydroxy naphthol blue indicator and titr. from pink to deep blue end point, using mag. stirrer. Add last few mL EDTA soln dropwise. Molarity EDTA soln = mg $CaCO_3$/(mL EDTA × 100.09).

(e) *Buffer soln.*—pH 10. Dissolve 67.5 g $NH_4Cl$ in 200 mL $H_2O$, add 570 mL $NH_4OH$, and dil. to 1 L.

(f) *Potassium hydroxide-potassium cyanide soln.*—Dissolve 280 g KOH and 66 g KCN in 1 L $H_2O$.

(g) *Potassium cyanide soln.*—2%. Dissolve 2 g KCN in 100 mL $H_2O$.

### B. Apparatus

(a) *Titration stand.*—Fluorescent illuminated.

(b) *Ion exchange column.*—Approx. 20 × 600 mm, fitted with coarse porosity fritted glass disk and Teflon stopcock. Place 30–40 g moist Amberlite IR-4B resin (anion exchange resin with high phosphate capacity) from fresh bottle in 600 mL beaker and exhaust with three 250 mL portions 5% $Na_2CO_3$ or NaOH. Wash with $H_2O$ until excess base is removed. Treat resin with three 250 mL portions 5% HCl (3 + 22), mixing thoroly after each treatment. Rinse with $H_2O$ until color is removed, and transfer with $H_2O$ to column. Column is ready for use after draining $H_2O$ to top of resin column. (Exchange capacity for phosphate is ca 1500 mg; therefore, number of aliquots can be passed thru column before regeneration is necessary. Rinse column with ca 250 mL $H_2O$ before each use until eluate is colorless.)

### C. Preparation of Sample

Transfer 2 g well mixed sample to 100 mL Pt or porcelain dish. Ash at ≤525° until apparently C-free (gray to brown). Cool, add 20 mL $H_2O$, stir with stirring rod, and add 10 mL HCl cautiously under watch glass. Rinse off watch glass into dish and evap. to dryness on steam bath. Add 50 mL HCl (1 + 9), heat on steam bath 15 min, and filter thru quant. paper into 200 mL vol. flask. Wash paper and dish thoroly with hot $H_2O$. Cool filtrate, dil. to vol., and mix.

### D. Determination

Transfer 50 mL aliquot prepd sample to 250 mL beaker and adjust to pH 3.5 with 10% KOH soln added dropwise, using pH meter and mag. stirrer. Pass sample thru resin column (column is in Cl form), collecting effluent in 250 mL vol. flask and adjusting flow rate to 2–3 mL/min. Wash column with two 50 mL portions $H_2O$, passing first portion thru at same rate as sample soln and second at 6–7 mL/min. Finally, pass enough $H_2O$ freely thru column to dil. to vol. Mix thoroly. Pipet two 100 mL aliquots into 400 mL beakers.

*Titration 1 (calcium + magnesium).*—Adjust first aliquot to pH 10 (using pH meter and mag. stirrer) with pH 10 buffer soln, (ca 5 mL). Add 2 mL 2% KCN soln, and 200 mg Calmagite indicator, and titr. immediately with 0.01*M* EDTA soln thru red to deep blue end point, using mag. stirrer.

*Titration 2 (calcium).*—Adjust second aliquot to pH 12.5–13.0 (using pH meter and mag. stirrer) with KOH-KCN soln (ca 10 mL). Add 0.100 g ascorbic acid and 200–300 mg hydroxy naphthol blue indicator. Titr. immediately with 0.01*M* EDTA soln thru pink to deep blue end point, using mag. stirrer.

$$\% \text{ Ca} = Y \times F \times 0.4008 \times 10 \times 100/\text{mg sample}$$

$$\% \text{ Mg} = (X - Y) \times F \times 0.2431 \times 10 \times 100/\text{mg sample}$$

where $X$ and $Y$ = mL EDTA soln from titrns *1* and *2*, and $F$ = molarity EDTA soln/0.01.

Refs.: JAOAC **49,** 287(1966); **50,** 663, 787(1967).

CAS-7440-70-2 (calcium)
CAS-7439-95-4 (magnesium)

## 932.20 Calcium Gluconate in Drugs
### Colorimetric Method
### Final Action

(Applicable to prepns whose aq. solns are neut. and which do not contain salts of other optically active hydroxy acids. *Caution:* See safety notes on uranyl acetate and toxic dusts.)

Weigh two 0.5 g portions Ca gluconate or two 1 g portions powd tablets contg ≤50% of the salt. If chocolate or fatty base is present, wash samples several times on hardened filter with absolute ether, then warm residue until ether is driven off.

Transfer each portion to sep. 25 mL vol. flasks, add 15 mL $H_2O$, and warm until Ca salt dissolves. (Samples contg cocoa will have undissolved residue.) Cool mixt. to room temp.

To one flask (No. 1) add 3.5 g *finely pulverized uranyl acetate,* stopper, and shake mech. 1 hr. (If agitation is not vigorous enough, >1 hr of shaking may be required.) Let other flask (No. 2) stand. If sample contains chocolate, add little alumina cream, **925.46B(b)**, to each flask. Cool to room temp., dil. flask No. 1 to vol. with *uranyl acetate soln* (10 g shaken with 95 mL $H_2O$ until satd and then filtered), and flask No. 2 with $H_2O$. Filter, and polarize each soln in 200 mm tube, using 50 mm tube contg *1.8% $K_2Cr_2O_7$ soln* as light filter. If soln is too dark to read in 200 mm tube, make reading in 100 mm tube and multiply result by 2. If $X$ = rotation in °S of Soln No. 2 and $Y$ = rotation of Soln No. 1, with 1 g sample: % $Ca(C_6H_{11}O_7)_2$ = 4.34 × $(Y - X)$; and with 0.5 g sample: % $Ca(C_6H_{11}O_7)_2$ = 8.52 × $(Y - X)$.

Refs.: JAOAC **15,** 456, 461(1932); **16,** 379(1933); **17,** 425(1934).

CAS-299-28-5 (calcium gluconate)

## 977.29 Calcium, Potassium, and Sodium in Electrolyte Replenishers
### Atomic Absorption Spectrophotometric–Flame Photometric Method
### First Action 1977
### Final Action 1979

(Applicable to Ringer's and Lactated Ringer's Injections)

### A. Reagents

(Use $H_2O$ of ≥0.5 megohm resistivity for all rinsing and diln. Use borosilicate volumetric glassware, including pipets, meeting NIST tolerances. Use ≥4 mL single transfer pipets for all dilns, except that Class A 5 mL Mohr pipets may be used to complete intermediate and final dilns. Clean all glassware with $HNO_3$ (1 + 3) until washings show no Na at 589 nm when compared with $H_2O$ used in Na detn.)

(a) *Buffer soln.*—0.25*M* EDTA + 25 mg $La_2O_3$/mL. (Required in AA detn of Ca and K only.) Transfer 73.1 g EDTA into 1 L erlenmeyer. Add ca 100 mL $H_2O$, and shake cautiously with just enough $NH_4OH$ to dissolve. Transfer 25.0 g low-Ca $La_2O_3$ to 500 mL erlenmeyer, add 25 mL $H_2O$, and very cautiously dissolve with 70% $HClO_4$. Cool both solns. Pour $HClO_4$ soln into EDTA soln, and wash mixt. into 1 L vol. flask. Dissolve completely and adjust to pH slightly alk. to *Me orange,* using $NH_4OH$ or 70% $HClO_4$, as needed. Dil. to vol. with $H_2O$, mix, and store in clean, dry polyethylene or Teflon bottle.

(b) *Calcium std soln.*—10 mg/mL. Dry $CaCO_3$ (low in alkalis) ≥2 hr at 285° (*Caution:* Higher temp. may convert $CaCO_3$, in part, to CaO), cool in desiccator, and transfer 24.975 g to

1 L vol. flask. Add ca 150 mL $H_2O$ and acidify cautiously with 45 mL HCl from freshly opened bottle. Cool to room temp., dil. to vol. with $H_2O$, and mix. Store in clean, dry polyethylene or Teflon bottle.

(**c**) *Potassium std soln.*—1 mg/mL. Dry KCl ≥2 hr at 500–600°, cool in desiccator, and transfer 1.9070 g to 1 L vol. flask. Dissolve and dil. to vol. with $H_2O$, mix, and store in clean, dry polyethylene or Teflon bottle.

(**d**) *Sodium std soln.*—10 mg/mL. Dry NaCl ≥2 hr at 500–600°, cool in desiccator, and transfer 25.420 g to 1 L vol. flask. Dissolve and dil. to vol. with $H_2O$, mix, and store in clean, dry polyethylene or Teflon bottle.

(**e**) *Mixed cation std soln.*—(5 μg Ca + 10 μg K + 200 μg Na)/mL. Pipet 10 mL Ca std soln, (**b**), into 200 mL vol. flask, dil. to vol. with $H_2O$, and mix. Pipet 10 mL this dild soln into 1 L vol. flask, pipet in 10 mL K std soln, (**c**), and 20 mL Na std soln, (**d**), dil. to vol. with $H_2O$, and mix. Store in clean, dry polyethylene or Teflon bottle. Stable ≥1 month. (Proportionate vols may be prepd.)

### *B. Instrument Suitability*

(*Caution:* See safety notes on AAS and flame photometers.)

Use spectrophtr in AA or flame emission mode. Keep *A* readings between 0.100 and 0.820 unit, or emission readings between 20 and 95% *T* by adjusting sample dilns, if necessary.

Prep. sufficient std solns to bracket sample detns. Readings of all stds must be on linear portion of std curve. Linearity is detd by running intermediate test std which must agree to within 1% of reading indicated by straight line relationship between the bracketing std points.

Spectrophtr must pass following precision test: Read, sequentially, low std, sample, and high std. Repeat twice and average readings of each soln. Results are acceptable if each individual reading differs from av. value for particular soln by ≤1.4%.

### *C. Determination*

(**a**) *Calcium.*—(*1*) *For absorption.*—Pipet 5 mL sample and 4 mL buffer soln into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix. Prep. "median range std" by dilg mixed cation std soln, including 4.00 mL buffer soln in each 100 mL std soln. Analyze sample and std solns at 422.7 nm, and est. no. and concn of stds needed for final detn as required in **977.29B**, par. 2. (*2*) *For emission.*—Proceed as in (*1*), omitting buffer soln.

(**b**) *Potassium.*—(*1*) *For absorption.*—Pipet 5 mL sample to 100 mL vol. flask, dil. to vol. with $H_2O$, and mix. Pipet 10 mL this soln into 50 mL vol. flask, pipet in 2 mL buffer soln, dil. to vol. with $H_2O$, and mix. Prep. "median range std" by dilg mixed cation std soln, including 2.00 mL buffer soln in each 50 mL final soln. Analyze sample and std solns at 766.5 nm, and est. no. and concn of stds needed for final assay as required in **977.29B**, par. 2. (*2*) *For emission.*—Proceed as in (*1*), omitting buffer soln.

(**c**) *Sodium.*—Pipet 5 mL sample into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix. Pipet 4 mL this soln into 500 mL vol. flask, dil. to vol. with $H_2O$, and mix. Prep. "median range std" by dilg mixed cation std soln. Analyze sample and std solns at 589.0 nm, and est. no. and concn of stds needed for final assay as required in **977.29B**, par. 2.

(**d**) *Calculations.*—Det. concn of each cation from std curve. Calc. concn of each cation as mg Cl salt/100 mL sample.

Ref.: JAOAC **60,** 929(1977).

CAS-7440-70-2 (calcium)
CAS-7440-09-7 (potassium)
CAS-7440-23-5 (sodium)

## 949.14★ Calcium, Phosphorus, and Iron in Vitamin Preparations

**Titrimetric Method**

**Final Action**
**Surplus 1977**

*See* **36.058–36.059**, 13th ed.

## 938.13★ Effervescent Potassium Bromide with Caffeine in Drugs

**Volhard Titration**

**Final Action**
**Surplus 1975**

*See* **36.057–36.058**, 12th ed.

## 941.19 Elixir of Five Bromides

**Final Action**

### *A. Preparation of Dilution*

Transfer 50 mL sample to 1 L vol. flask, dil. to vol., and mix. Measure aliquots of this diln at original temp. of sample.

### *B. Determinations*

(**a**) *Ammonium bromide.*—Place 200 mL aliquot of diln in Kjeldahl flask; add small piece of *paraffin* and excess 10% NaOH soln (ca 5 mL). Distil $NH_3$ into excess std acid (40 mL 0.1*N* usually is enough). Titr. excess acid with 0.1*N* NaOH, using Me red. 1 mL 0.1*N* acid = 0.00979 g $NH_4Br$.

(**b**) *Calcium bromide.*—Pipet 100 mL aliquot of diln into casserole or Pt dish and evap. to dryness. Ignite at dull red (ca 525°) until org. matter is thoroly charred. Cool, add 5 mL HCl (1 + 3) to dissolve Ca salts, filter, and wash well with hot $H_2O$. Return filter and unoxidized C to casserole or dish and ignite at 600° until residue is white. Treat residue with 5 mL HCl (1 + 3), filter, and wash with hot $H_2O$, combining filtrates.

Det. Ca as in **910.01**, and reserve filtrate for detn of Na, K, and Li. If 0.1*N* $KMnO_4$ is used, 1 mL = 0.0100 g $CaBr_2$.

(**c**) *Lithium bromide.*—Dil. filtrate and washings from Ca detn to 200 mL and mix. Evap. 100 mL aliquot to dryness and drive off all $NH_4$ salts by heating to faint red (ca 525°) in Pt dish. Treat residue with little $H_2O$, filter into Pt dish, add few mL HCl, and evap. to dryness.

Complete conversion of alkali bromides to chlorides by treating residue with Cl-$H_2O$ and evapg to dryness. Repeat addn and evapn of Cl-$H_2O$ twice more, or until there is no apparent darkening of soln due to liberation of Br.

Dissolve mixed chlorides in min. amt of cold $H_2O$ (ca 1.5 mL is more than enough for 0.5 g salts), in tall 200 mL beaker. Add 1 drop HCl, and then gradually add 20 mL absolute alcohol, dropping alcohol into center of beaker (not on sides) while rotating soln. (NaCl and KCl should be pptd in perfectly uniform granular condition.) In similar manner add 60 mL ether (sp gr 0.716–0.717 at 25°) and let mixt. stand ca 5 min or until ppt is well agglomerated and supernate is almost clear, rotating mixt. occasionally. Filter with suction thru weighed gooch into erlenmeyer, using bell jar arrangement, washing beaker thoroly with mixt. of alcohol and ether (1 + 5), and collecting all ppt on gooch with aid of policeman. Thoroly wash ppt on gooch, set crucible aside, and rinse funnel with alcohol-ether mixt. to wash any adhering Li soln into flask contg filtrate. Evap. filtrate to dryness on steam bath, using air current.

Treat residue with 10 mL absolute alcohol, warming if necessary, so that practically all residue dissolves. If slight film remains on bottom and sides of flask, remove with policeman. Then, while rotating soln in flask, add 50 mL ether (sp gr 0.716–0.717 at 25°), followed by 1 drop HCl. Let stand 30 min, rotating soln frequently. When fine ppt has agglomerated (only very small amt is usually pptd), filter into tall beaker with suction thru gooch contg first ppt. Wash combined ppts with the ether-alcohol mixt., taking same precautions as in first pptn. Air dry gooch and contents; then dry in oven, ignite gently, cool, and weigh to obtain combined wt NaCl and KCl. Reserve crucible and contents for K detn.

Evap., on steam bath, ether-alcohol filtrate and washings contg the Li. Dissolve residue in little $H_2O$, add slight excess of $H_2SO_4$ (1 + 1), and transfer to weighed porcelain or Pt dish. Evap. as far as possible on steam bath and then gently ignite residue over flame. (By placing dish on triangle over asbestos gauze and using low flame, soln can be evapd without spattering.) Finally ignite carefully over full flame, cool, and weigh. If charring has occurred, repeat ignition with $H_2SO_4$. Calc. to LiBr, using factor 1.5800.

**(d)** *Sodium bromide*.—Remove combined KCl and NaCl from gooch by washing with hot $H_2O$, dil. to 50 mL, and use 5 mL aliquot for detn of Na. Proceed as in **941.03**, beginning ". . . add 100 mL Mg uranyl acetate soln . . ." Calc. to NaBr, using factor 0.0688.

**(e)** *Potassium bromide*.—To 25 mL aliquot of soln of KCl and NaCl, add enough Pt soln (0.105 g $H_2PtCl_6$/mL) to convert KCl and NaCl to $K_2PtCl_6$ and $Na_2PtCl_6$, and evap. to dryness. Treat residue with 80% alcohol by vol., filter, and wash until excess of $H_2PtCl_6$ and $Na_2PtCl_6$ is removed. Dry filter and ppt, dissolve residue in hot $H_2O$, and transfer to weighed Pt dish. Evap. on steam bath, dry 30 min in oven at 100°, cool, and weigh as $K_2PtCl_6$. Calc. to KBr, using factor 0.4898.

**(f)** *Total bromine*.—Transfer 20 mL of diln to 500 mL flask. Add 100 mL $H_2O$, 2 mL $HNO_3$, and excess of 0.1*N* $AgNO_3$ (usually 30 mL). Titr. excess $AgNO_3$ with 0.1*N* $NH_4SCN$, using Fe alum indicator. 1 mL 0.1*N* $AgNO_3$ = 0.00799 g Br.

Refs.: JAOAC **24,** 842(1941); **25,** 847(1942).

CAS-12124-97-9 (ammonium bromide)
CAS-7789-41-5 (calcium bromide)
CAS-7550-35-8 (lithium bromide)
CAS-7758-02-3 (potassium bromide)
CAS-7647-15-6 (sodium bromide)

## 941.20★ Elixir of Three Bromides

**Final Action**
**Surplus 1975**

*See* **36.061–36.062**, 12th ed.

## 984.37 Fluoride in Drug Tablets and Solutions

### Fluoride-Selective Electrode Method

**First Action 1984**
**Final Action 1986**

### A. Principle

F in tablets and solns is detd by F-selective electrode, using total ionic strength adjustment buffer (TISAB) to eliminate complexation of F with polyvalent cations and direct potentiometry to measure concn of F ions in soln. (*Caution:* Store all samples and stds in plastic containers because F reacts with glass.)

### B. Reagents

**(a)** *Total ionic strength adjustment buffer (TISAB)*.—TISAB II (Orion Research Inc. Cat. No. 94-09-09); alternatively, in ca 500 mL $H_2O$ in 1 L beaker, dissolve 57 mL HOAc, 58 g NaCl, and 4 g CDTA (cyclohexylene dinitrilo tetraacetic acid or 1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid). Cool and adjust pH to 5.0–5.5 with 5M NaOH. Cool to room temp., transfer to 1 L vol. flask, and dil. to vol. with $H_2O$.

**(b)** *Fluoride std solns*.—(*1*) *$10^{-2}M$*.—Transfer 209.9 mg accurately weighed Ref. Std NaF, previously dried 4 h at 150°, to 500 mL vol. flask, dissolve, and dil. to vol. with $H_2O$. (*2*) *$10^{-3}M$*.—Pipet 25.0 mL of $10^{-2}$M soln to 250 mL vol. flask. Dil. to vol. with $H_2O$. (*3*) *$10^{-4}M$*.—Pipet 25.0 mL $10^{-3}$M soln to 250 mL vol. flask. Dil. to vol. with $H_2O$.

### C. Apparatus

(Equiv. app. may be substituted.)

**(a)** *Meter*.—Expanded-scale mV pH meter or selective ion meter.

**(b)** *F-selective electrode*.—F-combination electrode filled with internal filling soln.

**(c)** *Magnetic stirrer*.—Suitable mag. stirrer with asbestos pad placed on top to reduce heat transfer. Use teflon-coated stir bar.

### D. Preparation of Samples

**(a)** *Tablets*.—Det. no. of tablets equiv. to 10 mg F and completely disperse in 500 mL vol. flask contg ca 400 mL $H_2O$ by heating on steam bath and shaking intermittently. Cool to room temp. and dil. to vol. with $H_2O$.

**(b)** *Solutions*.—Pipet aliquot of soln contg 1–2 mg F into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix.

Do not let solns remain in glass >1 h.

### E. Determination

Pipet 20.0 mL of each std and sample prepn into sep. plastic beakers. Pipet 20.0 mL TISAB into each beaker. Use potentiometer equipped with F-selective electrode. Immerse electrode in $10^{-3}$M std soln and stir at const. rate with mag. stirrer. Take mV measurement when potential has stabilized within ±0.1 mV. Repeat with $10^{-2}$M and $10^{-4}$M std solns and with sample prepn. Using 2-cycle semi-log graph paper, prep. std curve plotting molar F std concn on log axis vs mV potential on linear axis. Det. molar F concn ($C_i$) in sample prepn from std curve. Prep. sep. mV measurements for each std soln and std curve for each sample detn.

### F. Calculations

Tablets:

$$\text{mg F/tablet} = C_i \times (19 \text{ mg F/mL}) \times 500 \text{ mL/no. of tablets} = 9500\ C_i/\text{no. of tablets}$$

Solns:

$$\text{mg F/aliquot} = C_i \times (19 \text{ mg F/mL}) \times 100 \text{ mL} = 1900\ C_i$$

where $C_i$ = molar concn in sample prepn as detd from std curve.

Ref.: JAOAC **67,** 682(1984).

## 935.66★ Hypophosphites in Sirups

**Final Action**
**Surplus 1975**

(Applicable in absence of phosphates; if phosphates are present, make suitable correction.)

### A. Method I

*See* **36.063**, 12th ed.

### B. Method II

(Not applicable in presence of other reducing agents or phenolic compds)

*See* **36.064–36.065**, 12th ed.

## 932.21 Iodine in Drugs
### Titrimetric Method
### Final Action

Transfer sample contg ≤0.1 g iodide (0.05 g is ample) to crucible, preferably Ni. If sample contains only slight amt of org. material, add 1 g starch. Add 2–3 g solid KOH. If sample is solid, add 10–15 mL alcohol before adding KOH. Alkali must be thoroly mixed with sample to prevent loss of I in furnace (either stir, leaving stirring rod in crucible, or heat and swirl on steam bath until KOH is in soln). Dry and char thoroly. (Use as low temp. as possible to prevent loss of I; ≤525°.) Ext charred mass with hot $H_2O$, filter into erlenmeyer, and wash well with hot $H_2O$.

Neutze filtrate with $H_2SO_4$ (1 + 1), make alk. again with 4% NaOH soln, and add 1 mL excess. Heat to bp and slowly add satd $KMnO_4$ soln until $KMnO_4$ color remains after several min of boiling. Then add ca 0.5 mL excess, continue boiling ca 5 min, and let cool. Add enough $KMnO_4$ to completely oxidize all iodide to iodate so that $KMnO_4$ color, not brown $MnO_2$ color, is present at end of boiling period. Add few mL alcohol and place on steam bath. ($KMnO_4$ color should be bleached; if it is not, add little more alcohol.) When ppt has settled, filter, and wash with hot *1% $NH_4Cl$ soln*. If filtrate is not clear, digest on steam bath until the $MnO_2$ can be retained on filter. After cooling, add 1–2 g KI, acidify with HCl, and titr. with 0.1*N* $Na_2S_2O_3$. 1 mL 0.1*N* $Na_2S_2O_3$ = 0.00277 g KI, 0.00250 g NaI, or 0.00212 g I.

Refs.: JAOAC **15,** 419(1932); **32,** 555(1949).

CAS-7553-56-2 (iodine)

## 941.21 Iodine in Ointments
### Titrimetric Method
### Final Action

(**a**) *Free iodine*.—Weigh (to 1 mg) ca 2 g ointment, and transfer to 250 mL I flask. Melt on $H_2O$ bath (≤70°), add 30 mL $CHCl_3$, mix well, and then add 30 mL $H_2O$. (All of base should be dissolved in $CHCl_3$ before $H_2O$ is added.) Titr. with 0.1*N* $Na_2S_2O_3$, using starch indicator (mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring const., and immediately discontinue heating; add ca 1 mL Hg, shake, and let soln stand over the Hg). Approach end point dropwise, shaking flask vigorously to ensure that all I has been extd from $CHCl_3$ layer. 1 mL 0.1*N* $Na_2S_2O_3$ = 0.01269 g I.

(**b**) *Potassium iodide*.—Pour liqs from free I detn, (**a**), into 500 mL I flask, rinsing flask with 200 mL $H_2O$, added in several portions. (It is desirable to maintain this vol. within rather narrow limits.) Add 0.5 mL *0.2% alc. p-ethoxychrysoidin indicator* and 1–4 drops 0.1*N* NaOH (to neutze). (Aq. layer should now be clear yellow.) Titr. with 0.1*N* $AgNO_3$, approaching end point dropwise and swirling frequently. ($AgNO_3$ soln causes turbidity due to formation of colloidal AgI and development of reddish-brown color similar to that observed in over-titrd Volhard detn. End point, which is produced by 1 drop $AgNO_3$ soln, is characterized by flocculation of colloidal AgI and complete disappearance of reddish brown tinge, leaving almost clear, pale yellow supernate.) mL 0.1*N* $AgNO_3$ − mL 0.1*N* $Na_2S_2O_3$, (**a**) = mL consumed by iodide originally present. 1 mL 0.1*N* $AgNO_3$ = 0.0166 g KI.

Ref.: JAOAC **24,** 833(1941).

CAS-7553-56-2 (iodine)
CAS-6881-11-0 (potassium iodide)

## 977.30 Iron in Drugs
### Spectrophotometric Method
### First Action 1977
### Final Action 1979

(Applicable to drugs listed in Table **977.30**. Rinse all glassware with deionized $H_2O$ before use to avoid Fe contamination from tap $H_2O$.)

### A. Principle

Sample is dissolved in dil. HCl or $H_2O$ and dild to concn of 3 mg/100 mL. $Fe^{+2}$ is detd by complexing with α,α'-dipyridyl at pH 4.5 and measuring *A* at 523 nm. Total Fe is detd by reducing $Fe^{+3}$ to $Fe^{+2}$ with ascorbic acid and complexing with dipyridyl. $Fe^{+3}$ is detd by difference.

### B. Reagents

(**a**) *Dipyridyl soln*.—Dissolve 0.1 g α,α'-dipyridyl in $H_2O$ and dil. to 100 mL. Soln is stable up to 4 months if stored in cool, dark place.

(**b**) *Iron std solns*.—(*1*) *Stock soln*.—0.3 mg Fe/mL. Accurately weigh ca 0.3 g std Fe powder (99.999%), dissolve in 100 mL $H_2O$ and 20 mL HCl by heating on steam bath, if necessary, dil. to 1 L with $H_2O$, and mix. (Complete soln may require as long as 5 hr heating.) (*2*) *Working soln*.—0.03 mg/mL. Dil. 50.0 mL stock soln to 500 mL with $H_2O$.

(**c**) *Acetate buffer soln*.—pH 4.5. Dissolve 273 g $NaOAc.3H_2O$ in $H_2O$, add 240 mL HOAc, and dil. to 2 L with $H_2O$.

### C. Preparation of Sample

(Complete immediately to avoid oxidn of $Fe^{+2}$ to $Fe^{+3}$.)

(**a**) *Powders*.—Accurately weigh sample contg ca 60 mg Fe, transfer to 200 mL vol. flask, and dissolve and dil. to vol. with initial solv. specified in Table **977.30**. Dil. 10.0 mL aliquot to 100.0 mL with specified diln solv.

(**b**) *Tablets*.—Accurately weigh portion powd tablets or individual tablets contg ca 60 mg Fe into 200 mL vol. flask, add 100 mL $H_2O$ and 4 mL HCl, heat on steam bath 30 min (Fe gluconate and $FeSO_4$ do not require heat; place 5–10 min in ultrasonic bath), cool to room temp., and dil. to vol. with $H_2O$. Filter thru Whatman No. 1 paper, or equiv. Dil. 10.00 mL filtrate to 100.0 mL with $H_2O$.

(**c**) *Elixirs, sirups, and injections*.—Pipet sample (use "to contain" pipet and rinse, if sample is viscous) contg ca 60 mg Fe into 200 mL vol. flask, dil. to vol. with $H_2O$, and mix. Pipet 10 mL into 100 mL vol. flask, add 2 mL HCl, and dil. to vol. with $H_2O$.

**Table 977.30 Conditions for Analysis of Various Iron Preparations**

| Preparation | Initial Solv. | Diln Solv. | Color Develop. Temp. |
|---|---|---|---|
| Ferrous ammonium sulfate, powder | (1) | $H_2O$ | Room |
| Ferrous sulfate, powder, tablets | (1) | $H_2O$ | Room |
| Ferrous gluconate, powder, tablets | (1) | $H_2O$ | Room |
| Ferrous fumarate, powder, tablets | (1) | $H_2O$ | Heat |
| Ferric ammonium citrate, powder | (1) | $H_2O$ | Heat |
| Ferric glycerophosphate, powder | (1) | $H_2O$ | Room |
| Iron cacodylate, powder, injection[a] | (1) | $H_2O$ | Heat |
| Iron peptonate, powder | $H_2O$ | (2) | Heat |
| Soluble ferric pyrophosphate, powder | $H_2O$ | (2) | Room |
| Ferrous sulfate, elixir | $H_2O$ | (2) | Room |
| Iron sorbitex, injection (1.00 mL sample) | $H_2O$ | (2) | Heat |
| Iron dextran, injection (1.00 mL sample) | $H_2O$ | (2) | Heat |

Solv. (1) = 100 mL $H_2O$ contg 4 mL HCl, dil. to vol. with $H_2O$
Solv. (2) = 2 mL HCl, dil. to vol. with $H_2O$
[a] For iron cacodylate injection, use sample contg 5–6 mg Fe and use initial diln directly for detn.

### D. Determination

(**a**) *Ferrous iron*.—Pipet duplicate 10 mL aliquots sample soln (1 as sample blank) and 10 mL working std soln, contg ca 300 μg Fe, into sep. 100 mL vol. flasks. Transfer 10 mL reagent blank soln, prepd by dilg 4 mL HCl to 2 L with $H_2O$, into fourth 100 mL vol. flask. To all solns, add 15 mL buffer soln and ca 20 mL $H_2O$, and mix. To 1 sample soln, std, and reagent blank, add 5 mL dipyridyl soln. To std soln, add 20–25 mg *USP ascorbic acid powder*. Dil. all solns to vol. with $H_2O$, and mix. Let stand 3 hr.

Record *A* of working std soln, sample soln, and sample blank soln (no addn of dipyridyl soln) from 700 to 500 nm against reagent blank soln, setting spectrophtr at 0 *A* at 523 nm against reagent blank soln. Use *A* at max., ca 523 nm.

$$\% \text{ Fe in powder} = [(A - A_0)/A'] \times C \times (200/W)$$

where $A$, $A_0$, and $A'$ refer to sample, sample blank, and std, resp.; $C$ = concn working std soln in μg/mL; and $W$ = mg sample.

$$\text{mg Fe/tablet} = [(A - A_0)/A'] \times C \times (2/W) \times T$$

where $T$ = av. mg/tablet.

$$\text{mg Fe in original aliquot elixir, sirup, or injection taken for assay} = [(A - A_0)/A'] \times C \times 2$$

$$\text{mg Fe compd} = (\text{mg Fe} \times \text{MW})/55.85$$

where MW = molecular wt of Fe compd.

(**b**) *Total iron*.—Proceed as in (**a**), adding ascorbic acid to sample, sample blank, and std solns. Develop color 3 hr at room temp. or 1 hr, without delay, on steam bath as specified in Table **977.30**, heating before dilg solns to vol. Det. *A* as in (**a**), and calc. % total Fe or mg/dose or aliquot.

(**c**) *Ferric iron*.—$Fe^{+3}$ = total Fe − $Fe^{+2}$, all expressed in same units.

Ref.: JAOAC **60,** 1350(1977).

CAS-1332-98-5 (ferric ammonium citrate)
CAS-1301-70-8 (ferric glycerophosphate)
CAS-10058-44-3 (ferric pyrophosphate)
CAS-10045-89-3 (ferrous ammonium sulfate)
CAS-141-01-5 (ferrous fumarate)
CAS-229-29-6 (ferrous gluconate)
CAS-7782-63-0 (ferrous sulfate)
CAS-9004-66-4 (iron dextran)
CAS-1338-16-5 (iron sorbitex)

## 978.27 Ferrous Sulfate in Drugs

### Semiautomated Method

**First Action 1978**
**Final Action 1980**

### A. Principle

$FeSO_4$ in tablets, capsules, or liqs is dissolved in 2% $H_2SO_4$, mixed with *o*-phenanthroline in acetate buffer to form stable $Fe^{+2}$ complex, and *A* is measured in flowcell at 502 nm.

### B. Apparatus

(**a**) *Automatic analyzer*.—With following modules (Technicon Instruments Corp.): Sampler II with 30/hr (3:1) cam; 2 proportioning pumps (I or II); manifold; colorimeter I, with 15 mm tubular flowcell and matched 502 nm filters, or spectrophtr; compatible recorder (*see* Fig. **978.27**), or equiv.

(**b**) *Shaker*.—Wrist action.

(**c**) *Ultrasonic generator*.—150 watt.

### C. Reagents

(Use deaerated, deionized $H_2O$ thruout.)

(**a**) *o-Phenanthroline reagent*.—35 mg/100 mL. Dissolve 350 mg *o*-phenanthroline.$H_2O$ in 500 mL $H_2O$, dil. to 1 L with $H_2O$, and add 10 drops wetting soln, (**c**).

(**b**) *Sulfuric acid*.—2% (w/v). Dil. 11.4 mL $H_2SO_4$ to 1 L with $H_2O$.

(**c**) *Acetate buffer*.—pH 4.6–4.7. Dissolve 136.08 g NaOAc in mixt. of 57 mL HOAc and 500 mL $H_2O$. Dil. to 2 L with $H_2O$, add 20 drops of wetting soln, (**d**), and mix well.

(**d**) *Wetting soln*.—30% soln (w/v) polyoxyethylene lauryl ether in $H_2O$ (Brij-35, Technicon No. T21-0110).

(**e**) *Iron std soln*.—60 mg Fe/100 mL. Accurately weigh ca 60 mg Fe wire and transfer to 100 mL vol. flask. Add 10 mL $H_2O$ and 1.3 mL $H_2SO_4$, heat on steam bath until dissolved, and dil. to vol. with $H_2O$.

### D. Preparation of Sample

Place individual tablet or capsule, liq. aliquot, or weighed composite in accurately measured vol. 2% $H_2SO_4$ to give Fe concn of 0.6 mg/mL. Use ultrasonic generator to disintegrate solid dosage formulations. After complete disintegration, agitate 15 min on mech. shaker. Let settle 2 hr.

### E. Analytical System

Sample is withdrawn and dild with air-segmented stream of $H_2O$ in double mixer, resampled, and mixed with acetate buffer. *o*-Phenanthroline reagent is added and, after mixing in double mixer, soln is debubbled and passed thru 15 mm flowcell, where *A* is measured at 502 nm.

### F. Start-Up and Shut-Down Operations

Place all lines in resp. solns and pump until steady baseline is obtained (ca 15 min). To shut down system, place all lines in $H_2O$ and pump 10 min. Remove lines from $H_2O$ reservoir and pump system dry. If irregular bubble pattern occurs during sample run, pump soln contg 10 drops wetting soln/L $H_2O$ thru system ca 5 min before finally flushing with $H_2O$ for shutdown.

### G. Determination

Fill sample cups in following order: 3 cups std soln, 5 cups sample soln, 1 cup std soln, 5 cups sample soln, etc., ending with 3 cups std soln. (First 2 cups of std soln are used to equilibrate system, but are not included in calcns.) Start sampler. After last cup has been sampled, let system operate until steady baseline is obtained. Draw tangent to initial and final baselines. Subtract baseline to det. net *A* and *A'* for each sample

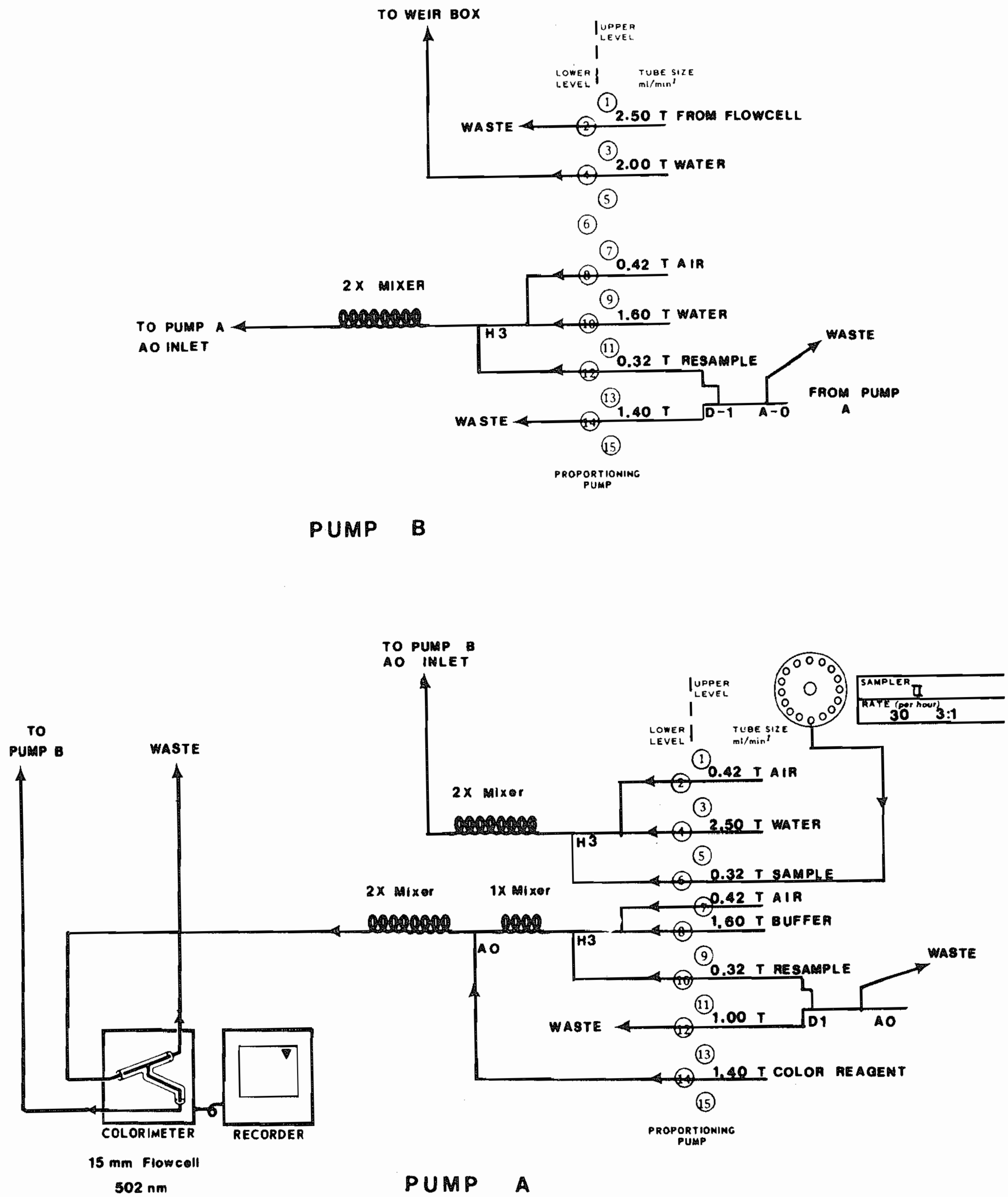



**FIG. 978.27—Flow diagrams for semiautomated analysis for ferrous sulfate**

and std peak, resp. Discard values for first 2 and last std peaks and calc. av. std $A'$.

$$\text{mg Fe in portion taken} = (A/A') \times C \times D$$

where $C$ = concn of std in mg/mL and $D$ = diln factor.

Ref.: JAOAC **61,** 968(1978).

CAS-7720-78-7 (ferrous sulfate)

## 983.27 Mercury in Mercury-Containing Drugs

### Atomic Absorption Spectrophotometric Method

**First Action 1983**
**Final Action 1985**

***A. Principle***

Samples are digested in $H_2O$–HCl–$HNO_3$, and Hg is detd by AAS using air–$C_2H_2$ flame or flameless technic (low Hg levels).

### B. Apparatus

Rinse all glassware before use with $HNO_3$ (1 + 1) followed by $H_2O$. For low Hg levels, decontaminate boiling flasks before use as follows: Add 5 mL $H_2O$–HCl–$HNO_3$ (4 + 3 + 1), place on steam bath 20 min, and rinse with $H_2O$.

(**a**) *Atomic absorption spectrophotometer.*—Equipped with air–$C_2H_2$ flame, or equipped with Hg hollow cathode lamp and gas flow-thru cell (Fig. **986.15B**), 25 (id) × 115 nm with quartz windows cemented in place. Operating conditions: wavelength 253.7 nm, slit width 160 μm, lamp current 3 mA, and sensitivity scale 2.5.

(**b**) *Diaphragm pump.*—Neptune Dyna-Pump, or equiv. Coat diaphragm and internal parts of pump with acrylic-type plastic spray. Use 16 gage Teflon tubing for all connections.

(**c**) *Gas inlet adapter.*—24/40 ₮(Kontes Glass Co. No. K-181000).

(**d**) *Digestion flask.*—250 mL flat-bottom boiling flask with 24/40 ₮ joint.

### C. Reagents

(**a**) *Reducing soln.*—Mix 50 mL $H_2SO_4$ with ca 300 mL $H_2O$. Cool to room temp. and dissolve 15 g NaCl, 15 g hydroxylamine sulfate, and 25 g $SnCl_2$ in soln. Dil. to 500 mL.

(**b**) *Diluting soln.*—To 1 L vol. flask contg 300–500 mL $H_2O$, add 58 mL $HNO_3$ and 67 mL $H_2SO_4$. Dil. to vol. with $H_2O$.

(**c**) *Magnesium perchlorate.*—Drying agent placed in filter flask (Fig. **986.15B**). Replace as needed. (*Caution:* $Mg(ClO_4)_2$ is explosive when in contact with org. substances.)

(**d**) *Mercury stock soln.*—1000 μg/mL. Dissolve 0.1354 g $HgCl_2$ in 100.0 mL $H_2O$.

(**e**) *Digestion soln.*—$H_2O$–HCl–$HNO_3$ (4 + 3 + 1). Prepare immediately before use.

(**f**) *$K_2Cr_2O_7$ soln.*—5%, aq.

### D. Sample Preparation

(**a**) *Ointments.*—Mix sample thoroly and accurately weigh portion contg ca 5 mg Hg into 50 mL beaker. Add 5 mL $H_2O$–HCl–$HNO_3$ (4 + 3 + 1). Cover with watch glass and heat on steam bath 30 min. Cool to room temp., swirl beaker to coagulate fat, and decant soln and three 10 mL $H_2O$ rinses into 50 mL vol. flask. Add 2 mL 5% $K_2Cr_2O_7$, dil. to vol., and mix. Prep. reagent blank, beginning "Add 5 mL $H_2O$-HCl-$HNO_3$ . . .".

(**b**) *Tinctures.*—Pipet aliquot contg ca 5 mg Hg into 50 mL vol. flask. Place on steam bath, and evap. almost to dryness in current of air. Add 5 mL $H_2O$-HCl-$HNO_3$ (4 + 3 + 1), and heat on steam bath 30 min. Blow air into flask 2–3 min, while swirling contents, to expel N oxides. Cool to room temp., add ca 30 mL $H_2O$ and 2 mL $K_2Cr_2O_7$ soln, dil. to vol. with $H_2O$, and mix. Prep. reagent blank, beginning "Add 5 mL $H_2O$–HCl–$HNO_3$ . . .".

(**c**) *Injectables.*—Pipet aliquot contg ca 5 mg Hg into 50 mL vol. flask, add 5 mL $H_2O$–HCl–$HNO_3$ (4 + 3 + 1), and proceed as in (**b**), beginning ". . . and heat on steam bath . . .".

(**d**) *Preservatives and solns* (*or samples contg low levels of Hg*).—Pipet duplicate aliquots contg 0.5 μg Hg (0.1 mL Eppendorf pipet, or equiv., dilg sample if necessary), into sep. decontaminated 250 mL boiling flasks, add 5 mL $H_2O$–HCl–$HNO_3$ (4 + 3 + 1) to each flask, and heat on steam bath 30 min. Cool to room temp., and add 95 mL dilg soln, (**b**). Prep. 2 reagent blanks, beginning "Add 5 mL $H_2O$–HCl–$HNO_3$ . . .".

### E. Standard Preparation

(**a**) *0 and 100 μg Hg/mL std solns* (*for samples a, b, and c*).—Pipet 0 and 5 mL 1000 μg/mL Hg stock soln into 50 mL vol. flasks, add 5 mL $H_2O$–HCl–$HNO_3$ (4 + 3 + 1), ca 30 mL $H_2O$, and 2 mL $K_2Cr_2O_7$ soln, dil. to vol. with $H_2O$, and mix.

(**b**) *0.5 μg Hg std soln* (*for sample d*).—Dilute 1000 μg/mL Hg stock soln to 5 μg/mL. Pipet duplicate 0.1 mL aliquots of this soln (Eppendorf pipet or equiv.) into sep. decontaminated 250 mL boiling flasks, (**d**). Add 5 mL $H_2O$–HCl–$HNO_3$ (4 + 3 + 1), and heat on steam bath 30 min. Cool to room temp., add 95 mL dilg solution, (**b**), and mix.

### F. Determination

(**a**) *Samples a, b, and c.*—Operate atomic absorption spectrophtr with air–$C_2H_2$ flame according to manufacturer's specifications. Zero instrument with 0 μg/mL Hg std soln, and measure $A$ of 100 μg/mL Hg std soln, blank soln, and sample solns, using 4× scale expansion.

(**b**) *Sample d.*—Adjust output of pump to ca 2 L air/min by regulating speed of pump with variable transformer. Connect app. as in Fig. **986.15B**, except for gas inlet adapter. With pump working and spectrophr zeroed, add 20 mL reducing soln to dild aliquot. Immediately connect gas inlet adapter and aerate ca 3 min. (Adjust aeration time to obtain max. $A$.) Record $A$, disconnect pressure on "out" side of pump, and open vent on filter flask to flush system. Analyze in following sequence: reagent blank, 0.5 μg Hg std soln, sample solns, and 0.5 μg/mL std soln.

### G. Calculations

(**a**) Flame AAS:

$$\text{mg Hg/g or mL} = (A - A_B) \times C'/(A' \times W \times 20)$$

(**b**) Flameless AAS:

$$\text{mg Hg/g or mL} = (A - A_B)/(A' - A_B) \times (C'/V) \times F \times 1/1000$$

where $A$, $A_B$, and $A'$ = absorbance of sample, blank, and std solns, resp.; $C'$ = concn of std soln (μg/mL, flame AAS; μg, flameless AAS); $W$ = wt (g) or vol. (mL) of sample taken; $V$ = vol. sample (mL) added to 250 mL boiling flask; $F$ = diln factor if sample was dild.

Ref.: JAOAC **66,** 1203(1983).

CAS-7439-97-6 (mercury)

## 957.19 Mercury in Drugs
### Gravimetric Method
### Final Action 1965

(Applicable to Hg in phenylmercuric chloride, $HgI_2$, nitromersol, HgO ointment, and calomel tablets.)

### A. Reagents

(**a**) *Strychnine sulfate soln.*—Approx. 0.01$M$; 4.3 g/500 mL.

(**b**) *Valser's reagent.*—Dissolve 10 g KI in $H_2O$ and dil. to 100 mL. Sat. with $HgI_2$ (ca 14 g) and filter.

### B. Apparatus

(**a**) *Digestion flask.*—Acetylation or r-b, 100 mL, fitted to $H_2O$-cooled straight-tube condenser with ₮ joint.

(**b**) *Gooch crucibles.*—Fitted with 21 mm filter paper disks, covered with thin layer of asbestos and dried at 105°. Use to filter and weigh ppt of strychnine.HI.$HgI_2$.

### C. Preparation of Samples

(*Caution:* See safety notes on bromine.)

Accurately weigh (avoid use of metal containers) or measure sample contg 20–100 mg Hg (optimal ca 50 mg) and treat as follows:

(**a**) *Solns of organic mercurials.*—Transfer sample to beaker and evap. just to dryness with low heat (60–70°) and air current. Dissolve residue in ca 5 mL 10% NaOH soln and transfer to digestion flask. Rinse beaker with four 3–4 mL portions $H_2O$ and add rinsings to digestion flask. Add excess liq. Br to soln and connect flask to condenser. Boil 4–5 min and add 3 mL HCl thru top of condenser. Continue to heat soln until Br collects in condenser tube. Remove heat and cool until Br returns to soln in digestion flask.

Alternately heat and cool until Br has almost completely dissipated. (After 3 intervals of heating, flow of $H_2O$ thru condenser may be discontinued to aid in removing Br.) Let flask cool, and rinse inside of condenser with ca 5 mL $H_2O$. Disconnect flask and rinse tip of condenser with small stream of $H_2O$ from wash bottle. Filter thru 9 cm paper into 150 mL beaker, and rinse flask and filter with four 5 mL portions $H_2O$.

(**b**) *Ointments.*—Transfer sample to digestion flask and add 5 mL HCl (1 + 3) followed by 5 mL satd Br-$H_2O$. Add small pieces of porcelain, SiC, or few glass beads to prevent bumping. Connect flask to condenser and fit flask over hole cut in asbestos board so that bottom extends just below undersurface of board. Heat over low flame, maintaining slow and continuous boiling ca 10 min, and then cool to room temp. Disconnect flask and decant aq. portion thru 9 cm paper into 150 mL beaker. Take precautions to retain all ointment base in flask. Rinse neck of flask into filter with few drops of $H_2O$ from wash bottle. Add 1 mL HCl (1 + 3), 1 mL satd Br-$H_2O$, and 8 mL $H_2O$ to flask and reflux. Again cool contents of flask and decant aq. phase thru filter.

Repeat refluxing and decanting with two 10 mL portions $H_2O$ and finally rinse condenser tube into flask with ca 5 mL $H_2O$. Disconnect flask, rinse condenser tip, and decant rinsings thru filter. Rinse filter with 2 small portions $H_2O$ from wash bottle.

Test for complete removal of Hg by adding 5 mL $H_2O$ and 2 drops HCl (1 + 3) to digestion flask and refluxing as before. Pass this soln thru original filter into 50 mL beaker. To filtrate add 1 drop 10% KI soln and 1 drop strychnine sulfate soln. No turbidity should be produced. If extn is incomplete, repeat refluxings with $H_2O$ until all Hg is removed. Reserve all test solns showing presence of Hg to add to major portion after pptn of Hg.

(**c**) *Calomel tablets.*—Det. av. wt/tablet. Grind to fine powder and transfer accurately weighed portion to digestion flask. Add 10 mL satd Br-$H_2O$ and 5 mL HCl (1 + 3). Connect flask to reflux condenser and gently boil contents until most of Br vapors collect in condenser. Discontinue heating until Br returns to soln in flask. Repeat alternate heating and cooling until Br vapors are dissipated. Cool flask and contents to room temp. and rinse condenser tube with ca 10 mL $H_2O$. Disconnect flask and rinse condenser tip into flask. Filter soln thru gooch into 150 mL beaker. Rinse flask with three 5 mL portions $H_2O$ and pass rinsings thru crucible, and finally rinse crucible with fine stream of $H_2O$.

(**d**) *Tablets containing purgative drugs.*—If tablets contain purgative drugs, add 10 mL alcohol to weighed sample in flask. Heat on steam bath with gentle agitation until alcohol begins to boil. Remove flask, cool under tap, and filter supernate thru gooch fitted with asbestos mat. Retain as much of insol. residue in flask as possible. Rinse flask and contents with three 10 mL portions alcohol and two 5 mL portions $H_2O$, and decant thru crucible as above. Remove asbestos mat with fine wire or needle and transfer to flask. Rinse crucible with 10 mL satd Br-$H_2O$ and 5 mL HCl (1 + 3), and add rinsings to flask. Connect flask to condenser, and treat as in (**c**).

### D. Determination

Add 10 mL 10% KI soln to filtrate, and if necessary, evap. on steam bath under air current to ca 50 mL. If soln has not previously been acidified, add 3 mL HCl (1 + 3). Add 1% $NaHSO_3$ soln until I color is discharged, and keep soln free from I color by addn of $NaHSO_3$ soln until final filtration is made. Add strychnine sulfate soln slowly from buret or pipet until ppt coagulates and settles rapidly. (Strychnine sulfate soln may be added as rapidly as it will flow from buret if theoretical amt is used, based on 1 mL soln for each 4 mg Hg expected to be present.) Avoid undue excess of strychnine because of slight solubility of its hydriodide.

Let ppt settle and test for complete pptn by adding 2–3 drops strychnine sulfate soln to clear supernate. If pptn is incomplete, indicated by cloudiness around the drops, add strychnine sulfate soln in 1 mL increments until pptn is complete. Let ppt remain in beaker with occasional stirring 0.5–1 hr.

Decant supernate thru weighed gooch, **957.19B(b)**. Wash ppt into crucible with fine stream of $H_2O$. Completely transfer ppt to crucible, and wash residue and crucible with three 5 mL portions $H_2O$. Scrub beaker thoroly with policeman. Transfer crucible and holder to another small suction flask and wash residue with 2–3 mL $H_2O$. Test filtrate for complete removal of strychnine by addn of Valser's reagent. If necessary, continue washing ppt with small portions $H_2O$ until last washings give no more than faint opalescence upon addn of Valser's reagent. Always test main filtrate by addn of ca 1 mL strychnine sulfate soln to assure complete pptn of Hg. If pptn was incomplete, repeat detn. Dry crucible 1 hr at 105°, cool in desiccator, and weigh. Calc. % Hg compd in sample on basis of MW of 916.74 for ppt of strychnine.HI.$HgI_2$.

Ref.: JAOAC **40**, 819(1957).

CAS-7546-30-7 (calomel)
CAS-7774-29-0 (mercuric iodide)
CAS-7439-97-6 (mercury)
CAS-133-58-4 (nitromersol)
CAS-100-56-1 (phenylmercuric chloride)

## 934.09 Merbromin in Drugs

### A. Tests for Purity —Procedure

(**a**) Acidify portion of merbromin soln with 10% $H_2SO_4$ and filter off ppt. Filtrate is only slightly yellow.

(**b**) Pass $H_2S$ (*Caution:* See safety notes on hydrogen sulfide.) into portion of filtrate. No ppt or coloring occurs.

(**c**) Add few mL 10% $HNO_3$ to another portion of filtrate and add $AgNO_3$ soln. No ppt forms.

### B. Total Solids in Solution —Final Action

Pipet 10 mL merbromin soln into tared, extra-wide-form weighing bottle and evap. to dryness on steam bath. Let dry overnight in open bottle in desiccator contg $H_2SO_4$. Weigh.

### C. Determination of Mercury —Final Action

(*Caution:* See safety notes on wet oxidation, sulfuric acid, flammable solvents, toxic solvents, carbon disulfide, carbon tetrachloride, hydrogen sulfide, permanganates, and asbestos.)

Pipet 10 mL ca 2% merbromin soln into 500 mL tall beaker and evap. to dryness on steam bath (or accurately weigh ca 0.2 g powder). Dissolve residue in 4 mL $H_2O$ and slowly add, with const mixing, 10 mL $H_2SO_4$. Incline beaker and cautiously add small portions finely powd $KMnO_4$, mixing after each addn, until deep purple color shows that considerable excess has been added. Let stand 30 min, mixing occasionally. Mixt. should still be purple.

Add 100 mL $H_2O$ and mix thoroly. Add small portions finely powd oxalic acid, mixing after each addn, until soln is clear. Filter thru small filter into 400 mL beaker, wash original beaker and filter until filtrate measures ca 200 mL, and pass *$H_2S$* thru soln 20 min. Warm on steam bath until ppt of HgS settles quickly after stirring, and again pass $H_2S$ thru warm soln 5 min. Immediately filter soln into weighed gooch; thoroly wash ppt on filter with $H_2O$, 3 times with alcohol, and then with 4 or 5 portions $CCl_4$ or $CS_2$, letting liq. run thru crucible without suction; finally wash with ether. Dry ppt to const wt at 100° and weigh as HgS. HgS $\times$ 0.8622 = Hg.

Qual. test dried ppt for Hg and other heavy metals. If slow filtration occurs during washing with $H_2O$, let ppt drain, and wash once with alcohol; then continue as directed.

Refs.: JAOAC **17,** 75, 432(1934).

CAS-129-16-8 (merbromin)

## 931.12 Calomel in Ointments

### Titrimetric Method

### Final Action

Accurately weigh ca 1 g ointment, transfer to 250 mL g-s erlenmeyer, and treat with ca 50 mL $CHCl_3$. When base is dissolved, decant thru dry, closely packed asbestos mat in Caldwell crucible (*Caution:* See safety notes on asbestos.), using light suction. Wash flask and contents several times with 20–30 mL portions $CHCl_3$, decanting thru crucible. Let any residual $CHCl_3$ in flask evap., and transfer asbestos mat and contents to flask, wiping sides of crucible and mouth of flask with damp piece of filter paper and adding it to flask. Add 2.5 g KI and 30 mL std 0.1*N* I, **939.13** (stdzd against $Na_2S_2O_3$), stopper, and mix well. Let stand ca 1.5 hr or until soln of calomel is complete, agitating frequently and fairly vigorously. Titr. with 0.1*N* $Na_2S_2O_3$, **942.27B**, adding 1 or 2 mL excess and using starch indicator, (mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste, add ca 200 mL boiling $H_2O$ with stirring, and discontinue heating; add ca 1 to 6 Hg, shake, and let soln stand over Hg). When all traces of I disappear, back-titr. with std I soln to blue color. 1 mL 0.1*N* I = 0.02360 g $Hg_2Cl_2$.

Ref.: JAOAC **14,** 312(1931).

CAS-7546-30-7 (calomel)

## 927.11 Calomel in Tablets

### Titrimetric Method

### Final Action

Count and weigh representative number of tablets. Powder tablets and accurately weigh well mixed sample contg 0.19–0.26 g (3–4 grains) $Hg_2Cl_2$. Transfer to 200 mL g-s erlenmeyer, add ca 50 mL $H_2O$, acidify with HOAc, and after sol. fillers dissolve, decant with aid of suction thru tightly packed asbestos mat placed on plate of Caldwell crucible (*Caution:* see safety notes on asbestos.). Wash once with $H_2O$ by decantation and then successively with alcohol and ether. Transfer removable plate holding mat and insol. material to original flask, washing into flask any insol. material adhering to sides of crucible. Add 2.5 g KI, 10 mL $H_2O$, and then 30 mL std 0.1*N* I soln, **939.13A**. Complete detn as in **931.12**.

Refs.: JAOAC **10,** 367(1927); **11,** 343(1928); **12,** 280(1929).

CAS-7546-30-7 (calomel)

## 929.11 Mercurous Iodide in Tablets

### Titrimetric Method

### Final Action

Accurately weigh well mixed powd sample contg 0.19–0.26 g (3–4 grains) $Hg_2I_2$. Transfer sample to 200 mL g-s flask, and proceed as in **927.11**, omitting addn of $H_2O$ after the KI. 1 mL 0.1*N* I = 0.03275 g $Hg_2I_2$.

*Note:* Some com. tablets are difficult to filter thru asbestos mat without loss of $Hg_2I_2$. Placing few drops of alumina cream on mat before filtration is started (wash free from $NH_3$), satisfactorily prevents loss, tho it retards filtration. *Alumina cream.*—Prep. cold satd soln of alum in $H_2O$. Add $NH_4OH$ with constant stirring until soln is alk. to litmus, let ppt settle, and wash by decantation with $H_2O$ until wash $H_2O$ gives only slight test for sulfates with $BaCl_2$ soln. Pour off excess $H_2O$ and store residual cream in g-s bottle.

Ref.: JAOAC **12,** 280(1929).

CAS-7783-30-4 (mercurous iodide)

## 935.67 Mercury in Mercurial Ointments

### Titrimetric Method

### Final Action

(*Caution:* See safety notes on distillation and nitric acid.)

After mixing ointment thoroly with glass rod, avoiding contact with metals, weigh 1 g sample into erlenmeyer. Add 20 mL $H_2O$ and 20 mL $HNO_3$, and heat gently over small flame until red fumes cease to evolve. Cool, and decant aq. soln from ointment base into separator. Wash ointment base with 50 mL boiling $H_2O$, cool, and decant into separator. Repeat washing until all Hg is removed.

Shake combined solns in separator with 50 mL ether. Transfer aq. soln to erlenmeyer. Wash ether soln with three 10 mL portions $H_2O$ until Hg is removed, adding washings to flask. Add 3 mL $FeNH_4(SO_4)_2$ soln, **931.11A(b)**, and titr. with 0.1*N* $NH_4SCN$. 1 mL 0.1*N* $NH_4SCN$ = 0.01003 g Hg.

Ref.: JAOAC **18,** 520(1935).

CAS-7439-97-6 (mercury)

## 957.20★ Mercury in Ointments of Mercuric Nitrate

### Titrimetric Method

### Final Action
### Surplus 1977

*See* **36.090**, 13th ed.

## 955.51★ Nitrites in Tablets

### Hydrazine Method

**Final Action**
**Surplus 1975**

(Applicable in presence or absence of nitrates or chlorides)

*See* **36.080–36.081**, 12th ed.

## 925.58 Silver Protein in Drugs

### Titrimetric Method

**Final Action**

### A. Total Silver

(*Caution:* See safety notes on distillation and nitric acid.)

Place 1 g sample, accurately weighed, in 500 mL Kjeldahl flask; add 15 mL $H_2SO_4$ and then 10 mL $HNO_3$. Place on steam bath few min, with occasional rotation, to ensure homogeneous mixt., and boil to white fumes. Add more $HNO_3$, boil again to clear colorless soln, and cool. Add 100 mL $H_2O$ and boil until free of N oxides. Cool, dil. to 300 mL, add 5 mL $HNO_3$ and 5 mL $FeNH_4(SO_4)_2$ soln, **931.11A(b)**, and titr. with 0.1*N* $NH_4SCN$. 1 mL 0.1*N* $NH_4SCN$ = 0.01079 g Ag.

### B. Ionizable Silver Compounds

Weigh strip of com. dialyzing tubing 55 mm wide and ca 30 cm long, wet with $H_2O$ until uniformly pliable, shake free of adhering $H_2O$, and partially dry by rolling in clean paper towel. Reweigh while still moist and place in 250 mL beaker. (Sheets of dialyzing parchment paper may be used in place of tubing. Over one end of glass tube 10 cm long and ca 2.5 cm od, fold and secure with rubber band sq piece of parchment paper in form of sack large enough to hold sample soln. Dialyzing material should be kept in humid container to prevent breaking when handled.)

Weigh 1 g sample, dissolve in 15 mL $H_2O$, and transfer to dialyzing tube. Calc., and add enough $H_2O$ to beaker to make 100 mL (this ensures 20 mL in dialyzing tube and 80 mL in beaker). Adjust tubing to form "U" in beaker, cover with watch glass, and keep cool and in dark 24 hr.

(**a**) *Qualitative test.*—Test few mL clear, colorless soln from beaker for Ag ions by addn of few drops HCl (1 + 3) and trace of $HNO_3$.

(**b**) *Determination.*—If Ag ions are present, remove 50 mL clear, colorless soln (representing 0.5 g sample) from beaker, dil. to 100 mL, and add 2 mL $FeNH_4(SO_4)_2$ soln, **931.11A(b)**, and 2 mL colorless $HNO_3$. Titr. with 0.01*N* $NH_4SCN$ and calc. to % by wt ionizable Ag. 1 mL 0.01*N* $NH_4SCN$ = 0.001079 g Ag.

Refs.: JAOAC **8,** 551(1925); **9,** 312(1926); **10,** 374(1927).

CAS-7440-22-4 (silver)

## ANTIHISTAMINES

## 974.39★ Selected Drug Combinations

### Ion Exchange Chromatography

**First Action 1974**
**Final Action 1978**
**Surplus 1989**

(Applicable to 14 antihistamines, antitussive agents, expectorants, and sedatives, alone or combined)

*See* **36.108–36.114**, 14th ed.

## 945.69 Meperidine in Drugs

**Final Action**

### A. Distillation Method★ —Surplus 1970

*See* **36.093–36.095**, 11th ed.

### B. Extraction Method

Accurately weigh portion of powd sample contg ca 0.1 g meperidine, and macerate 2 hr with 10 mL $H_2O$ and 1 mL 1*N* $H_2SO_4$. Decant liq. thru small filter into separator. Macerate residue 20 min with 5 mL $H_2O$, filter thru same filter, and wash residue and filter with small portions of $H_2O$.

Sat. soln with NaCl; then add 5 mL 1*N* NaOH and ext with 25 mL and six 20 mL portions ether as in **960.53A**. Wash combined ether exts with two 5 mL portions $H_2O$; ext this $H_2O$ with 10 mL ether and add this ether to main ether ext. Ext ether soln first with 20.0 mL 0.02*N* $H_2SO_4$, and then successively with 10 and 5 mL $H_2O$. Combine $H_2SO_4$ and $H_2O$ exts in beaker and warm on $H_2O$ bath until no ether odor is detected. Cool soln, and titr. excess acid with 0.02*N* NaOH, using Me red. 1 mL 0.02*N* $H_2SO_4$ = 0.005676 g meperidine.HCl, $C_{15}H_{21}O_2N.HCl$.

Refs.: JAOAC **28,** 711(1945); **31,** 540(1948).

CAS-50-13-5 (meperidine hydrochloride)

## 978.28 Mephentermine Sulfate in Drugs

### Spectrophotometric Method

**First Action 1978**
**Final Action 1981**

### A. Principle

Mephentermine sulfate is sepd on ion exchange column, eluted with alcoholic HCl, and measured by UV spectrophotometry.

### B. Apparatus

(**a**) *Chromatographic tube.*—Glass, 150 × 12 (id) mm, fitted with replaceable coarse fritted glass disk, Teflon stopcock, and Buna-N "O" ring seal (Kontes Glass Co., No. K-422280, or equiv.).

(**b**) *Ion exchange resin.*—*See* **974.39C(d)**.

### C. Reagents

(**a**) *Alcoholic hydrochloric acid.*—(*1*) 1.5*N*.—Mix 1 part HCl with 7 parts alcohol-$H_2O$ (1 + 1). (*2*) 6*N*.—Mix 1 part HCl with 1 part alcohol-$H_2O$ (1 + 1).

(**b**) *Mephentermine sulfate std soln.*—0.5 mg/mL. Accurately weigh ca 25 mg mephentermine sulfate, previously dried 1 hr at 105°, into 50 mL vol. flask. Dissolve and dil. to vol. with 1.5*N* alcoholic HCl, (**a**)(*1*), and mix.

### D. Preparation of Column

Prep. slurry of 2 g resin, (**b**), with 20–25 mL alcohol-$H_2O$ (1 + 1), and transfer to tube, (**a**), with stopcock closed and plug of glass wool under fritted disk. Let resin settle by gravity; then top with small pledget of glass wool. (Column need not be tamped.) Drain solv., wash column with three 10–20 mL portions alcohol-$H_2O$ (1 + 1) followed by 20 mL $H_2O$, and discard all washings. Prevent column from drying out before use by maintaining head of 2–3 mL alcohol-$H_2O$ (1 + 1) or $H_2O$.

When using column for first time and upon completion of sepns, wash thoroly with 15–20 mL 6*N* alcoholic HCl, (**a**)(*2*), to recondition resin. With stopcock closed, stir resin to obtain slurry, let settle, and drain. Repeat twice. Finally, wash resin with alcohol-$H_2O$ (1 + 1) until excess acid is removed and then with three 10–20 mL portions $H_2O$. Store under $H_2O$ when not in use.

Perform blank detn on new column, beginning in **978.28E** with "Elute mephentermine with three 5 mL portions . . ." If UV spectrum has $A > 0.05$ at 258 nm, recondition column again or prep. new column, using different bottle of resin.

### E. Preparation of Sample

(**a**) *Tablets*.—Grind tablets to pass No. 80 sieve. Accurately weigh amt powder contg ca 25 mg mephentermine sulfate into 50 mL beaker. Add 10 mL $H_2O$, heat on steam bath 2–3 min, cool, and filter thru 9 cm Whatman No. 541 paper into prepd column, **978.28D**. Column flow rate should be ca 2–3 drops/sec. Thoroly wash filter paper with three 5–10 mL portions $H_2O$, letting each portion pass into column before addn of next. Wash column with 25 mL alcohol-$H_2O$ (1 + 1). Discard all washings. Elute mephentermine with three 5 mL portions 1.5*N* alcoholic HCl, then with 30 mL 1.5*N* alcoholic HCl. Let each portion just enter column before adding next, introducing eluant down sides of column so as not to disturb resin. Collect eluate in 50 mL vol. flask, rinse liq. from tip of column into flask, and dil. to vol.

(**b**) *Elixir*.—Add sample vol. contg ca 25 mg mephentermine sulfate to prepd column, **978.28D**, and proceed as in (**a**), beginning "Elute mephentermine with three 5 mL portions . . ." If sample contains parabens, wash column with 25 mL alcohol before eluting.

### F. Determination

Scan sample and std solns from 220–300 nm against 1.5*N* alcohol-HCl as ref. blank. Draw baseline connecting min. *A* at ca 254 and 262 nm, and use max. *A* at 258 nm to calc. potency of mephentermine sulfate:

$$\text{mg Mephentermine sulfate/tablet} = (A/A') \times (X/\text{g sample}) \times W$$

where $A$ and $A'$ refer to sample and std, resp.; $X$ = av. g/tablet; and $W$ = mg std in 50.0 mL 1.5*N* alcoholic HCl; or

$$\text{mg Mephentermine sulfate/mL} = (A/A') \times (F/\text{mL sample}) \times W$$

where $F$ = diln factor.

Ref.: JAOAC **61,** 60(1978).

CAS-1212-72-2 (mephentermine sulfate)

## 959.15 Methapyrilene in Expectorants
**Spectrophotometric Method**
**Final Action 1965**

(*See also* **974.39**.)

### A. Reagent

*Methapyrilene hydrochloride std soln*.—0.015 mg methapyrilene.HCl/mL. Transfer 60 mg methapyrilene.HCl, accurately weighed, to 200 mL vol. flask. Dissolve in ca 0.1*N* $H_2SO_4$ and dil. to vol. with ca 0.1*N* $H_2SO_4$. Transfer 5 mL aliquot to 100 mL vol. flask and dil. to vol. with ca 0.1*N* $H_2SO_4$.

### B. Determination

Pipet 10 mL sample into separator, make alk. with $NH_4OH$, and ext with four 20 mL portions $CHCl_3$. Combine $CHCl_3$ exts in 100 mL vol. flask and dil. to vol. with $CHCl_3$. Transfer aliquot contg 1–3 mg methapyrilene to small beaker and evap. just to dryness on steam bath with air current. Dissolve residue in ca 0.1*N* $H_2SO_4$, transfer to 100 mL vol. flask, and dil. to vol. with ca 0.1*N* $H_2SO_4$. Det. *A* of this soln and *A'* of std against ca 0.1*N* $H_2SO_4$ blank at 315 nm.

$$\text{mg Methapyrilene.HCl/100 mL sample} = (A \times 1.5 \times 100 \times 10)/(A' \times \text{vol. aliquot}).$$

Ref.: JAOAC **42,** 466(1959).

CAS-135-23-9 (methapyrilene hydrochloride)

## 959.16 Pyrilamine in Cough Sirup
**Spectrophotometric Method**
**Final Action 1965**

(*See also* **974.39**.)

### A. Reagent

*Pyrilamine std soln*.—0.015 mg pyrilamine maleate/mL. Transfer 150 mg pyrilamine maleate to 500 mL vol. flask, dissolve in ca 0.1*N* $H_2SO_4$, and dil. to vol. with ca 0.1*N* $H_2SO_4$. Transfer 5 mL aliquot to 100 mL vol. flask and dil. to vol. with ca 0.1*N* $H_2SO_4$.

### B. Determination

Proceed as for detn of methapyrilene, **959.15B**, but measure *A* at 314 nm.

$$\text{mg Pyrilamine maleate/100 mL sample} = (A \times 100 \times 1.5 \times 10)/)A' \times \text{mL aliquot})$$

where $A$ and $A'$ refer to sample and std, resp.

Ref.: JAOAC **42,** 466(1959).

CAS-91-84-9 (pyrilamine)
CAS-59-33-6 (pyrilamine maleate)

## 981.24 Chlorpheniramine Maleate in Drug Tablets
**Semiautomated Method**
**First Action 1981**
**Final Action 1982**

### A. Principle

Method is automated version of general USP XIX assay for salts of org. nitrogenous bases. Sample is made basic, extd with isooctane, resampled, re-extd with acid, and measured at 265 nm.

### B. Apparatus

(**a**) *Automatic analyzer*.—AutoAnalyzer with following modules: Sampler II with cam or timing clock set at 30/hr with sample to wash ratio of 2:1, proportioning pump III, and manifold; *see* Figure **981.24** (Technicon Instrument Co.).

(**b**) *Spectrophotometer*.—Zeiss spectrophotometer, Model PM2 DL (Carl Zeiss West Germany, PO Box 1369/1380, D-7082, Oberkochen, West Germany), or equiv., fitted with Helma 10 mm, 18-180 μL flowcell (Helma Cells, PO Box 544, Jamaica, NY 11424), or equiv.

(**c**) *Recorder*.—Texas Instrument Servo/Riter II (Texas Instruments, Inc., 24500 Hwy 290, PO Box 144, Sypress, TX 77429), or equiv.

(**d**) *Shaker*.—Model BT, wrist action (Burrell Corp.), or equiv.

(**e**) *Ultrasonic generator*.—Model II, 150 watt (Heat Systems-Ultrasonic Inc., 38 E Mall, Plainview, NY 11803), or equiv.

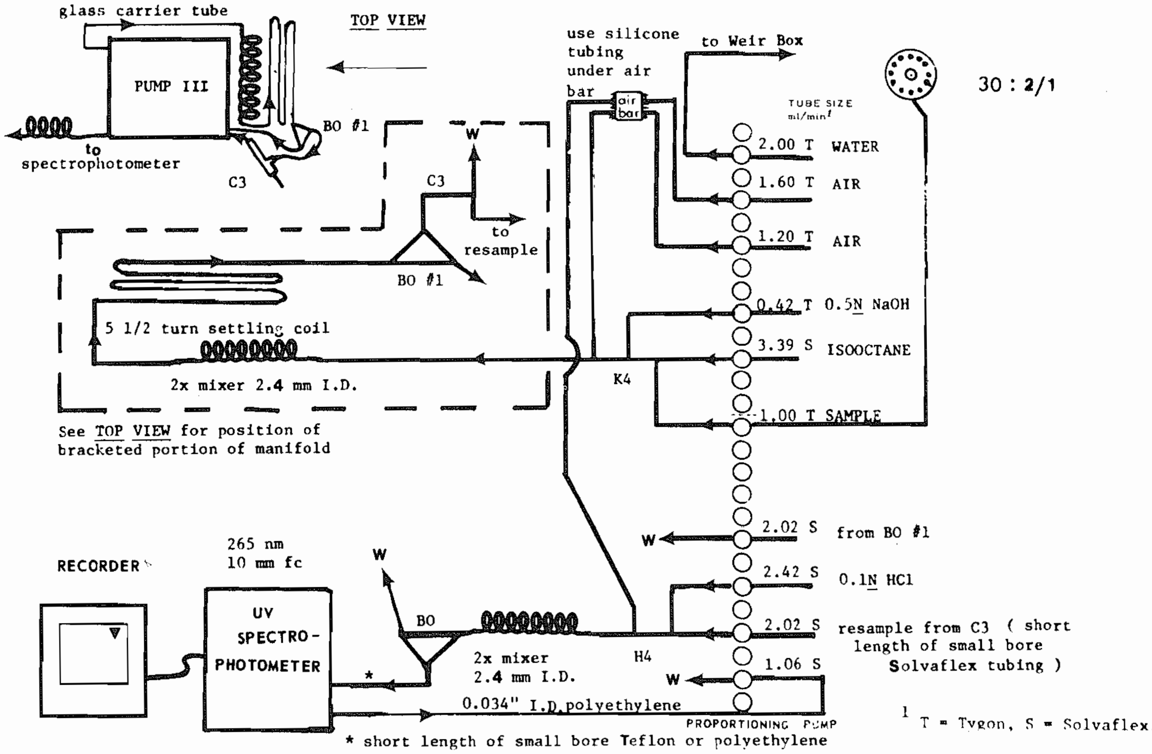



**FIG. 981.24—Schematic of semiautomated analysis of chlorpheniramine tablets**

### C. Reagents

(**a**) *Hydrochloric acid soln.*—0.1*N*. Dil. 34 mL HCl to 4 L with $H_2O$.

(**b**) *Sodium hydroxide soln.*—0.5*N*. Dil. 55 mL 50% w/w NaOH to 2 L with $H_2O$.

(**c**) *Chlorpheniramine maleate std soln.*—0.08 mg/mL. Accurately weigh ca 80 mg USP Ref. Std Chlorpheniramine Maleate and transfer to 100 mL vol. flask. Dissolve and dil. to vol. with 0.1*N* HCl. Transfer 10.0 mL aliquot to 100 mL vol. flask and dil. to vol. with 0.1*N* HCl. Soln is stable ≥2 weeks.

### D. Preparation of Sample

Disintegrate individual tablets or disperse weighed composite in accurately measured vol. 0.1*N* HCl to give concn of 0.08 mg/mL based on amt of drug claimed. Use ultrasonic generator or shake mech. ca 30 min to assure tablet disintegration. Let particulates settle completely, overnight if necessary, before sampling. Use clear portion of sample soln for analysis.

### E. Analytical System

Isooctane and sample soln are brought together on manifold, 0.5*N* NaOH is added, and stream is segmented with air. Phases are passed thru mixing coil and org. phase is sepd, resampled, and brought together with 0.1*N* HCl. Stream is segmented with air and passed thru mixing coil, and aq. phase is drawn thru flowcell where *A* is measured at 265 nm.

### F. Start Up

Place all *aq.* reagent lines in respective reagents and pump 5 min; then pump isooctane until steady baseline is obtained.

### G. Shut Down

Remove isooctane line first; 5 min later, remove other lines. Pump system dry.

### H. Determination

Fill sample cups in following order: 3 cups std soln, 5 cups sample soln, 1 cup std soln, etc. Place 2 cups std soln at end of each run. (Extra cups of std solns at start and end of sampling pattern will eliminate carryover effect in transitions from wash soln to std soln and vice versa. Two extra cups at start and 1 extra cup at end should suffice, but det. number needed for equilibrium by experiment. System should give uniform response for ≥2 std cups before sampling pattern is started.) Start Sampler II. After last cup has been sampled, let system operate until steady baseline is obtained. Draw tangent line to initial and final baselines. Subtract baseline to determine net *A* and *A'* for each sample and std peak, resp. Discard values for first 2 and last std peak. All std peaks can be averaged or stds flanking sample pattern can be used.

$$\text{mg chlorpheniramine maleate taken} = (A/A') \times C \times D$$

where $C$ = concn of std in mg/mL, and $D$ = diln factor for sample. If ground composite is used, av. tablet wt and sample wt must be used in formula.

If peak shape or steady state slope creep upward excessively, check for isooctane in flowcell. Remove all lines and pump dry. Pump alcohol thru isooctane line 5 min; then pump dry. Start up system as above.

Ref.: JAOAC **62,** 1197(1979).

CAS-113-92-8 (chlorpheniramine maleate)

## 958.10 Antihistamines in Drugs in Presence of Aspirin, Phenacetin, and Caffeine

### Spectrophotometric Method

### Final Action 1965

(Applicable to thonzylamine.HCl, pheniramine maleate, and chlorpheniramine maleate in combination with APC)

#### *A. Preparation of Standard Solutions*

Prep. sep. std solns of thonzylamine.HCl, pheniramine maleate, and chlorpheniramine maleate by dissolving 250 mg antihistamine salt, accurately weighed, in 50.0 mL $H_2O$. Pipet 5 mL of each soln into sep. 100 mL vol. flasks and dil. to vol. with ca 0.1*N* $H_2SO_4$. Transfer 10 mL of each acid soln to sep. 100 mL vol. flasks and dil. to vol. with ca 0.1*N* $H_2SO_4$. (Concn = 2.5 mg/100 mL.) Det. $A'$ of thonzylamine.HCl at 314 nm, pheniramine maleate at 265 nm, and chlorpheniramine maleate at 264 nm.

#### *B. Determination*

Place accurately weighed powd sample contg ca 10 mg antihistamine in 125 mL separator. Add 15 mL $H_2O$ and ca 0.5 mL $H_2SO_4$ (1 + 1). Ext with $CHCl_3$, using 30, 20, 20, and 20 mL portions. Re-ext by passing $CHCl_3$ exts successively thru 2 separators, each contg 10 mL ca 0.1*N* $H_2SO_4$, shaking vigorously each time. Discard $CHCl_3$ and combine aq. solns.

Make combined solns alk. with 10% NaOH and ext with 30, 20, 20, and 20 mL portions $CHCl_3$. Again pass $CHCl_3$ exts successively thru 2 separators, each contg 20 mL ca 0.1*N* $H_2SO_4$, shaking vigorously each time. Discard $CHCl_3$, combine acid aq. solns, and dil. to vol. with ca 0.1*N* $H_2SO_4$ in 100 mL vol. flask. Transfer 25 mL aliquot to 100 mL vol. flask and dil. to vol. with ca 0.1*N* $H_2SO_4$. Det. *A* at wavelength of max. absorption against ca 0.1*N* acid as ref.

% Thonzylamine.HCl

$$= (A \times 2.5 \times 4 \times 100)/(A' \times \text{mg sample})$$

% Pheniramine or chlorpheniramine maleate

$$= 1.018 \times (A \times 2.5 \times 4 \times 100)/(A' \times \text{mg sample})$$

where 1.018 corrects for absorbance of maleate moiety of std.

Ref.: JAOAC **41,** 495(1958).

CAS-113-92-8 (chlorpheniramine maleate)
CAS-132-20-7 (pheniramine maleate)
CAS-63-56-9 (thonzylamine hydrochloride)

## Pseudoephedrine HCl and Triprolidine HCl or Chlorpheniramine Maleate in Drug Combinations

*See* **981.26.**

# ALKANOLAMINES

## 956.08 Norepinephrine in Drug Preparations of Epinephrine

### Spectrophotometric Method

### Final Action

#### *A. Apparatus*

(**a**) *Chromatographic tubes*.—Fuse 6 cm length of 5–6 mm tubing to piece of 25 mm tubing ca 25 cm long (25 × 200 mm test tube may be used). Constrict stem slightly ca 2 cm below seal. Com. tubes with dimensions ±10% are satisfactory. Pack wad of Pyrex glass wool in base as support.

(**b**) *Tamping rod*.—Flatten end of glass rod to circular head with clearance of ca 1 mm in tube (**a**). Or use disk of stainless steel, Al, etc., of diam. ca 1 mm less than id of column, (**a**), attached to 30–45 cm (12–18") rod.

(**c**) *Hypodermic syringe*.—1 mL without needle, graduated in 0.01 mL.

#### *B. Reagents*

(**a**) *Diatomaceous earth*.—*See* **960.53B**.

(**b**) *Glass wool*.—Pyrex No. 3950.

(**c**) *Benzene*.—Distil reagent grade benzene in all-glass app. Shake distillate with $H_2O$ 2–3 min and filter benzene layer thru paper. Use this $H_2O$-satd solv. unless dry benzene is specified. (*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, and benzene.)

(**d**) *Concentrated phosphate buffer*.—pH 6. Dil. 50.0 mL 0.2*M* $KH_2PO_4$ soln, **941.17B(b)**, and 5.64 mL 0.2*M* NaOH, **941.17B(d)**, to 100 mL with $H_2O$.

(**e**) *Iodine-potassium iodide soln*.—Dissolve 2 g I and 6 g KI in $H_2O$, and dil. to 100 mL.

(**f**) *Norepinephrine std soln*.—0.100 mg norepinephrine base/mL. Dissolve 19.9 mg *l*-norepinephrine (levarterenol) bitartrate.$H_2O$ in exactly 100 mL $H_2O$. Discard after 8 hr.

#### *C. Preparation of Sample*

(**a**) *Aqueous solns of epinephrine.HCl containing bisulfite and chlorobutanol*.—If soln is 0.1% with respect to "total epinephrine" (epinephrine + norepinephrine), pipet 30 mL sample into 125 mL separator provided with tightly fitting stopper and stopcock. If soln is more concd, use sample contg 30 mg "total epinephrine," and dil. to 30 mL with $H_2O$.

(**b**) *Suspensions of epinephrine in oil*.—Mix suspension by gentle swirling and agitation; add to separator accurately measured vol. contg ca 30 mg epinephrine and 25 mL pet ether, and swirl until oily base dissolves. Add 10 mL 0.05*N* $H_2SO_4$ and ext epinephrine by shaking 1 min. Drain aq. layer into 125 mL separator, and wash pet ether layer with two 10 mL portions $H_2O$. Add washes to acid ext, wash combined aq. layers with two 10 mL portions $CCl_4$, and discard $CCl_4$. Rinse stopper and mouth of separator with few drops $H_2O$ and let rinsings drain into separator. Proceed as in **956.08D**, beginning "Add 2.10 g $NaHCO_3$ . . ."

(**c**) *Ointments of epinephrine bitartrate (petrolatum base)*.—Transfer to separator accurately weighed sample contg ca 60 mg epinephrine bitartrate. Add 25 mL benzene and swirl until ointment base dissolves. Proceed as in (**b**), beginning "Add 10 mL 0.05*N* $H_2SO_4$ . . ." except if bisulfite is present, it must be removed with I, as below, before proceeding with acetylation.

#### *D. Acetylation*

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Add 25 mL $CCl_4$ and shake vigorously to ext chlorobutanol. After layers sep. completely, drain and discard solv., and repeat extn with two 25 mL portions $CCl_4$. After each extn, drain as much solv. as possible. Rinse stopper and mouth of separator with few drops of $H_2O$, and let rinsings drain into separator. Add 4 drops starch indicator, **949.15A(b)**; then, while swirling, destroy $NaHSO_3$ by adding I-KI soln, (**e**), *dropwise* until soln remains blue. Immediately discharge blue color by adding 0.1*N* $Na_2S_2O_3$ dropwise. Add 2.10 g $NaHCO_3$ (prevent it from contacting wet mouth of separator) and swirl few sec to dissolve most of $NaHCO_3$. Immediately, using hypodermic syringe, rapidly inject into separator exactly 1 mL $Ac_2O$ (prevent reagent from contacting mouth of funnel). Stopper separator at once and shake vigorously until evolution of $CO_2$ stops (ca 7–8 min). Release pressure as necessary by momentarily inverting separator and cautiously opening stopcock.

Let mixt. stand 5 min; then ext with six 30 mL portions $CHCl_3$. Filter each ext thru $CHCl_3$-washed compact pledget of absorbent cotton into beaker, and evap. combined exts to small vol. or to dryness on steam bath under air current. Quant. transfer residue with small portions $CHCl_3$ to tared 50 mL beaker and continue evapn until solv. is removed. Dry 30 min at 105°, let cool in desiccator, and weigh. Wt mixed amorphous triacetyl derivatives of epinephrine and norepinephrine × 0.5923 = $E$ = "total epinephrine."

### E. Chromatographic Separation of Acetylation Product

(*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, benzene, and chloroform.)

Place wad of glass wool in chromatgc tube and compress it tightly at juncture of tube and stem, using packing rod.

Place 10 g diat. earth and ca 175 mL benzene in 250 mL beaker. While stirring vigorously and continuously, add 7.0 mL $H_2O$, dropwise, to produce uniform solid phase. Transfer to chromatgc tube ca $^1/_{10}$ of the solid, under benzene, and compress it firmly and evenly with packing rod. While keeping column of benzene above solid in tube, add remainder of solid in beaker in ca 5 equal portions and compress each portion firmly and evenly before adding next. Properly prepd column is ca 65 mm high and permits flow of ca 2–4 mL benzene/min under head of 8 cm solv. With wad of absorbent cotton affixed to stiff wire, remove any solid adhering to tube above column. Keep layer of benzene above column until used.

To beaker contg mixt. of triacetyl derivatives, add exactly 6 mL *dry* benzene. Warm gently and dissolve residue completely by stirring and swirling. Cover beaker with watch glass to retard evapn, and cool to room temp.

Remove supernate benzene from tube by careful aspiration, pipet onto top of column accurately measured aliquot of soln of derivative equiv. to 20–25 mg total epinephrine, and immediately place graduate under tube. As soon as last of benzene soln is absorbed by column, rinse down wall of tube with three 2 mL portions benzene, delivered conveniently from pipet. Let each rinse be completely absorbed before adding next portion. Then carefully add benzene into tube to ht of ca 8 cm above top of column, and maintain level of the benzene with suitable constant level device. After 160 mL effluent (contg triacetylepinephrine) collects, thoroly rinse tip of tube with $CHCl_3$ and discard effluent and rinsings, or reserve for qual. tests.

Remove layer of benzene above column by aspiration, place clean receiver under tube, and let $CHCl_3$ pass thru column until 100 mL effluent (contg triacetylnorepinephrine) collects. Evap. effluent to dryness and transfer to 50 mL beaker, confining residue near bottom of beaker.

### F. Determination of Norepinephrine

Add exactly 10 mL 0.50*N* HCl to residue of triacetylnorepinephrine, warm gently, and dissolve by stirring and rubbing with rubber policeman. Pour soln into g-s test tube (15 × 150 mm is convenient), place tube in boiling $H_2O$ bath, and stopper loosely. After 5 min, stopper tightly and maintain 30 addnl min at 100°. Remove tube and cool to room temp., lifting stopper slightly from time to time to keep vac. from forming.

Thoroly mix contents and transfer 1 mL aliquot to another g-s test tube. Neutze acid by adding exactly 42 mg $NaHCO_3$. (Ensure that *all* $NaHCO_3$ is delivered to bottom of tube, and that none adheres to wall above acid layer.) After effervescence stops, add 1.5 mL $H_2O$ and 2.5 mL buffer, (**d**). Mix by swirling, and add 4 drops 0.1*N* I. Swirl, and after exactly 3 min (timed by stopwatch) from addn of I, add 6 drops 0.1*N* $Na_2S_2O_3$, stopper, and mix thoroly. Measure $A$ at 520 nm, 3 ± 0.5 min after addn of $Na_2S_2O_3$ soln, in 1 cm cells against the pH 6 buffer blank in Beckman Model DU spectrophtr, or equiv.

Transfer 1.5 mL aliquot std soln to g-s test tube. Add 1 mL $H_2O$ and 2.5 mL pH 6 buffer, (**d**), and swirl. Develop color and measure $A'$ at 520 nm as above.

Calc. amt norepinephrine in sample originally taken for analysis, and from this value and $E$, **956.08D**, calc. % norepinephrine in "total epinephrine."

Ref.: JAOAC **39**, 639(1956).

CAS-51-43-4 (epinephrine)
CAS-51-42-3 (epinephrine bitartrate)
CAS-55-31-2 (epinephrine hydrochloride)
CAS-51-41-2 (norepinephrine)

## 965.42★ Phenylephrine Hydrochloride in Drugs

**Acetylation Method**
**First Action 1965**
**Surplus 1970**

*See* **36.108–36.111**, 11th ed.

## 969.49 Phenylephrine Hydrochloride in Drugs

**Colorimetric Method**
**First Action 1969**
**Final Action 1970**

(Not applicable in presence of tetracycline, acetaminophen, salicylamide, phenolic compds, and Zn salts)

### A. Reagents

Prep. (**a**), (**b**), and (**d**)(*2*) fresh on day of use.

(**a**) *4-Aminoantipyrine hydrochloride soln.*—(Eastman Kodak, No. 6535) 3% in $H_2O$.

(**b**) *Potassium ferricyanide soln.*—4% $K_3Fe(CN)_6$ in $H_2O$.

(**c**) *Sodium borate soln.*—2% $Na_2B_4O_7.10H_2O$ in $H_2O$.

(**d**) *Phenylephrine hydrochloride std solns.*—(*1*) *Stock soln.*—Approx. 2.5 mg/mL. Weigh ca 125 mg phenylephrine.HCl to nearest 0.1 mg into 50 mL vol. flask and dil. to vol. with $H_2O$. Soln is stable several months under refrigeration. (*2*) *Working std soln.*—Approx. 0.25 mg/mL. Dil. 5 mL stock soln to 50 mL with $H_2O$.

### B. Preparation of Samples

(**a**) *Tablets and capsules.*—Det. av. wt/unit. Grind tablets to powder and mix, or mix contents of capsules. Weigh portion contg ca 12.5 mg phenylephrine.HCl into 50 mL vol. flask, add ca 30 mL $H_2O$, and shake vigorously. Dil. to vol., shake again, and filter if soln is not clear.

(**b**) *Powders for oral suspensions, oral suspensions, sirups, solns, etc.*—Reconstitute powders for oral suspension as directed on label or use solns, sirups, and oral suspensions as is. Transfer aliquot contg ca 12.5 mg phenylephrine.HCl into 50 mL vol. flask and proceed as in (**a**).

### C. Determination

(Reaction is time dependent; assay samples one at a time.)

Transfer 2 mL aliquot sample soln to 50 mL vol. flask (omit sample for reagent blank), add 1.0 mL 4% $K_3Fe(CN)_6$ soln, and swirl. Dil. to ca 48 mL with $Na_2B_2O_7$ soln and add 1.0 mL aminoantipyrine soln. Immediately dil. to vol. with $Na_2B_2O_7$ soln and shake vigorously. Immediately det. $A$ of soln at 490 nm against reagent blank, in matched 1 cm cells.

Calc. sample concn, $S = CFA/A'$, where $C$ = mg std/mL, $F$ = diln factor, and $A$ and $A'$ refer to sample and std, resp.

Report mg phenylephrine.HCl/tablet, capsule, or vol. liq. dose.

Refs.: J. Pharm. Sci. **52,** 802(1963). JAOAC **52,** 500(1969).

CAS-61-76-7 (phenylephrine hydrochloride)

## 971.37 Phenylephrine Hydrochloride in Drugs

**Automated Method**

**First Action 1971**
**Final Action 1973**

### A. Principle

Oxidn products of phenylephrine with $K_3Fe(CN)_6$ form colored complex with 4-aminoantipyrine in borate soln. Method is automated version of **969.49** and is not applicable in presence of tetracycline, acetaminophen, salicylamide, phenolic compds, and Zn salts.

### B. Apparatus

*Automatic analyzer.*—AutoAnalyzer with following modules (available from Technicon Corp.): (*1*) *Sampler II.*—With 50/hr (2:1) cam. (*2*) *Proportioning pump.* (*3*) *Colorimeter.*—With 15 mm tubular flowcell and matched 490 nm filters. (*4*) *Recorder.* (*5*) *Manifold.*

Assemble app. as shown in Fig. **971.37A**. Make sample and resample pump tubes as short as possible by cutting each end 6 mm from color-coded shoulders. Sample line is 20 cm. Reduce vol. at point of debubbling and flowcell by constricting lower arm of C-5 debubbler with Tygon tubing to give push fit with 0.015 × 2.5″ polyethylene tubing leading to flowcell as shown in Fig. **971.37B**.

Prewash system with $H_2O$ before placing reagent lines in their appropriate reagent container. When tubes are pumping satisfactorily and system is equilibrated (ca 15 min), adjust colorimeter and recorder to produce steady baseline.

### C. Reagents

*See* **969.49A(a)**, **(b)**, **(c)**, **(d)**(*1*) (2.5 mg/mL), and in addn: *Wetting agent.*—Add 0.5 mL polyoxyethylene lauryl ether (Brij 35) to 1 L $H_2O$.

### D. Preparation of Sample

Proceed as in **969.49B**, except weigh portion contg ca 125 mg in **(a)** and transfer aliquot (dild if necessary) contg 125 mg in **(b)**.

### E. Stream Flow

*See* Fig. **971.37A**. Sample solns are withdrawn from sample cups, segmented with air, and dild in manifold. Stream is debubbled and resampled. Resultant stream is buffered with air-segmented stream of borate soln. $K_3Fe(CN)_6$ is added and mixed, and color reagent is added and mixed. Stream is debubbled and $A$ is measured at 490 nm.

### F. Determination

Fill 2 mL sample cups with prepd solns and aspirate at 2:1 sample-to-wash ratio, picking up sample with 0.016″ stainless steel probe. Include 2 std solns (2.5 mg/mL) at beginning and end of each run of 5 samples in duplicate (10 detns). Draw

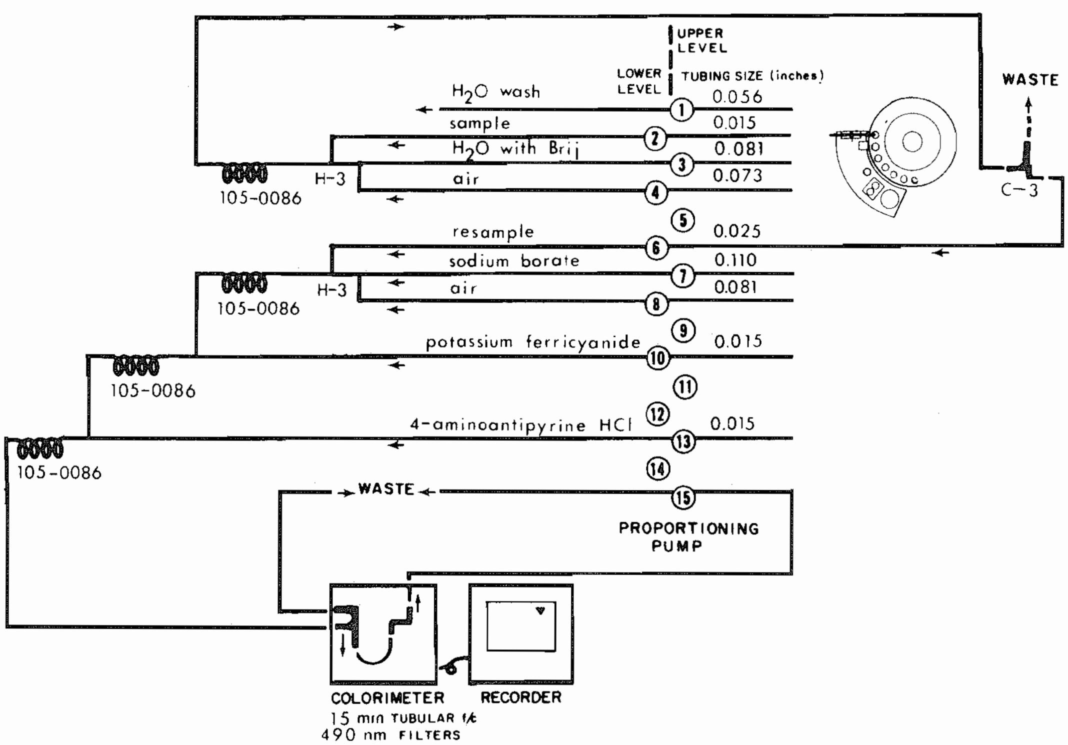



**FIG. 971.37A—Flow diagram for phenylephrine hydrochloride**

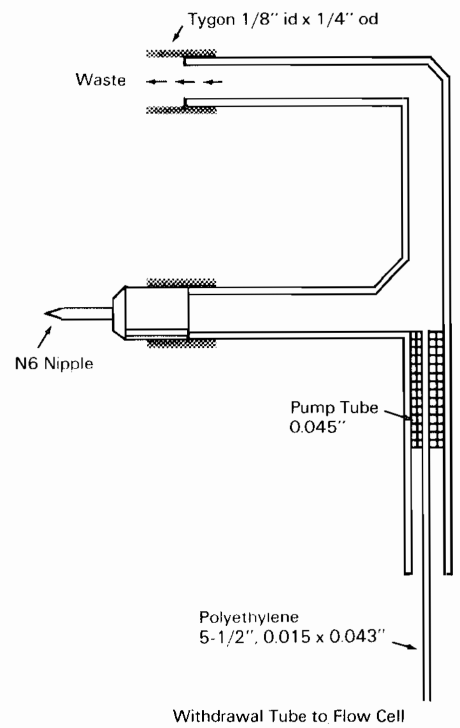



**FIG. 971.37B—Assembly of debubbler**

line between baseline at beginning and end of run, if necessary. Subtract baseline $A$ from max. $A$ to obtain net $A$ ($\Delta A$) for each peak. Calc. mg phenylephrine.HCl/unit dose as in **969.49C** from $\Delta A$, using av. of duplicate detns.

Ref.: JAOAC **54,** 596, 600(1971).

CAS-61-76-7 (phenylephrine hydrochloride)

## 970.78 Phenylephrine Hydrochloride in Drugs

**Ion-Pair Column Partition Method**

**First Action 1970**
**Final Action 1971**

(Not applicable in presence of phenolic nitrogenous bases)

### A. Apparatus and Reagents

(**a**) *Recording spectrophotometer.*—With matched 1 cm cells.

(**b**) *Chromatographic tubes.*—Fuse 6 cm length of 5–6 mm tubing to piece of 25 mm tubing ca 25 cm long (25 × 200 mm test tube may be used). Constrict stem slightly ca 2 cm below seal. Com. tubes with dimensions ±10% are satisfactory. Pack wad of Pyrex glass wool in base as support.

(**c**) *Phosphate buffer.*—pH 5.80 ± 0.05. Mix 1 vol. 1*M* $K_2HPO_4$ (174 g/L) and 4 vols 1*M* $KH_2PO_4$ (136 g/L) and adjust pH with either component.

(**d**) *Phosphate-citrate buffer.*—pH 5.10 ± 0.05. Mix 2 vols 1*M* $K_2HPO_4$ and 1 vol. 1*M* citric acid (192 g $C_6H_8O_7$ or 210 g $C_6H_8O_7.H_2O$/L) and adjust pH with either component.

(**e**) *Diatomaceous earth.—See* **960.53B**.

(**f**) *Bis-(2-ethylhexyl) hydrogen phosphate (DEHP) soln.*—Reagent grade. 2.4% v/v in $H_2O$-satd ether. Prep. fresh daily.

(**g**) *Sulfuric acid.*—0.1*N*, ether-satd. Prep. fresh daily.

(**h**) *Chloroform and ether.*—$H_2O$-satd. Prep. fresh daily and use thruout detn.

(**i**) *Phenylephrine hydrochloride std solns.*—(*1*) *Stock soln.*—1 mg/mL. Accurately weigh ca 100 mg USP Phenylephrine.HCl Ref. Std in 100 mL vol. flask and dil. to vol. with $H_2O$. (*2*) *Working soln.*—0.04 mg/mL. Dil. 2 mL stock soln to 50 mL with 0.1*N* NaOH and use to obtain spectrum between 400 and 200 nm (or as far as instrument permits) along with sample detn.

### B. Preparation of Samples

(**a**) *Samples containing about 1 mg phenylephrine.HCl/mL sirup.*—Pipet 4.0 mL pH 5.8 buffer into 10 mL vol. flask. Carefully add sirup to vol. Do not wet flask above mark.

(**b**) *Samples containing more than 1 mg phenylephrine.HCl/mL sirup.*—Dil. to 1 mg/mL and proceed as in (**a**).

(**c**) *Tablets.*—Weigh ground sample contg ca 2 mg phenylephrine into 50 mL beaker. If components of tablets are $H_2O$-sol., add 2 mL $H_2O$, warm slightly to dissolve, and add 1 mL pH 5.8 buffer. If some components are not $H_2O$-sol. (e.g., acetaminophen), add 1 mL dimethylsulfoxide, warm to dissolve, and then add 2 mL pH 5.8 buffer. For tablets contg antacids (e.g., $Mg(OH)_2$ and $Al(OH)_3$), heat powd sample contg ca 2 mg phenylephrine with 5 mL alcohol and 1 mL HCl to dissolve alk. material; add 10 mL *n*-BuOH and evap. to dryness. Dissolve residue in 1 mL dimethylsulfoxide and add 2 mL pH 5.8 buffer.

(**d**) *Capsules.*—Take portion of contents contg ca 2 mg phenylephrine and proceed as in (**c**). Grind sample if necessary.

### C. Determination

Pack small glass wool plug in base of chromatgc tube as support. Transfer mixt. of 1 g diat. earth with 0.8 mL pH 5.1 buffer to tube and tamp to uniform mass. Mix 4 g diat. earth with 3.0 mL aliquot prepd sample and carefully transfer directly above pH 5.1 layer, tamping gently. Dry-wash beaker with 1 g diat. earth, add to column, and tamp. Cover with small glass wool pad. Pass 75 mL $CHCl_3$ thru column followed by 125 mL ether, and discard eluates. Place 125 mL separator contg ca 20 mL 0.1*N* $H_2SO_4$ as receiver under column. Elute column with 50 mL DEHP-ether soln and then with 25 mL ether, collecting in same separator. Shake separator and transfer aq. phase to 50 mL vol. flask contg 6 mL 1*N* NaOH. Re-ext ether with 15 mL 0.1*N* $H_2SO_4$. Combine exts and dil. to vol. with $H_2O$. Obtain spectrum between 200 and 400 nm on same day as elution.

### D. Calculations

Det. corrected $A$ ($\Delta A$) of both std and sample as follows: Construct baseline representing background $A$ extension obtained from 400 to ca 250 nm. (Constructed baseline $A$ value at wavelength of max. $A$, ca 290 nm, is designated as $A_B$.) Subtract $A_B$ from total $A_{max}$ observed at wavelength peak.

For std, calc. $a = \Delta A'/bc$, where $b$ = cell pathlength (1 cm), and $c$ = concn in g/L.

For samples, calc. $c = (\Delta A \times F)/ab$, where $F$ = diln factor.

Report mg phenylephrine.HCl/tablet, capsule, or vol. liq. dose.

Ref.: JAOAC **53,** 120(1970).

CAS-61-76-7 (phenylephrine hydrochloride)

## 958.11 Phenylpropanolamine Hydrochloride in Drugs

**Spectrophotometric Method**

**Final Action 1965**

### A. Apparatus

*See* **967.31A**.

### B. Reagents

(**a**) *Chloroform.*—*A* at 258.5 nm, measured against $H_2O$ blank, <0.200.

(**b**) *Diatomaceous earth.*—*See* **960.53B**.

### C. Preparation of Column

Fix pledget of glass wool in stem of chromatgc tube above constriction. Clamp tube vertically. In small beaker mix 3 g diat. earth and 2 mL $H_2O$. Transfer to tube with metal spatula and press down evenly with packing rod.

### D. Determination

(**a**) *Capsules and tablets.*—To 150 mL beaker transfer accurately weighed amt of powd sample contg ca 50 mg phenylpropanolamine.HCl. Add 5 mL $NH_4OH$ (1 + 4) and mix by gentle swirling. Add 5 g diat. earth and mix with metal spatula. Transfer to tube without loss thru powder funnel, in 4 or more portions, pressing down each portion evenly with packing rod. When removing funnel from tube each time, tap it lightly in tube to remove loosely adhering particles; then hang it in beaker of such size that it does not touch bottom. After using packing rod, scrape off most of adhering material into tube with spatula, and tap rod and spatula over mouth of tube. When laying down implements, place them in position such that their ends do not touch anything. Finally use smooth, intact rubber policeman to sweep material from beaker and funnel into tube. Rub beaker, spatula, and packing rod with three ca 1 g portions diat. earth, sweeping each portion thru funnel into tube, using rubber policeman. Press down each portion with packing rod.

Place 100 mL vol. flask in receiving position. Wash down inside of tube with $CHCl_3$, adding enough (ca 20 mL) to moisten column and produce only few drops of eluate. Elute with 95 mL $CHCl_3$, wash tip of tube with little $CHCl_3$, and dil. to vol. with $CHCl_3$. Measure *A* at 258.5 nm, 2–5 min after pouring into silica cell, against portion of same $CHCl_3$ used for elution.

To 150 mL beaker transfer ca 50 mg pure phenylpropanolamine.HCl, accurately weighed. Proceed as with sample, beginning "Add 5 mL $NH_4OH$ (1 + 4) . . ." Det. *A* of sample and std eluates at ca same time, on same setting of wavelength dial. Use same cell for both eluates, and same cell for both blanks. Calc. phenylpropanolamine.HCl content.

(**b**) *Aqueous solns.*—Prep. column as in **958.11C**. Into 150 mL beaker pipet vol. sample contg ca 50 mg phenylpropanolamine.HCl, or pipet 10 mL, whichever is less. Add 1 mL $NH_4OH$ and mix by gentle swirling. Add number of g diat. earth equal to total number mL of liq. and mix with metal spatula. Proceed as in (**a**), beginning "Transfer to tube without loss . . .

Ref.: JAOAC **41,** 499(1958).

CAS-154-41-6 (phenylpropanolamine hydrochloride)

## 958.12 Phenylpropanolamine Hydrochloride in Drugs

**Extraction Method**

**Final Action**

Proceed as in **957.21C–D**.

Refs.: JAOAC **41,** 509(1958); **58,** 852(1975).

## 973.70 Phenylalkanolamine Salts Including Phenylpropanolamine Hydrochloride and Ephedrine Sulfate in Elixirs and Sirups

**Spectrophotometric Method**

**First Action 1973**
**Final Action 1974**

(Applicable to individual phenylalkanolamines when only one is present, except for phenylephrine, which does not interfere and which is not detd by this method.)

### A. Principle

Phenylalkanolamine is eluted with $CH_2Cl_2$ from weakly basic diat. earth column, retained on weakly acidic column, and converted to benzaldehyde by on-column periodate reaction. Benzaldehyde is detd by UV spectrometry and is proportional to amt alkanolamine salt in sample.

### B. Reagents and Apparatus

(**a**) *Phosphate-chloride soln.*—Dissolve 5 g $KH_2PO_4$ and 7.5 g KCl in 100 mL $H_2O$.

(**b**) *Sodium metaperiodate soln.*—Dissolve 2 g $NaIO_4$ in 20 mL $H_2O$. Store in dark.

(**c**) *Water-saturated methylene chloride.*—Sat. ca 400 mL spectral grade $CH_2Cl_2$ by shaking 1 min with equal vol. $H_2O$. Use thruout.

(**d**) *Diatomaceous earth.*—*See* **960.53B**.

(**e**) *Phenylalkanolamine salt std soln.*—0.4 mg/mL. Accurately weigh ca 100 mg phenylalkanolamine salt and dissolve and dil. to 250 mL with $H_2O$.

(**f**) *Recording spectrophotometer.*—With matched 1 cm cells.

(**g**) *Chromatographic tubes.*—Fuse 6 cm length of 5–6 mm tubing to piece of 25 mm tubing ca 25 cm long (25 × 200 mm test tube may be used). Constrict stem slightly ca 2 cm below seal. Com. tubes with dimensions ±10% are satisfactory. Pack wad of Pyrex glass wool in base as support.

(**h**) *Tamping rod.*—Flatten end of glass rod to circular head with clearance of ca 1 mm in tube (**a**). Or use disk of stainless steel, Al, etc., of diam. ca 1 mm less than id of column, (**a**), attached to 30–45 cm (12–18") rod.

### C. Preparation of Sample and Chromatographic Columns

*Sample.*—Accurately dil. sample with $H_2O$ to final concn of ca 0.4 mg/mL.

*Column I.*—Add 2.0 mL dild sample to 300 mg $K_2HPO_4$ in 150 mL beaker. Swirl to dissolve. Add 3 g diat. earth, mix, transfer quant. to column, and tamp. Dry-wash beaker with 1 g diat. earth, add wash to column, and tamp. Cover with small glass wool plug. If acidic compds such as acetaminophen or theophylline are present, underlay sample-diat. earth mixt. with mixt. of 3 g diat. earth and 2 mL 10% NaOH.

*Column II.*—Mix 3 g diat. earth and 2 mL phosphate-chloride soln, and transfer to column. Tamp and cover with small glass wool pad.

*Column III.*—Mix 0.5 mL $H_2O$ and 1 g diat. earth, transfer to column, and tamp. Mix 3 g diat. earth and 2 mL $NaIO_4$ soln, transfer to column, tamp, and cover with glass wool pad.

### *D. Preparation of Standard*

Prep. sep. column *III* as above. Mix 2.0 mL phenylalkanolamine salt std soln and 3 g diat. earth, transfer quant. to column, and tamp. Dry-wash beaker with 1 g diat. earth, transfer wash to column, tamp, and cover with glass wool pad. Place 100 mL vol. flask under column. Wet column with 10 mL $CH_2Cl_2$. With pipet, evenly distribute 1.0 mL $NH_4OH$ onto surface of column packing. Elute column with four 25 mL portions $CH_2Cl_2$; let each portion sink entirely into surface. Rinse tip of column into flask and dil. eluate to vol. with $CH_2Cl_2$. Elute with addnl 25 mL $CH_2Cl_2$ and collect eluate for use as blank.

### *E. Determination*

Mount columns so that eluate from *I* flows onto *II*. Elute combined columns with four 25 mL portions $CH_2Cl_2$; let each portion sink entirely into surface of both columns. Rinse tip of Column *I* into *II* with $CH_2Cl_2$ and discard *I*. Elute Column *II* with addnl 25 mL $CH_2Cl_2$. Discard all eluates.

Mount Column *II* above *III* and place 100 mL vol. flask under *III*. With pipet, evenly distribute 1.0 mL $NH_4OH$ onto surface of Column *II* packing. Elute combined columns with four 25 mL portions $CH_2Cl_2$; let each portion sink entirely into surface of each column. Rinse tip of Column *II* into *III* with ca 1 mL $CH_2Cl_2$. Discard Column *II* and continue to elute Column *III* until 100 mL eluate is collected.

Scan spectra of sample and std eluates from 350 to 230 nm, against column blank eluate. If liq. is cloudy, let soln clear (ca 1 min) before detg $A$. Calc. net $A$ for sample and std solns, $\Delta A$ and $\Delta A'$, resp., at min. $A$, ca 267 nm, and max. $A$, ca 246 nm.

$$\text{mg Phenylalkanolamine salt/mL} = (\Delta A/\Delta A') \times C \times F$$

where $C$ = mg std/mL and $F$ = diln factor.

Refs.: JAOAC **56,** 100(1973); **58,** 852(1975).

CAS-134-72-5 (ephedrine sulfate)
CAS-154-41-6 (phenylpropanolamine hydrochloride)

## PHENETHYLAMINES

## 954.13★ Amphetamine Drugs

**Final Action 1965**
**Surplus 1973**

### *A. Titrimetric Method*

*See* **38.116**, 12th ed.

### *B. Confirmatory Gravimetric Determination*

*See* **38.117**, 12th ed.

## 954.14★ Amphetamine Drugs

**Stereochemical Composition**

**Final Action**
**Surplus 1977**

*See* **38.127–38.128**, 13th ed.

## 972.47 Amphetamine in Drugs

**Gas Chromatographic Method**

**First Action 1972**
**Final Action 1973**

### *A. Apparatus*

(**a**) *Gas chromatograph.*—Equipped with flame ionization detector. Operate at sensitivity such that proline derivative from 10 μg *d*-amphetamine sulfate gives peak 70–90% full scale. Adjust injection zone and detector temps to 230°.

(**b**) *Column.*—Glass, 2 m × 4 mm id, packed with 1% Carbowax 20M on 80–100 or 100–120 mesh Gas-Chrom Q (Applied Science). Condition column 24 hr at 210° before use. Operate at 185° and adjust N carrier gas flow rate so that proline derivative of *d*-amphetamine is eluted in ca 15 min (ca 60 mL/min).

(**c**) *Chromatographic tube.*—22 × 300 mm, without stopcock.

### *B. Reagents*

(**a**) *Diatomaceous earth.—See* **960.53B**.

(**b**) *Proline reagent.*—(Prep. reagent at <30°.) To 1.0 g *l*-proline (Sigma Chemical Co.) in 125 mL g-s conical flask, add 5 g trifluoroacetic anhydride and swirl until proline is dissolved. Evap. excess trifluoroacetic anhydride under stream of dry N. Add 5 mL thionyl chloride, let stand 15 min, and evap. excess under stream of dry N. Dissolve residue in 100 mL $CH_2Cl_2$ and refrigerate when not in use. Properly prepd reagent will give *l*-amphetamine ratio, **972.47D**, ≤0.02 with *d*-amphetamine sulfate. (*Caution:* Trifluoroacetic anhydride and thionyl chloride are toxic. Wear rubber gloves and eye protection and use effective fume removal device for evapn.)

(**c**) *Std soln I.*—0.5 mg USP Ref. Std Dextroamphetamine Sulfate/mL $H_2O$.

(**d**) *Std soln II.*—0.5 mg *dl*-amphetamine sulfate (Sigma Chemical Co., No. A1263)/mL $H_2O$. Recrystallize *dl*-amphetamine sulfate from alcohol and dry under vac. at 100° before use.

### *C. Preparation of Wash Column*

Mix 3.0 mL 1*N* NaOH with 5.0 g diat. earth and transfer to chromatgc tube contg small glass wool plug. Place glass wool pad on top of diat. earth and tamp firmly.

### *D. Preparation of Standard Curve*

Prep. series of *l*-amphetamine sulfate stds contg total of ca 10 mg amphetamine sulfate in 20 mL $H_2O$ in 125 mL separators as follows: 0% *l*-amphetamine sulfate from 20 mL std soln *I* and 0 mL std soln *II*; 12.5%, 15 mL *I* and 5 mL *II*; 25%, 10 mL *I* and 10 mL *II*; 37.5%, 5 mL *I* and 15 mL *II*; and 50%, 0 mL *I* and 20 mL *II*. Treat each soln as follows: Add 5 mL 1*N* NaOH and ext with two 25 mL portions $CHCl_3$. Filter $CHCl_3$ exts thru absorbent cotton into dry 150 mL beaker. Add 3 mL proline reagent, (**b**), and let stand 30 min; then transfer to wash column. Collect eluate in 150 mL beaker. Pass addnl 25 mL $CHCl_3$ thru column. Evap. on steam bath with aid of N stream, dissolve residue in 5 mL $CHCl_3$, and transfer to g-s flask. Inject 5 μL and calc.

$$l\text{-amphetamine ratio} = H_l \times R_l / [(H_d \times R_d) + (H_l \times R_l)]$$

where $H$ and $R$ = peak ht and retention time, resp., of *d*- and *l*-amphetamine derivatives. Plot *l*-amphetamine ratio against % *l*-amphetamine.

### *E. Determination*

Transfer finely powd sample contg ca 10 mg amphetamine sulfate to 100 mL beaker, add 2 g diat. earth, and mix. Add 3.0 mL 1*N* NaOH and mix to uniform slurry. Add 3.0 g diat.

earth, mix well, and pack into chromatgc tube contg small glass wool plug. Place 150 mL beaker under column and elute with 100 mL $CHCl_3$. Proceed as in **972.47D**, beginning "Add 3 mL proline reagent, (**b**), . . ." Calc. *l*-amphetamine ratio and det. *l*-amphetamine from std curve.

Ref.: JAOAC **55,** 146(1972).

CAS-51-63-8 (*d*-amphetamine sulfate)
CAS-60-13-9 (*dl*-amphetamine sulfate)

### Amphetamine in Presence of Antihistamines and Barbiturates and Other Drugs

*See* **974.39**.

### Mephentermine Sulfate in Drugs

*See* **978.28.**

### 957.21 Phenethylamines in Drugs

**Spectrophotometric Method**

**Final Action 1965**

(Applicable to amphetamine, methamphetamine, mephentermine, phenylpropylmethylamine (Vonedrine®), ephedrine, and phenylpropanolamine (Propadrine®))

#### *A. Apparatus*

*Spectrophotometer*.—Suitable for measurement in region 250–270 nm; with 1 cm cells of quartz or fused Si (preferably matched pair); or recording spectrophtr.

#### *B. Preparation of Standard Solution*

Accurately weigh 500–700 mg phenethylamine salt of known purity, transfer to 100 mL vol. flask, and dissolve in 0.1*N* $H_2SO_4$. Dil. to vol. with the $H_2SO_4$ and mix well.

#### *C. Determination*

Accurately weigh powd sample contg 25–50 mg amine base and transfer to 40–50 mL g-s centrf. tube contg 3–3.5 g NaCl and 6–7 glass beads. Dissolve sample by adding 5 mL 1*N* $H_2SO_4$, and swirl gently to aid escape of any liberated $CO_2$. Test for acidity with litmus paper, adding more acid if necessary. Pipet in 25 mL $CHCl_3$ and 4 mL 2*N* NaOH, stopper securely, and shake 3–5 min. To second 40–50 mL centrf. tube contg 3–3.5 g NaCl and 6–7 glass beads, add 10 mL std soln, **957.21B**. Swirl to dissolve salt, pipet in 25 mL $CHCl_3$ and 1 mL 2*N* NaOH, stopper securely, and shake 3–5 min.

Centrf. tubes 3–5 min at 1500–1800 rpm. Withdraw 10 mL clear $CHCl_3$ layer by closing upper end of 10 mL pipet with index finger while lowering tip thru aq. layer. Wipe off outer portion of pipet, and transfer 10 mL $CHCl_3$ layer to second 40–50 mL centrf. tube contg 25 mL 0.1*N* $H_2SO_4$ and 6–7 glass beads. Stopper securely, shake, and centrf. as above.

Prep. acid blank soln by shaking 25 mL 0.1*N* $H_2SO_4$ with 3–5 mL $CHCl_3$ and centrfg to obtain clear acid soln. Read *A* of portions of clear acid soln obtained from aliquots of std and of sample solns against acid blank prepd as above in ref. cell, using 1 cm cells in range 252–255 for first min., 256–258 for max., and 260–262 for second min.

#### *D. Calculations*

Calc. *A* difference ($\Delta A$) between *A* at maximum and *A* of 2 minima: $\Delta A = A_{max} - 0.5(A_{min\,1} + A_{min\,2})$.

Calc. absorptivity differential ($\Delta a$) produced by 1 g/L (1 mg/mL) of std amine base or salt:

$$\Delta a_{std} = \Delta A_{std} \times 100/\text{wt std}$$

where $\Delta A_{std}$ = *A* difference for std soln; 100 = mL std soln measured; and wt = mg std in aliquot measured.

$$\text{mg Amine/unit of sample} = (\Delta A_{sample} \times 25 \times 25 \times \text{av. wt of unit in mg})/(\Delta a_{std} \times 10 \times \text{wt sample in mg})$$

Refs.: JAOAC **40,** 824(1957); **41,** 509(1958); **48,** 170(1965); **49,** 166(1966).

CAS-300-62-9 (amphetamine)
CAS-299-42-3 (ephedrine)
CAS-100-92-5 (mephentermine)
CAS-537-46-2 (methamphetamine)
CAS-14838-15-4 (phenylpropanolamine)
CAS-93-88-9 (phenylpropylmethylamine)

## AMINOBENZOATES

### 968.40 Benzocaine in Drugs

**Colorimetric Method**

**First Action 1968**
**Final Action 1969**

#### *A. Principle*

Benzocaine is diazotized with $NaNO_2$, excess nitrite is removed with $NH_4$ sulfamate, and product is coupled with *N*-1-naphthylethylenediamine.2HCl. Colored soln has max. at 540 nm. Method is not applicable in presence of sulfonamides. Benzocaine must be sepd from inorg. I to avoid interference. Antipyrine in 10-fold excess does not interfere.

#### *B. Reagents*

*Benzocaine std solns*.—(*1*) *Stock soln*.—0.25 mg/mL. Dissolve 25.0 mg benzocaine USP in 25–50 mL $H_2O$ in 100 mL vol. flask. Add 3 mL HCl, shake gently, and dil. to vol. with $H_2O$. (*2*) *Working soln*.—5 μg/mL. Pipet 10 mL stock soln into 100 mL vol. flask and dil. to vol. with $H_2O$. Pipet 20 mL of this soln into 100 mL vol. flask and dil. to vol. with $H_2O$.

#### *C. Preparation of Standard Curve*

Pipet 0.0, 2.0, 6.0, and 10.0 mL working std soln into sep. 25 mL vol. flasks. To each flask add 1 mL HCl (1 + 1). Dil. to 15 mL with $H_2O$, add 1 mL 0.1% $NaNO_2$ soln, prepd fresh daily, mix, and let stand 5 min, swirling several times during standing. Add 1 mL 0.5% $NH_4$ sulfamate soln, and let stand 5 min, swirling several times during standing. Add 1 mL colorless 0.1% *N*-1-naphthylethylenediamine.2HCl soln, (prep. fresh weekly and store in dark glass bottle in refrigerator), let stand 15 min, swirling several times during standing, and dil. to 25.0 mL with $H_2O$.

Det. *A* of each soln in matched 1 cm cells in spectrophtr at 540 nm against $H_2O$ as ref. (Avoid collection of N bubbles on cell walls.) To obtain $\Delta A$, subtract reading of soln contg *no* std from each of other std readings. Plot $\Delta A$ against benzocaine concn.

#### *D. Preparation of Samples*

(**a**) *Liquid preparation in water-soluble bases*.—Weigh sample contg 100 mg benzocaine, transfer to 250 mL vol. flask, and add 75 mL alcohol. Add 3 mL HCl, dil. to vol. with $H_2O$, and mix well. Pipet 10 mL into 100 mL vol. flask, dil. to vol.

with $H_2O$, and mix well. Pipet 10 mL of diln into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix well.

(**b**) *Tablets or troches.*—Weigh powd sample contg 7.5–10 mg benzocaine. Transfer to 100 mL beaker, wet with 2–3 mL alcohol, stir with glass rod to slurry, add 5 mL $H_2O$ and 2 mL HCl, stir, and let stand at room temp. 5 min. Dil. with 25 mL $H_2O$, transfer quant. to 100 mL vol. flask, and dil. to vol. If soln is cloudy, filter thru dry paper, discarding first 10 mL filtrate. Pipet 10 mL clear filtrate into 100 mL vol. flask, dil. to vol. with $H_2O$, and mix well.

(**c**) *Suppositories in water-soluble bases.*—Weigh sample contg 50–100 mg benzocaine into 100 mL beaker, add 15 mL $H_2O$ and 3 mL HCl, and let stand at room temp. 15 min, stirring occasionally with rod. Transfer quant. to 250 mL vol. flask, dil. to vol., and mix well. Pipet 10 mL into 100 mL vol. flask, dil. to vol., and mix well. Pipet 10 mL of diln into 100 mL vol. flask, dil. to vol., and mix well.

### *E. Determination*

Pipet 2 aliquots of final diln specified in **968.40D** contg 20–30 μg benzocaine into sep. 25 mL vol. flasks. Label one flask "sample" and the other "sample blank." Proceed as in **968.40C**, beginning "To each flask add 1 mL HCl (1 + 1)." except do not add $NaNO_2$ to "sample blank."

Det. *A* at 540 nm for each soln against $H_2O$ as ref. Subtract *A* of "sample blank" from "sample" reading. Det. μg benzocaine in aliquot from std curve.

$$\% \text{ Benzocaine} = (B \times F)/(W \times 10)$$

where $B$ = μg benzocaine from std curve, $F$ = diln factor, and $W$ = mg sample.

Ref.: JAOAC **51,** 612(1968).

CAS-94-09-7 (benzocaine)

## 968.41★ Benzocaine in Drugs

**Bromination Method**
**Final Action**
**Surplus 1975**

*See* **38.139**, 12th ed.

## 968.42 Benzocaine and Antipyrine in Drugs

**Spectrophotometric Method**
**First Action 1968**
**Final Action 1969**

(Applicable in presence of glycerol and propylene glycol bases)

### *A. Principle*

Benzocaine and antipyrine are extd by column partition chromatgy. Antipyrine is retained on $FeCl_3$ column and benzocaine on HCl column. Max. *A* are detd in $CHCl_3$ eluates at 272 nm for antipyrine and 283 nm for benzocaine. To identify compds, IR spectra of KBr dispersions are compared to stds.

### *B. Apparatus and Reagents*

(**a**) *Chromatographic tubes.*—Fuse 6 cm length of 5–6 mm tubing to piece of 25 mm tubing ca 25 cm long (25 × 200 mm test tube may be used). Constrict stem slightly ca 2 cm below seal. Com. tubes with dimensions ±10% are satisfactory. Pack wad of Pyrex glass wool in base as support.

(**b**) *Tamping rod.*—Flatten end of glass rod to circular head with clearance of ca 1 mm in tube (**a**). Or use disk of stainless steel, Al, etc., of diam. ca 1 mm less than id of column, (**a**), attached to 30–45 cm (12–18") rod.

(**c**) *Syringe.*—10 mL syringe with 14 gage 4" laboratory cannula.

(**d**) *Ferric chloride.*—9%. Dissolve 9 g anhyd. $FeCl_3$ in $H_2O$ and dil. to 100 mL.

(**e**) *Diatomaceous earth.*—*See* **960.53B**.

(**f**) *Chloroform, washed.*—Shake 4 vols $CHCl_3$ with 1 vol. $H_2O$.

(**g**) *Mixed solvent.*—$H_2O$-satd $CHCl_3$-ether-isooctane (10 + 25 + 65).

(**h**) *Antipyrine std soln.*—1 mg/100 mL. Accurately weigh antipyrine std, previously dried 2 hr at 60°, and dil. with $CHCl_3$ to give concn of 0.1 mg/mL. Pipet 10 mL into 100 mL vol. flask contg 10 mL MeOH and 1.0 mL mixed solv., and dil. to vol. with washed $CHCl_3$.

(**i**) *Benzocaine std soln.*—0.4 mg/100 mL. Accurately weigh USP Benzocaine Ref. Std, previously dried 3 hr over $P_2O_5$, and dil. with $CHCl_3$ to concn of 0.04 mg/mL. Pipet 10 mL into 100 mL vol. flask contg 10 mL MeOH and 1.0 mL mixed solv., and dil. to vol. with washed $CHCl_3$.

(**j**) *Photometric blank.*—Pipet 10 mL MeOH and 1 mL mixed solv. into 100 mL vol. flask, dil. to vol. with washed $CHCl_3$, and mix.

(**k**) *Potassium bromide.*—Anhyd. spectrophtric grade.

### *C. Sample Density*

(Altho sample is weighed because of viscosity of prepns, report results on wt/vol. basis.)

Slowly withdraw 10 mL sample with syringe, keeping air bubbles to min., and transfer to previously weighed 10 mL vol. flask without touching sides of flask above mark. Let any air bubbles present rise before filling to final vol. Weigh flask and contents, and calc. sample density.

### *D. Preparation of Chromatographic Columns*

Loosely pack small amt glass wool uniformly in base of 3 chromatgc tubes to support diat. earth.

*Bottom column.*—Mix 3 g diat. earth with 2 mL 2*N* HCl to form uniform fluffy mixt. Transfer mixt. to column and tamp firmly to uniform mass.

*Middle column.*—Mix 5 g diat. earth with 3 mL $FeCl_3$ soln and transfer to column as above.

*Top column.*—Accurately weigh sample contg 20 mg antipyrine into 100 mL beaker. Add 2 mL $H_2O$ and then 3 g diat. earth. Mix thoroly, transfer to column, and tamp firmly to uniform mass. Dry-wash beaker with 1 g diat. earth and tamp as above.

Top each column with small loose pad of glass wool.

### *E. Determination*

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

(**a**) *Separation of antipyrine and benzocaine.*—Arrange 3 columns in series. Rinse beaker that contained sample with 50 mL mixed solv. and transfer to top column. Elute columns with 3 addnl 25 mL portions mixed solv. Discard eluate. (Middle column contains antipyrine and bottom column contains benzocaine.) Sep. columns, and elute middle and bottom columns sep. with four 25 mL portions washed $CHCl_3$, collecting eluates in 100 mL vol. flasks. Dil. to vol. with washed $CHCl_3$.

(**b**) *Determination of antipyrine.*—Pipet 5 mL from antipyrine flask into 100 mL vol. flask contg 10 mL MeOH and dil. to vol. with washed $CHCl_3$. Det. *A* of final diln against photometric blank, (**i**), at 272 nm, using 1 cm cells. Similarly, det. $A'$ of std antipyrine soln and calc. amt of antipyrine in sample.

**(c)** *Determination of benzocaine.*—Pipet aliquot of eluate contg 0.4 mg benzocaine into 100 mL vol. flask contg 10.0 mL MeOH and dil. to vol. with washed $CHCl_3$. Det. $A$ of final diln against photometric blank, **(i)**, at 283 nm. Similarly, det. $A'$ of std benzocaine soln and calc. amt of benzocaine in sample.

**(d)** *Identification of antipyrine and benzocaine.*—Transfer remaining $CHCl_3$ eluates from antipyrine and benzocaine sepn to sep. 150 mL beakers. Place beakers in 30–40° $H_2O$ bath and evap. to dryness with gentle air current. (*Caution:* Benzocaine is volatile.)

Prep. KBr disk of each residue, using 0.8 mg residue and 200 mg KBr. Record IR spectrum of each between 2 and 16 μm and qual. compare these spectra with IR spectra of antipyrine and benzocaine stds.

Ref.: JAOAC **51,** 496, 624(1968).

CAS-60-80-0 (antipyrine)
CAS-94-09-7 (benzocaine)

## 949.15 Butacaine Sulfate in Drugs

**Final Action**

### *A. Reagents*

**(a)** *Potassium iodide soln.*—20%. Prep. fresh.

**(b)** *Starch indicator.*—Make 1.5 g sol. starch into paste with few mL $H_2O$, and add slowly, with stirring, to 300 mL boiling $H_2O$.

**(c)** *Picrolonic acid soln.*—2.5% in alcohol.

**(d)** *Potassium bromide-bromate soln.*—0.1N. Prep. as in **947.13A**. Stdze as follows: Transfer 30 mL to I flask, and add 25 mL $H_2O$, 5 mL 20% KI soln, and 5 mL HCl. Shake thoroly and titr. with 0.1N $Na_2S_2O_3$, using starch indicator (mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring const., and immediately discontinue heating. Add ca 1 mL Hg, shake, and let soln stand over the Hg).

### *B. Determination*

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

**(a)** *Ointments containing butacaine sulfate in petrolatum or other greasy base.*—Into 125 mL separator accurately weigh sample contg ca 50 mg butacaine sulfate. Add 25 mL benzene and swirl until ointment base dissolves; then add 10 mL HCl (1 + 7) and shake separator gently ca 1 min. Let layers sep., drain aq. phase into second separator, and repeat extn 4 times with 10 mL portions $H_2O$. Wash combined aq. exts with 5 mL $CCl_4$ and discard washing. Neutze soln with $NH_4OH$, add 2 mL excess, and ext butacaine base by shaking with five 15 mL portions $CHCl_3$. Filter each ext thru cotton pledget into 100 mL beaker, and evap. combined exts on steam bath under air current until no $CHCl_3$ odor remains.

Rinse down beaker wall with 2 mL alcohol delivered from pipet, warm until oily base dissolves completely, and add 1 drop HCl. Tilt and rotate beaker to wet with acidic soln any liq. on wall of beaker, and add 1 drop Me red. If soln does not react strongly acid, add addnl HCl dropwise. Dil. with few mL $H_2O$, and wash quant. into 500 mL I flask with more $H_2O$.

To soln add, from pipet, 10 mL KBr-$KBrO_3$ soln, dil. to 200 mL with $H_2O$, and add 10 mL HCl. Immediately stopper flask and swirl 5 min or until ppt coagulates. After 5 min, add 5 mL KI soln to flask, stopper, and shake vigorously. Rinse stopper and neck of flask with little $H_2O$, and titr. soln with 0.1$N$ $Na_2S_2O_3$, **942.27A**, until color is discharged. Add 15 mL starch indicator and 20 mL $CHCl_3$, stopper flask, and shake vigorously. Continue titrn, vigorously shaking stoppered flask after each addn of $Na_2S_2O_3$ soln. Add $Na_2S_2O_3$ soln dropwise as end point approaches. (During titrn, mixt. passes thru series of color changes; at end point aq. phase is colorless and emulsified $CHCl_3$ layer is nearly so.) 1 mL 0.1$N$ KBr-$KBrO_3$ = 0.00889 g $(C_{18}H_{30}N_2O_2)_2.H_2SO_4$.

To isolate bromination product for identification, transfer titrd mixt. to separator, make alk. with $NH_4OH$, and shake vigorously. Drain emulsified $CHCl_3$ layer, and to break emulsion, filter with suction thru 0.5 cm layer Hyflo Super-Cel (or similar filter-aid) supported on paper in buchner. Shake aq. phase remaining in separator with 25 mL $CHCl_3$, and pass $CHCl_3$ ext thru filter. Transfer combined filtrates to separator, filter $CHCl_3$ layer thru cotton pledget into beaker, and evap. on steam bath under air current.

To oily residue of dibromobutacaine add 2 mL picrolonic acid soln and stir. Filter ppt on Hirsch funnel, wash with 2–3 mL alcohol, dry at 105°, and det. capillary mp, alone and in admixt. with authentic dibromobutacaine picrolonate (mp 158–160° with decomposition). If ppt does not form on adding picrolonic acid soln to bromination product, seed with small crystal of dibromobutacaine picrolonate; if ppt still does not form, butacaine is absent.

**(b)** *Tablets.*—Det. av. wt/tablet. To 125 mL separator add accurately weighed, finely powd tablet mixt. contg ca 200 mg butacaine sulfate, add 25 mL $H_2O$, and swirl separator until sample dissolves. Add 2 mL $NH_4OH$ and ext with six 15 mL portions $CHCl_3$. Shake each ext with 5 mL $H_2O$ in second separator, and then filter thru cotton pledget into beaker. (If emulsion forms in aq. phase in first separator, more than 6 extns may be required. Test for complete extn by evapg seventh ext on steam bath; if appreciable residue is obtained, dissolve it in $CHCl_3$, combine with previous exts, and continue extns until complete. If aq. phase in first separator tends to emulsify, break emulsion by addn of $Na_2SO_4$ or by other means.) Evap. filtrate to small vol. on steam bath and complete detn by one of following methods:

(*1*) Quant. transfer concd soln of butacaine base to tared 50 mL beaker with $CHCl_3$, remove solv. on steam bath in air current, dry 30 min at 105°, cool in desiccator, and weigh. Wt residue × 1.160 = wt butacaine sulfate, $(C_{18}H_{30}N_2O_2)_2.H_2SO_4$.

Gravimetric detn may be checked acidimetrically as follows: Rinse down wall of beaker with 2 mL neut. alcohol delivered from pipet, warm beaker on steam bath until butacaine base dissolves completely, add 1 drop Me red, and rinse down beaker wall with another 2 mL alcohol. Titr. soln with 0.1 $N$ $H_2SO_4$, **890.01**, almost to point of color change; rinse down wall of beaker with $H_2O$, dil. to ca 45 mL, and complete titrn. 1 mL 0.1$N$ $H_2SO_4$ = 0.0355 g $(C_{18}H_{30}N_2O_2)_2.H_2SO_4$.

(*2*) Det. gravimetrically as in (*1*); then proceed as in **(a)**, beginning "Rinse down beaker wall with 2 mL alcohol . . ." except use 50 mL instead of 10 mL KBr-$KBrO_3$ soln.

(*3*) Completely remove solv. on steam bath, and proceed as in (*1*), second par. Then wash titrd soln into 500 mL I flask, pipet in 50 mL KBr-$KBrO_3$ soln, dil. to 200 mL with $H_2O$, add 10 mL HCl, and proceed as in **(a)**, beginning "Immediately stopper flask . . ."

**(c)** *Crystals.*—Accurately weigh ca 200 mg sample into 125 mL separator, add 25 mL $H_2O$, and swirl separator until sample dissolves. Continue as in **(b)**, beginning "Add 2 mL $NH_4OH$ . . ."

**(d)** *Solns.*—Transfer to 125 mL separator aliquot contg ca 200 mg butacaine sulfate, and if necessary, dil. to 25 mL with $H_2O$. Proceed as in **(b)**, completing detn by **(b)**(*1*), (*2*), or (*3*) if chlorobutanol is absent, and only by **(b)**(*3*) if chlorobutanol is present.

Refs.: JAOAC **32,** 548(1949); **33,** 206(1950).

CAS-149-15-5 (butacaine sulfate)

## Procaine in Drugs
### Qualitative Tests

*See* **930.40**.

## 975.57 Procaine in Drugs
### Quantitative Methods
### First Action 1975
### Final Action 1976
#### *Method I—with or without Propoxycaine*

(Applicable in presence of parabens and phenolic vasoconstrictors)

#### *A. Apparatus and Reagents*

(**a**) *Chromatographic tubes.*—Fuse 6 cm length of 5–6 mm tubing to piece of 25 mm tubing ca 25 cm long (25 × 200 mm test tube may be used). Constrict stem slightly ca 2 cm below seal. Com. tubes with dimensions ± 10% are satisfactory. Pack wad of pyrex glass wool in base as support.

(**b**) *Tamping rod.*—Flatten end of glass rod to circular head with clearance of ca 1 mm in tube (**a**). Or use disk of stainless steel, Al, etc., of diam. ca 1 mm less than id of column, (**a**), attached to 30–45 cm (12–18″) rod.

(**c**) *Bromide-citrate buffer.*—Mix equal vols 0.5*M* Na citrate (147 g $2H_2O$/L) and 0.5*M* citric acid (105 g $1H_2O$/L). Adjust to pH 4.0 ± 0.2, using pH meter, by addn of appropriate citrate soln. Add 10.3 g NaBr/100 mL soln (1.0*M* Br) and mix.

(**d**) *Chloroform-isooctane solvent.*—65% $CHCl_3$ in isooctane. Do not sat. with $H_2O$ before use. Dil. 65 parts $CHCl_3$ in graduate to 100 parts with isooctane and mix.

(**e**) *Phosphate buffer.*—pH 7.0 ± 0.2. Mix equal vols 0.5*M* $KH_2PO_4$ (68.0 g/L) and 0.5*M* $K_2HPO_4$ (87.1 g/L).

(**f**) *Procaine hydrochloride and propoxycaine hydrochloride std solns.*—Prep. sep. aq. solns contg 10 μg/mL.

#### *B. Preparation of Sample and Column*

Pipet 1 mL sample soln contg 20 mg procaine.HCl or 4 mg propoxycaine.HCl into beaker, add 1.0 mL phosphate buffer, and mix. Add 3 g diat. earth, **960.53B**, and mix.

Place small glass wool plug in base of chromatgc tube. Mix 4 g diat. earth and 3 mL bromide-citrate buffer in small beaker, transfer to column, and tamp with gentle pressure. Mix 2 g diat. earth and 1 mL 0.1*N* NaOH, add to column, and tamp. Quant. transfer sample mixt. to column and tamp. Scrub beaker with 1 g diat. earth and 2–3 drops phosphate buffer, add to column, and tamp. Cover with pad of glass wool.

#### *C. Determination*

(Perform elution for propoxycaine even in its absence.)

(**a**) *Propoxycaine.*—Place 200 mL vol. flask under column and elute with 150 mL 65% $CHCl_3$ in isooctane. Dil. to vol. with $CHCl_3$ and mix. Pipet 10 mL aliquot into 100 mL beaker, add 4 drops HOAc, and evap. nearly to dryness on steam bath under gentle air current, then to dryness with reduced heat. Pipet 20 mL $H_2O$ into beaker and dissolve residue.

(**b**) *Procaine.*—Place 200 mL vol. flask under column and elute with 125 mL $CHCl_3$. Dil. to vol. with $CHCl_3$ and mix. Pipet 10 mL aliquot into 100 mL beaker and evap. to dryness as in (**a**), but without addn of HOAc. Dissolve residue in $H_2O$ and transfer quant. to 100 mL vol. flask, dil. to vol. with $H_2O$, and mix.

(**c**) *Spectrophotometry.*—Record spectra of sample and std solns from 350 to 250 nm and det. *A* of samples and *A*′ of stds at max., ca 302 and 290 nm, for propoxycaine and procaine, resp.

$$C = C' \ (A/A') \ D$$

where $C$ and $C'$ = concns (μg/mL) of sample and std, resp., and $D$ = appropriate diln factor.

Ref.: JAOAC **58,** 88, 93(1975).

CAS-59-46-1 (procaine)
CAS-51-05-8 (procaine hydrochloride)
CAS-86-43-1 (propoxycaine)
CAS-550-83-4 (propoxycaine hydrochloride)

#### *D. Method II★ —Surplus 1975*

(Dets as procaine any *p*-aminobenzoic acid formed by decomposition)

*See* **38.211**, 12th ed.

#### *E. Method III*

(Dets only undecomposed procaine)

*See* **961.18B**.

#### *F. Method IV★ —Surplus 1975*

(Applicable in presence of chlorobutanol, cocaine, codeine, heroin, lactose, and morphine)

*See* **32.096**, 10th ed.

## 976.32 Procainamide Hydrochloride in Drugs
### Spectrophotometric Method
### First Action 1976
### Final Action 1982

#### *A. Principle*

Procainamide is extd from acid soln with $CHCl_3$. After evapn of solv., residue is dissolved in alk. soln and max. *A* detd at ca 272 nm.

#### *B. Reagent*

*Procainamide hydrochloride std solns.*—*Stock soln.*—1 mg/mL. Assay std as in USP XX. Accurately weigh ca 100 mg procainamide.HCl (ICN-K&K Laboratories, Inc., A Div. of ICN Biomedicals, Inc., PO Box 28050, Cleveland, OH 44128-0250, No. 17158) into 100 mL vol. flask, and dissolve and dil. to vol. with 0.01*N* NaOH. *Working soln.*—1 mg/100 mL. Pipet 1 mL stock soln into 100 mL vol. flask and dil. to vol. with 0.01*N* NaOH. Prep. fresh daily.

#### *C. Preparation of Sample*

(**a**) *Capsules.*—Proceed as in **927.09B**. Mix, and transfer accurately weighed portion contg ca 100 mL procainamide.HCl to 125 mL separator. Add 10 mL HCl (1 + 9) and shake to disperse. Add 15 mL $H_2O$.

(**b**) *Tablets.*—Det. av. wt. Reduce tablets to fine powder.

(For tablets with coatings that do not reduce to powder, carefully peel off and discard coatings, and reduce tablets to fine powder.) Mix, and transfer accurately weighed portion contg ca 100 mg procainamide.HCl to 125 mL separator. Add 10 mL HCl (1 + 9) and shake to disperse. Add 15 mL $H_2O$.

(c) *Injections.*—Dil. soln, if necessary, with HCl (1 + 9) to give ca 100 mg procainamide.HCl/mL. Pipet 1 mL into 125 mL separator, add 10 mL HCl (1 + 9), and shake. Add 15 mL $H_2O$.

#### D. Determination

(*Caution:* See safety notes on distillation and chloroform.)

Ext sample soln with three 25 mL portions $CHCl_3$ and discard $CHCl_3$. To aq. soln add 5 mL $NH_4OH$ and ext with five 25 mL portions $CHCl_3$, collecting exts in 250 mL beaker and rinsing tip of separator into beaker after each extn. Evap. $CHCl_3$ to dryness on steam bath with air current. Quant. transfer residue to 100 mL vol. flask with 0.01*N* NaOH, and dil. to vol. with same solv. Pipet 1 mL dild soln into another 100 mL vol. flask and dil. to vol. with 0.01*N* NaOH. Record spectra of std and sample solns against 0.01*N* NaOH and det. *A* at max., ca 272 nm.

$$\text{mg Procainamide.HCl/capsule or tablet} = (A/A') \times C \times D \times (W/W')$$

where $A$ and $A'$ refer to sample and std solns, resp.; $C$ = concn of std soln in mg/mL; $D$ = sample diln factor in mL; $W$ = av. mg/capsule or tablet; and $W'$ = mg sample.

$$\text{For injections, mg procainamide.HCl/mL} = (A/A') \times C \times D/V$$

where $V$ = mL sample aliquot.

Ref.: JAOAC **59,** 807(1976).

CAS-614-39-1 (procainamide hydrochloride)

## SYNTHETICS

### 938.14 Phenazopyridine Hydrochloride in Drugs

**Titrimetric Method**

**Final Action**

#### A. Reagents

(a) *Titanium trichloride std soln.*—Prep. as in **948.28A** and stdze as in **948.28B**.

(b) *Light green SF yellowish soln.*—Dissolve 1 g (Use C.I. No. 42095.) in $H_2O$ and dil. to 1 L.

#### B. Preparation of Solution

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

(a) *Solns.*—To vol. contg ca 0.1 g phenazopyridine.HCl, add 10 mL 0.1*N* HCl and dil. to 100 mL.

(b) *Tablets and jelly.*—Accurately weigh sample (powd in case of tablets) contg ca 0.1 g phenazopyridine.HCl, add 10 mL 0.1*N* HCl, and dil. to 100 mL.

(c) *Ointments.*—Accurately weigh, in 100 mL beaker, sample contg ca 0.1 g phenazopyridine.HCl, stir with ether until ointment base dissolves, and wash into separator with ether and $H_2O$. Shake thoroly, and drain aq. layer into second separator contg 25 mL ether. Shake, and drain aq. layer into third separator contg 25 mL ether. Shake, and transfer aq. layer to 250 mL beaker. Wash ether layers with alternate 10 mL portions HCl (1 + 1) and $H_2O$ until no more color is removed, successively passing each portion of the HCl or $H_2O$ thru the 3 separators and finally into beaker. Nearly neutze combined acid exts with $NH_4OH$, cool, wash into separator, make ammoniacal, and ext with 25 mL portions $CHCl_3$ until no more color is removed, filtering $CHCl_3$ thru cotton pledget in stem of separator. Evap. combined $CHCl_3$ exts just to dryness, take up in 10 mL 0.1*N* HCl, and dil. to 100 mL.

#### C. Determination

Heat soln to bp, add 15 g *Na acid tartrate,* and boil 2 min. Add 10 mL light green SF yellowish soln and titr. hot with std $TiCl_3$ soln in current of $CO_2$. End point is change from green to pale yellow. Perform blank titrn with 10 mL 0.1*N* HCl, 90 mL $H_2O$, 15 g Na acid tartrate, and 10 mL light green SF yellowish soln, and subtract from vol. $TiCl_3$ previously found. 1 mL 0.1*N* $TiCl_3$ = 0.00624 g phenazopyridine.HCl, $C_{11}H_{11}N_5$.HCl.

Ref.: JAOAC **21,** 552(1938).

CAS-136-40-3 (phenazopyridine hydrochloride)

### 983.28 Amitriptyline in Drug Tablets and Injectables

**Liquid Chromatographic Method**

**First Action 1983**
**Final Action 1986**

#### A. Principle

Amitriptyline content of tablets and injectables is detd by liq. chromatography, using trifluoperazine as internal std and UV detection at 239 nm.

#### B. Apparatus and Reagents

(a) *Liquid chromatograph.*—Tracor Model 950 solvent pump (replacement Model 951), Model 970A variable wavelength detector (replacement Model 971), Model 26325 recorder (replacement Model 1200) (Tracor Instruments Inc.,), and 20 μL loop injector. *Operating conditions:* column temp., ambient; solv. flow rate, 1.33 mL/min; detector wavelength, 239 nm; attenuation, 16 AUFS; recorder, 1 mV; chart speed, 1 in./4 min.

(b) *Chromatographic column.*—Stainless steel, 300 × 3.9 mm id, packed with 10 μm μBondapak CN (Waters Associates, Inc.), or equiv.

(c) *Methanol.*—AR grade (Fisher Scientific Co.).

(d) *Mobile phase.*—MeOH–0.005M ammonium acetate (90 + 10).

(e) *Culture tubes.*—95 × 25 mm with screw cap (Kimble Glass Inc.).

(f) *Internal std soln.*—Accurately prepare ca 0.5 mg Trifluoperazine HCl Ref. Std/mL MeOH.

(g) *Std soln.*—0.04 mg/mL. Accurately weigh ca 10 mg USP Amitriptyline HCl Ref. Std and transfer to 250 mL vol. flask. Dissolve in 1 mL MeOH, add 25.0 mL internal std soln, dil. with MeOH, and mix.

#### C. Sample Preparation

(a) *Tablets.*—Weigh and finely powder ≥20 tablets. Accurately weigh and transfer amt of powd. equiv. to 10 mg amitriptyline HCl into screw-cap culture tube and add 25.0 mL internal std soln. Tumble on rotator 15 min at ca 30 rpm, and filter, if necessary. Dil. accurately measured vol. of soln with MeOH to ca 0.04 mg/mL.

(b) *Single tablet.*—Place one tablet in 95 × 25 mm screw-cap culture tube and crush to fine powd. with glass rod. Add 25.0 mL MeOH and mix. Tumble on rotator 15 min at ca 30 rpm, and filter, if necessary. Pipet accurately measured aliquot ($A$) of this soln, equiv. to 2 mg amitriptyline HCl, into 50 mL vol. flask, add 5.0 mL internal std soln, dil. to vol. with MeOH, and mix.

(**c**) *Injectables*.—Accurately pipet vol. of injectable, equiv. to 10 mg amitriptyline HCl, into 50 mL vol. flask, add 25.0 mL internal std soln, dil. to vol. with MeOH, and mix. Dil. accurately measured vol. of this soln with MeOH to ca 0.04 mg/mL.

### D. Determination

Equilibrate system with mobile phase at 1.33 mL/min, until baseline is steady. Inject measured vol. of std soln into chromatograph by microsyringe or sampling valve. Adjust injection vol. and operating conditions so amitriptyline HCl in std soln injection gives peak ht ca 60% full scale and retention time ca 7 min. Under these conditions, 5 replicate injections of std soln should give coefficient of variation of $\leq 3\%$ and resolution factor ($R$) between the 2 main peaks should be $\geq 1$. Make alternate injections of equal vols. of sample and std solns. Measure peak hts for amitriptyline HCl and internal std in sample and std solns, and det. response ratios.

### E. Calculations

$$\text{Tablets: mg/tablet} = RR/RR' \times C \times T/W$$

$$\text{Single tablet: mg/tablet} = RR/RR' \times C \times 5/A$$

$$\text{Injectables: mg/mL} = RR/RR' \times C/V$$

where $RR$ and $RR'$ = ratio of amitriptyline HCl peak ht to internal std peak ht for sample and std solns, resp.; $C$ = mg amitriptyline HCl in 250 mL std soln; $T$ = av. tablet wt, g; $W$ = wt sample taken, g; $A$ = aliquot taken, mL; $V$ = vol. injectable taken, mL.

Ref.: JAOAC **66,** 1196(1983).

CAS-549-18-8 (amitriptyline hydrochloride)

## Meperidine in Drugs

*See* **945.69**.

## 984.38 Methocarbamol in Drugs

### Liquid Chromatographic Method

**First Action 1984**
**Final Action 1988**

(Applicable to injectables and tablets)

### A. Principle

Methocarbamol is dissolved in aq. MeOH and detd by liq. chromatgy with acetanilide internal std, $H_2O$-MeOH mobile phase, and detection at 280 nm.

### B. Apparatus

(**a**) *Liquid chromatograph*.—Equipped with Model U6K injector, Model 6000A solv. delivery system, and Model 440 detector (Waters Associates, Inc.) or equiv. Operating conditions: flow rate 1.8 mL/min; 280 nm detector at 0.10 AUFS; temp. ambient; vol. injected 20 μL.

(**b**) *LC column*.—Zorbax ODS, 5–6 μm particle size, 4.4 mm id by 25 cm long (E.I. DuPont), or equiv.

(**c**) *Integrator*.—HP 3380A (replacement Models HP3394A or HP3396A) (Hewlett-Packard, Avondale Div.), or equiv.

(**d**) *Filters*.—Millipore type HAWP, pore size 0.45 μm (Millipore Corp.), or equiv.

### C. Reagents

(**a**) *Solvents*.—$H_2O$ and MeOH, each of suitable LC grade; degas before use.

(**b**) *Internal std*.—Acetanilide, 6 mg/mL $H_2O$–MeOH (75 + 25).

(**c**) *Methocarbamol std soln*.—Transfer ca 50 mg, accurately weighed, USP Methocarbamol Ref. Std to 100 mL vol. flask, add ca 23 mL MeOH and 10.0 mL internal std, dil. to vol. with $H_2O$, and mix thoroly.

(**d**) *LC mobile phase*.—$H_2O$–MeOH (75 + 25); degas.

### D. Sample Preparation

(**a**) *Injections*.—Transfer accurately measured vol. of methocarbamol injection equiv. to ca 500 mg to 100 mL vol. flask, add 25 mL MeOH and dil. to vol. with $H_2O$. Transfer 10.0 mL of this diln and 10.0 mL internal std to second 100 mL vol. flask, add 20 mL MeOH, and dil. to vol. with $H_2O$ (sample soln).

(**b**) *Tablets*.—Transfer accurately weighed portion of finely ground tablet composite equiv. to 250 mg methocarbamol to 200 mL vol. flask, add 50 mL MeOH, mix thoroly, and add $H_2O$ to vol. Transfer 20.0 mL of this initial diln plus 5.0 mL internal std to 50 mL vol. flask, add 6–7 mL of MeOH, and dil. to vol. with $H_2O$ (sample soln).

Filter sample soln thru Swinny filter (Millipore Cat. No. XX 30 012 00) contg 0.45 μm Millipore filter (HAWP 01300) and attached to Hamilton gas-tight syringe (No. 1010 W) fitted with Luer-lok tip.

### E. Standardization

Set mobile phase flow rate at 1.8 mL/min and inject 20 μL methocarbamol std soln. Retention time for methocarbamol (2nd peak) should be 19 ± 2 min. Resolution, $R = 2\ (t_2 - t_1)/(W_2 + W_1)$, should be not <6 for methocarbamol and internal standard peak, where $t_1$ = retention of internal std; $t_2$ = retention of methocarbamol; $W_1$ = width of base of internal std peak; $W_2$ = width of base of methocarbamol peak.

Adjust mobile phase ratios and/or flow rates as necessary to give required retention times and/or resolution.

### F. Determination

Make 20 μL injections of std and sample solns. Measure peak areas and calc. response ratios (methocarbamol peak to internal std peak) for std and sample solns. Calc. methocarbamol:

$$\text{Liqs, mg/mL} = (R/R') \times (C/V) \times 1000$$

$$\text{Tablets, mg/tab.} = (R/R') \times (C/W) \times 500 \times T$$

where $R$ and $R'$ = response ratios for sample and std solns, resp.; $C$ = concn methocarbamol std soln, mg/mL; $V$ = mL injection taken for analysis; $T$ = av. tablet wt, g; $W$ = sample wt, g.

Ref.: JAOAC **67,** 225(1984).

CAS-532-03-6 (methocarbamol)

## 926.17★ Nitroglycerin in Drugs

### Reduction Method

**Final Action**
**Surplus 1980**

*See* **39.104–39.106**, 13th ed.

## 958.13 Nitroglycerin in Drugs
### Infrared Spectrophotometric Method
### Final Action 1965

#### A. Reagent

*Nitroglycerin std.*—Absorbate on lactose contg ca 10% nitroglycerin. Stdze as in **958.14** except use $k$ = 89.04 for $NaNO_3$ and 74.87 for $KNO_3$. This product is stable indefinitely in tightly stoppered bottle.

#### B. Determination

(*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, and carbon disulfide.)

Transfer number of tablets contg ca 5 mg nitroglycerin to small separator. Dissolve or suspend in 5 mL $H_2O$, add 20 mL $CS_2$, shake 1 min, and let sep. Filter $CS_2$ layer thru pledget of cotton previously washed with $CS_2$ and collect in 100 mL beaker. Repeat extn with three 10 mL portions $CS_2$. Evap. combined exts to ca 3 mL, using gentle current of air, at temp. ≤50°. Transfer quant. to 5 mL vol. flask and dil. to vol. with $CS_2$.

Accurately weigh amt std absorbate contg ca 5 mg nitroglycerin. Transfer to small separator and ext as above.

Det. baseline $A_B$ of sample and std solns relative to $CS_2$ at 7.89 μm, drawing baseline between min. at 7.5 and 8.3 μm. Calc. nitroglycerin content of sample. Record spectra of sample and std solns from 2 to 15 μm and compare for identity of sample.

Ref.: JAOAC **41,** 504(1958).

CAS-55-63-0 (nitroglycerin)

## 960.54 Nitrate Esters in Drugs
### Infrared Spectrophotometric Method
### First Action 1960
### Final Action 1965

(Applicable to mannitol hexanitrate, erythrityl tetranitrate, or pentaerythrityl tetranitrate)

#### A. Apparatus

(**a**) *Recording infrared spectrophotometer.*—With two 1.0 mm liq. absorption cells with NaCl windows, preferably matched or of known *A* difference, and KBr disk holder.

(**b**) *Chromatographic tube.*—25 × 200 mm with 5 × 40 mm stem.

(**c**) *Die and hydraulic press.*—Suitable for prepg KBr disks.

#### B. Preparation of Standard Solution

Ext ester from com. absorbate (usually 10% on lactose or other inert diluent) with ether, filter, and evap. to dryness with aid of air current at temp. ≤50°. Dry in vac. desiccator 1 hr. Prep. std soln contg 0.5 mg ester/mL $CHCl_3$.

(*Caution:* Pure crystalline nitrate esters are very explosive, especially pentaerythrityl tetranitrate. Do not use sample contg >5 mg pure compd.)

#### C. Preparation of Sample

Reduce tablets to fine powder. Weigh sample contg ca 25 mg nitrate ester and transfer to 125 mL separator with ca 5 mL $H_2O$. Make distinctly acid with $H_2SO_4$ (1 + 9). Proceed as in (**a**) in absence of phenobarbital, or (**b**) in presence of phenobarbital.

(**a**) Add 10 mL $CHCl_3$ to separator, shake vigorously several min, and let sep. Transfer $CHCl_3$ layer to 50 mL vol. flask. Ext aq. soln with three addnl 10 mL portions $CHCl_3$ and transfer each ext to vol. flask. Dil. to vol. with $CHCl_3$, mix, and filter.

(**b**) Add 15 mL $CHCl_3$ to separator, shake vigorously several min, and let sep. Transfer $CHCl_3$ layer to chromatgc column contg 4 mL 1*M* $K_3PO_4$ soln adsorbed on 5 g diat. earth, **960.53B**, collecting eluate in 50 mL vol. flask. Ext. aq. soln with three addnl 10 mL portions $CHCl_3$, and pass each ext thru column, collecting eluate in vol. flask. Dil. to vol. with $CHCl_3$, mix, and filter.

#### D. Determination

(Store $CHCl_3$ to be used in IR measurements in stoppered flask.)

Transfer 5 mL aliquot $CHCl_3$ soln to 25 mL g-s erlenmeyer, evap. to dryness with aid of air current at temp. ≤50°, and complete drying in vac. desiccator. Add 5.00 mL $CHCl_3$ to residue, stopper flask tightly, and let stand 30 min with occasional shaking to ensure complete soln. Det *A* of std and sample solns against $CHCl_3$ at max. (ca 6.0 μm) and calc. amt of ester per tablet.

Evap. another portion $CHCl_3$ soln to dryness as above. Prep. KBr disk by grinding together in agate mortar 1 mg residue with 200 mg IR spectral grade KBr and pressing in die and hydraulic press. Record spectrum from 2 to 15 μm and compare with spectrum of std nitrate ester to det. identity of sample.

Ref.: JAOAC **43,** 259(1960).

CAS-7297-25-8 (erythrityl tetranitrate)
CAS-15825-70-4 (mannitol hexanitrate)
CAS-78-11-5 (pentaerythrityl tetranitrate)

## 958.14 Mannitol Hexanitrate; Mannitol Hexanitrate and Phenobarbital in Drugs
### Spectrophotometric Methods
### Final Action

(Ascorbic acid interferes. *See also* **960.54**.)

#### A. Reagents

(**a**) *Phenoldisulfonic acid.*—Heat 5 g colorless phenol, 30 mL $H_2SO_4$, and 15 mL fuming $H_2SO_4$ (ca 20% free $SO_3$) on steam bath 2 hr. (*Caution:* See safety notes on sulfuric acid and fuming acids.)

(**b**) *Nitrate std soln.*—Dissolve 100 mg $KNO_3$ or $NaNO_3$ in ca 1 mL $H_2O$ and dil. to 100 mL with HOAc.

(**c**) *Phenobarbital std soln.*—In 100 mL vol. flask dissolve 100 mg phenobarbital and dil. to vol. with HOAc. Pipet 5 mL of this soln and 15 mL HOAc into 100 mL vol. flask, dil. to vol. with $H_2O$, and filter, discarding first 5 mL filtrate.

#### B. Preparation of Sample

Transfer accurately weighed sample contg ca 30 mg mannitol hexanitrate to 50 mL vol. flask, and dil. to vol. with HOAc. Shake well and filter, discarding first 5 mL filtrate.

#### C. Determination of Mannitol Hexanitrate

Transfer 1.0 mL aliquots of sample, std, and HOAc (blank) to individual 100 mL vol. flasks, add 2.0 mL phenoldisulfonic acid to each, and let stand 15 min. Dil. with $H_2O$ to ca 60 mL, add $NH_4OH$ (ca 10 mL) until max. yellow color appears, cool to room temp., dil. to vol. with $H_2O$, and mix. Det. *A* of sample and std, *A'*, relative to blank at 408 nm.

% Mannitol hexanitrate = $(A \times R_2 \times k \times 50)/(A' \times R_1)$

where $R_1$ is mg sample, $R_2$ is mg std/mL, and $k$ is 88.66 for $NaNO_3$ and 74.56 for $KNO_3$ std.

### D. Determination of Phenobarbital

Pipet 10 mL aliquot sample soln into 50 mL vol. flask, dil. to vol. with $H_2O$, shake, and filter, discarding first 5 mL filtrate. Prep. blank by dilg 10.0 mL HOAc to 50 mL with $H_2O$ and filtering. Dil. sep. 20 mL aliquots of std, sample, and blank solns to 100 mL with $NH_4OH$ (1 + 9), adjusting to room temp. before dilg. to vol. (Final pH of soln, 9.0–9.6.) Det. $A$ of sample and std relative to blank at 240 nm, and calc. phenobarbital content.

Ref.: JAOAC **41,** 493(1958).

CAS-15825-70-4 (mannitol hexanitrate)
CAS-50-06-6 (phenobarbital)

## 964.25 Pentaerythrityl Tetranitrate in Drugs

### Spectrophotometric Method

### Final Action 1965

(Applicable in presence of meprobamate)

Accurately weigh powd sample contg ca 40 mg pentaerythrityl tetranitrate into 50 mL g-s vol. flask. Add ca 30 mL HOAc, shake ca 1 min, and dil. to vol. with HOAc. Filter, discarding first 5 mL filtrate. Proceed as in **958.14C**.

% Pentaerythrityl tetranitrate = $(A \times R' \times k \times 50)/(A' \times R)$

where $A$ and $A'$ refer to sample and std, resp.; $R$ and $R'$ are mg sample and mg std/mL, resp.; and $k$ is 92.99 for $NaNO_3$ or 78.18 for $KNO_3$ std.

Ref.: JAOAC **47,** 469(1964).

CAS-78-11-5 (pentaerythrityl tetranitrate)

## 970.79 Pentaerythrityl Tetranitrate and Meprobamate in Drugs

### Infrared Spectroscopic Method

### First Action 1970
### Final Action 1971

(*Caution:* PETN may explode when heated strongly, even when dissolved.)

### A. Apparatus

*See* **960.54A**.

### B. Reagents

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

(**a**) *Dilute phosphoric acid.*—3 + 1. Dil. 3 vols 85% $H_3PO_4$ with 1 vol. $H_2O$.
(**b**) *Water-washed benzene.*—Shake equal vols benzene and $H_2O$ 1 min in separator. Discard lower phase. Use within 2 days of prepn.
(**c**) *Water-washed chloroform.*—Shake equal vols. $CHCl_3$ and $H_2O$ 1 min in separator. Discard upper layer. Use within 2 days of prepn.
(**d**) *Anhydrous chloroform.*—Filter $H_2O$-washed $CHCl_3$ thru anhyd. $Na_2SO_4$.
(**e**) *Pentaerythrityl tetranitrate (PETN) std soln.*—10 mg/50 mL. Ext PETN from com. PETN (usually 10% on lactose or other inert diluent) with $CHCl_3$ to give ca 20 mg pure PETN. Filter and evap. to dryness under air current with little or no heat. Dry in vac. desiccator 2 hr. Accurately weigh ca 10 mg, using microbalance, dissolve in anhyd. $CHCl_3$, and dil. to 50 mL with this solv. Destroy excess PETN by dissolving in acetone and burning in large vessel behind safety barrier, using effective fume removal device.
(**f**) *Meprobamate std soln.*—80 mg/100 mL. Dissolve 80 mg USP Meprobamate Ref. Std in anhyd. $CHCl_3$ and dil. to 100 mL with this solv.
(**g**) *Diatomaceous earth.—See* **960.53B**.

### C. Determination

(Use $H_2O$-washed solvs unless designated anhyd. *Caution:* See safety notes on distillation, flammable solvents, benzene, and chloroform.)

Loosely pack small amt of fine glass wool in base of chromatgc tube to support diat. earth. Weigh 3 g diat. earth into 100 mL beaker, add 2.0 mL 1*N* NaOH, mix with metal spatula until fluffy, and pack uniformly in tube. Weigh 5 g diat. earth into 250 mL beaker, add 7.0 mL dil. $H_3PO_4$, mix until fluffy, and pack uniformly on column. (Do not pack too tightly as column will elute too slowly.) Accurately weigh portion powd sample contg ca 10 mg PETN into 150 mL beaker. Add 4 mL benzene and heat gently with swirling ca 1 min. Cool, add 4 g diat. earth, mix until fluffy, transfer quant. to column, and pack uniformly. Dry-wash beaker with 1 g diat. earth and 0.5 mL benzene, transfer to column, and pack uniformly. Wipe sample beaker and all app. used in column prepn with glass wool, and pack on column.

Pass 75 mL benzene thru column and collect eluate in 150 mL beaker until elution ceases. Rinse column tip with small portions benzene into beaker and set aside. This fraction contains PETN.

Place 250 mL beaker under column. Add 4.0 mL $H_2O$ to column and let it be absorbed. Pass 150 mL $CHCl_3$ thru column and collect eluate in 250 mL beaker. Rinse column tip with $CHCl_3$ into beaker. This fraction contains meprobamate.

Evap. each fraction on steam bath under gentle air current to ca 10 mL and take to dryness with little or no heat from steam bath. Place beakers in vac. oven 30 min at 30° and $\leq$380 mm (15″) Hg. Remove from oven. Add ca 10 mL anhyd. $CHCl_3$ to PETN beaker and heat gently to dissolve residue. Quant. transfer with anhyd. $CHCl_3$ to 50 mL vol. flask and dil. to vol. with anhyd. $CHCl_3$.

Dissolve meprobamate residue with ca 20–25 mL anhyd. $CHCl_3$. If theoretical wt of meprobamate in sample wt taken is 100 mg, transfer quant. to 100 mL vol. flask with anhyd. $CHCl_3$ and dil. to vol. with this solv. If theoretical wt of meprobamate is ca 200 mg, use 250 mL vol. flask and proceed as above.

Scan sample and std solns in 1.0 mm cells from 5.0 to 6.5 $\mu$m (2000–1540 $cm^{-1}$) on IR spectrophtr against anhyd. $CHCl_3$ as ref.

Calc. PETN by subtracting $A$ at 5.5 $\mu$m (1818 $cm^{-1}$) from $A$ at ca 6.02 $\mu$m (1660 $cm^{-1}$) and compare with std $A$. (*Note:* PETN sample solns may contain very small peak at ca 5.8 $\mu$m (1722 $cm^{-1}$). This is contaminant of meprobamate and does not interfere with PETN detn. Also, a peak may appear at ca 6.25 $\mu$m (1600 $cm^{-1}$). This is $H_2O$ peak. Disregard this peak in calcg PETN net $A$.)

Calc. meprobamate by subtracting $A$ at 5.5 $\mu$m (1818 $cm^{-1}$) from $A$ at ca 5.82 $\mu$m (1718 $cm^{-1}$) and compare with std $A$.

#### D. Identification

(a) *PETN*.—Prep. both std and sample KBr disks from respective assay solns. Evap. 4–5 mL of each soln in small mortar, add 200 mg KBr, mix thoroly, and press. Scan spectrum from 2 to 15 μm (5000–667 $cm^{-1}$). Compare sample and std curves. (*Note:* Sample IR curve may deviate from std curve. This deviation is caused by meprobamate contaminant. However, all major peaks in std and sample should be evident.)

(b) *Meprobamate*.—Prepare KBr disks as above from 1 mL sample and std assay solns. Scan and compare as in (a).

Ref.: JAOAC **53,** 594(1970).

CAS-57-53-4 (meprobamate)
CAS-78-11-5 (pentaerythrityl tetranitrate)

## 980.34 Nitroglycerin in Sublingual Drug Tablets

### Semiautomated Method

**First Action 1980**
**Final Action 1982**

#### A. Principle

Nitroglycerin is hydrolyzed in aq. alkali to nitrite which diazotizes procaine.HCl. Product is coupled with *N*-1-naphthylethylenediamine.2HCl, and *A* is measured at 550 nm.

#### B. Apparatus

(a) *Automatic analyzer*.—With following modules (Technicon Instruments Corp., or equiv.): Sampler with 30/hr (2:1) cam; proportioning pump; const temp. bath (50°) with one 40′ (12 m) × 1.6 mm id coil; colorimeter with 15 × 2.0 mm id flowcell and matched 550 nm filters, or spectrophtr with 10 mm flowcell; recorder; manifold (*see* Fig. **980.34**). Use glass interconnecting tubing thruout, unless otherwise specified.

(b) *Shaker*.—Wrist action.

#### C. Reagents

(Use deionized or distilled $H_2O$ thruout.)

(a) *Strontium hydroxide soln*.—1%. Add 20.0 g $Sr(OH)_2.8H_2O$ to 1.8 L recently boiled $H_2O$, and heat to dissolve. (Turbidity due to $SrCO_3$ may remain.) Cool, dil. to 2 L with $H_2O$, and mix. Let stand overnight, filter thru paper, and protect from $CO_2$.

(b) *Procaine soln*.—0.3%. Dissolve 3.0 g procaine.HCl in $H_2O$, dil. to 1 L, and mix.

(c) *Hydrochloric acid*.—(1 + 4).

(d) *Coupling reagent*.—*0.1% aq. N*-naphthylethylenediamine. 2HCl. Prep. fresh weekly and store in dark glass bottle in refrigerator.

(e) *Nitroglycerin std soln*.—15 μg/mL. Accurately weigh 75 mg 10% nitroglycerin lactose absorbate, stdzd as in **958.14**, except use $k$ = 89.04 for $NaNO_3$ and 74.87 for $KNO_3$, into 500 mL vol. flask. Add 300 mL $H_2O$, shake vigorously, and dil. to vol. with $H_2O$.

#### D. Preparation of Sample

Place individual tablet or weighed composite into suitable g-s container, and add accurately measured vol. $H_2O$ to give nitroglycerin concn ca 15 μg/mL. Shake vigorously mech. until drug is in soln (5–15 min).

#### E. Analytical System

Sample is withdrawn, segmented with air, and hydrolyzed with $Sr(OH)_2$ in 50° heating bath. Soln is resampled, segmented with air, and mixed with procaine.HCl, HCl, and coupling reagent in succession. After time delay, *A* is read at 550 nm.

#### F. Start-Up and Shut-Down Operations

Pump $H_2O$ thru all lines for 5 min; then place all lines in resp. solns and pump until steady baseline is obtained. For shut-down, pump $H_2O$ thru all lines for 5 min; then remove lines and pump dry.

#### G. Determination

Fill 2 mL sample cups in following order: 3 cups std soln, 5 cups sample soln, 1 cup std soln, 5 cups sample soln, etc., ending with 2 cups std soln. (First 2 cups of std soln are used to equilibrate system, but are not included in calcns.) Start Sampler II or IV. After last cup has been sampled, let system operate until steady baseline is obtained. Draw tangent to initial and final baselines. Subtract baseline to det. net *A* and *A′* for each sample and std peak, resp. Using av. of 2 stds which bracket sample peak, calc. nitroglycerin as follows:

$$\text{mg Nitroglycerin in portion taken} = (A/A') \times C \times D$$

where $C$ = concn of std in mg/mL and $D$ = diln factor.

Ref.: JAOAC **63,** 696(1980).

CAS-55-63-0 (nitroglycerin)

## 972.48 Dichlorophene in Drugs

### Spectrophotometric Method

**First Action 1972**
**Final Action 1974**

#### A. Principle

Dichlorophene is extd from acid soln with $CHCl_3$ and detd by measuring *A* in alc. NaOH soln at 305 nm.

#### B. Reagent

*Dichlorophene std solns*.—(*1*) *Stock soln*.—600 μg/mL. Dissolve 60 mg dichlorophene (Sigma Chemical Co., Inc.) in alcohol and dil. to 100 mL. (*2*) *Working soln*.—15 μg/mL. Dil. 5.0 mL stock soln to 200 mL with 0.1*N* NaOH. Prep. fresh daily.

#### C. Preparation of Sample

(a) *Soft gelatin capsules*.—Select representative number (5–20) of capsules. Treat each capsule, one at a time, as follows: Using sharp scalpel, cut capsule lengthwise and transfer to funnel placed in neck of 500 mL vol. flask. Thoroly rinse capsule contents, scalpel, and funnel with alcohol. Remove funnel and carefully transfer opened capsule to vol. flask. Dil. to vol. with alcohol and mix. Prep. soln contg ca 0.3 mg dichlorophene/mL by stepwise diln with alcohol.

(b) *Suspension*.—Mix thoroly. Using 10 mL "to contain" pipet with wide orifice (Duopette pipet, Scientific Products, Inc., No. P4615-1X, or equiv.), withdraw suspension to TC mark. Drain pipet into 250 mL vol. flask, using pressure applied from rubber bulb. Thoroly rinse pipet into flask with alcohol, dil. to vol., and mix. Prep. soln contg ca 0.3 mg dichlorophene/mL by stepwise diln with alcohol.

#### D. Determination

Transfer 10 mL aliquot prepd sample soln to 125 mL separator contg 10 mL $H_2O$. Add 3 mL 1*N* NaOH, mix well, and ext with 10 mL *n*-hexane. Let sep. and transfer lower aq. layer to second separator. Ext with another 10 mL portion *n*-hexane. Transfer aq. layer to third separator. Combine *n*-hexane exts in first separator and ext with 10 mL 50% alcohol. Discard hexane. Add alc. wash to aq. layer in third separator. Add 7

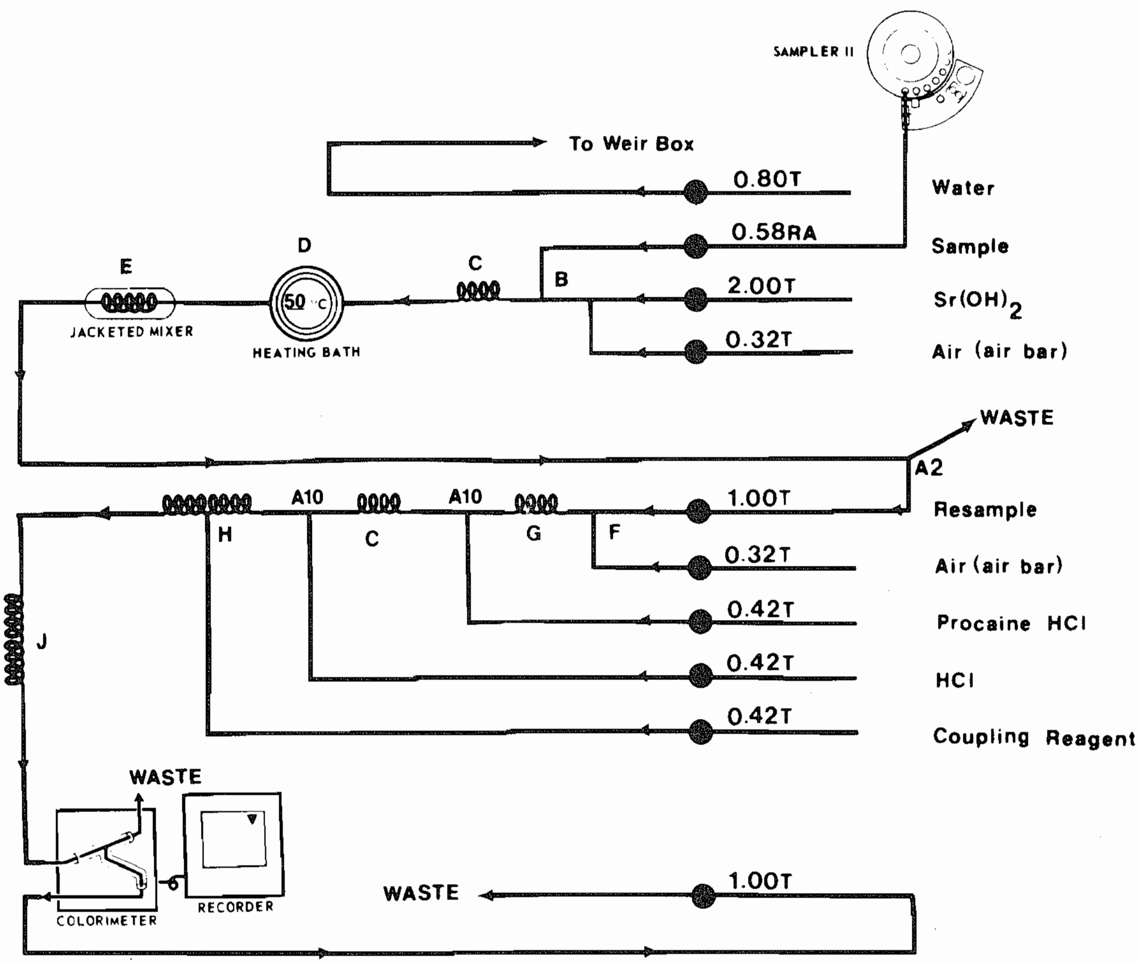



**FIG. 980.34—Flow diagram for semiautomated analysis of nitroglycerin: B, injection fitting, No. 116-0489-01; C, 10-turn coil, No. 157-0226; D, heating bath equipped with 40 ft × 1.6 mm id coil; E, jacketed coil (tap $H_2O$), 4 turns × 1 mm id; F, injection fitting, No. 116-0492-01; G, 5-turn coil, No. 170-0103-01; H, 20-turn coil with center tap, No. 157-B089; J, phasing coil, 28-turn × 2.4 mm id, No. 116-152-4; RA, red acidflex pump tube; T, tygon pump tube. Pump tube sizes in mL/min; Technicon part numbers—use these or equiv.**

mL 1*N* HCl and mix well. (Soln should be distinctly acid to litmus paper.) Ext acid soln with four 25 mL portions $CHCl_3$. Filter each ext thru $CHCl_3$-washed cotton into 250 mL beaker. Rinse funnel and cotton with ca 5 mL $CHCl_3$ and carefully evap. *just* to dryness on steam bath, using gentle air jet. Let beaker and contents cool. Dissolve residue of dichlorophene in 5.0 mL alcohol and transfer to 200 mL vol. flask, using 0.1*N* NaOH. Dil. to vol. with 0.1*N* NaOH and mix.

Record spectra of sample and std solns from 370 to 225 nm, using 1 cm cells and 5 mL alcohol dild to 200 mL with 0.1*N* NaOH as ref.

Det. *A* of std and sample solns at peak wavelength ca 305 nm and calc. dichlorophene content of sample.

### *E. Identification*

Dil. aliquots of sample assay soln, working std soln, and ref. soln with equal vols 0.1*N* NaOH. Obtain *A* between 370 and 225 nm.

Spectra should be similar and exhibit maxima at 245 and 305 nm. Ratio ($A_{245}/A_{305}$) of sample does not differ appreciably from that of std.

Ref.: JAOAC **55,** 163(1972).

CAS-97-23-4 (dichlorophene)

## 920.210 Methenamine in Tablets

### Titrimetric Method

### Final Action

### *A. Reagent*

(**a**) *Modified Nessler reagent.*—(*1*) Dissolve 10 g $HgCl_2$, 30 g KI, and 5 g acacia in 200 mL $H_2O$, and filter thru cotton; (*2*) dissolve 15 g NaOH in 100 mL $H_2O$. Mix 20 mL soln (*1*) with 10 mL soln (*2*).

(**b**) *Starch indicator.*—Mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring const., and immediately discontinue heating. Add ca 1 mL Hg, shake, and let soln stand over the Hg.

### *B. Determination*

Weigh 0.5 g powder, prepd as in **927.09A**, into r-b flask, and add 100 mL $H_2O$ and 25 mL HCl (1 + 2.5). Connect with reflux condenser (preferably of worm type) and boil gently 15 min. Cool, wash condenser tube with little $H_2O$, transfer contents of flask to 250 mL vol. flask, and dil. to vol.

Chill 30 mL Nessler reagent and add 10 mL aliquot of hydrolyzed sample soln. Wash neck of container with jet of $H_2O$ and let stand ≥1 min. Add 10 mL HOAc (1 + 1.5) so that inside of neck is completely washed by reagent, mix quickly and thoroly by rotating and tilting flask, and immediately add 20 mL 0.1$N$ I from buret or pipet. Titr. excess I with 0.1$N$ $Na_2S_2O_3$, adding 5–10 drops starch indicator toward end of titrn, until blue disappears. Final color of soln is pale straw-green. If preferred, end point may be detd by reappearance of faint blue when drop of the I soln is added. 1 mL 0.1$N$ I = 0.00117 g methenamine.

Ref.: JAOAC **3,** 374(1920).

CAS-100-97-0 (methenamine)

## 973.71 Methenamine and Methenamine Mandelate in Drugs

### Automated Method

**First Action 1973**
**Final Action 1974**

### *A. Principle*

Methenamine is hydrolyzed to HCHO and $NH_4^+$ in acid soln. Free HCHO condenses with chromotropic acid in strong acid soln to form colorless hydroxydiphenylmethane derivative which is further oxidized to colored *p*-quinoidal compd with max. $A$ at 570 nm. Method is applicable to methenamine, methenamine mandelate, and methenamine with $NaH_2PO_4$.

### *B. Reagents and Apparatus*

(Use deionized $H_2O$ or equiv. thruout.)

(**a**) *Dilute ammonia.*—Dil. 5 mL $NH_4OH$ to 1 L with $H_2O$.

(**b**) *Dilute sulfuric acid.*—72%. Slowly add $H_2SO_4$ to 600 mL $H_2O$ to total vol. of 1.5 L (when cool).

(**c**) *Chromotropic acid (CTA) color reagent.*—Suspend 500 mg CTA, di-Na salt (Sigma Chem Co., Inc., No. D5144, Eastman Kodak Co., or equiv.), in 20 mL $H_2O$ and *slowly* add 30 mL $H_2SO_4$ in small portions (overheating produces deep violet color and inactivates reagent). Cool, and mix into 1.5 L 72% $H_2SO_4$.

(**d**) *Methenamine std soln.*—Dissolve enough methenamine, previously dried over $P_2O_5$ and stdzd (USP), in dil. $NH_4OH$ to give concn of std appropriate for dosage level analyzed (*see* table).

(**e**) *Automatic analyzer.*—AutoAnalyzer with following modules (Technicon Instruments Corp.): Sampler II with 20/hr (2:1) cam; proportioning pump I; heating bath set at 90° with two 40′ coils, 1.6 mm id; colorimeter with 15 mm tubular flowcell and matched 570 nm filters; recorder with semilog paper; manifold (Fig. **973.71**). Wire down all tube connections carrying $H_2SO_4$.

### *C. Preparation of Sample*

Disintegrate uncoated tablets contg equiv. of 250–500 mg methenamine by intermittent shaking in 100 mL dil. $NH_4OH$. Crush coated tablets and hard uncoated tablets before addn of solv. Ultrasonic bath may be used to hasten soln. Dil. 1.0 mL sample soln to 50 mL with dil. $NH_4OH$.

Samples of all dosage levels can be prepd in 125 mL (4 oz) glass, snap-cap vials provided with Parafilm seal if appropriate sampling pump tube is used (*see* table sizes). A 1 + 50 diln may be accomplished with 1.00 mL Thomas-Seligson vac. diln pipet mounted beneath automatic 50.0 mL delivery pipet. Est. increase in vol. resulting from dissolving 1 tablet in 100 mL solv., using vol. flask and graduated pipet.

### *D. Analytical System*

*See* Fig. **973.71** and the table. Sample solns are withdrawn from sample cups, segmented with air, and dild in manifold with $H_2O$. CTA soln is added and stream is passed thru beaded coil into 90° heating bath for color development. Stream is cooled in $H_2O$-jacketed coil and equilibrated at room temp. in mixing coil. Stream is debubbled and passed into colorimeter equipped with 570 nm filters and 15 mm tubular flowcell for $A$ measurement. Inlet and outlet tubing of flowcell should be ca same id.

| Product | Dosage, mg/tablet | Pump Tube Size, mL/min | Std, mg/100 mL |
|---|---|---|---|
| Methenamine mandelate | 250 | 1.00 | 2.50 |
| Methenamine mandelate | 500 | 0.60 | 5.00 |
| Methenamine | 300, 325 | 0.60 | 6.00, 6.50 |
| Methenamine | 500 | 0.23 | 5.00 |
| Methenamine mandelate | 1000 | 0.23 | 10.00 |

### *E. Start-Up and Shut-Down Operations*

Turn on heating bath (3 hr), cooling $H_2O$ in jacketed coil (30 min), and colorimeter (30 min) in advance. Prewash system 5 min with $H_2O$ and then pump all reagents thru their resp. lines. Let equilibrate 20–30 min and adjust colorimeter and recorder to produce steady baseline. To shut down system, flush 10–15 min with $H_2O$ and pump all lines dry.

### *F. Determination*

Fill 8.5 mL sample cups with prepd solns and aspirate thru 0.034″ stainless steel probe at 20/hr with sample-to-wash ratio of 2:1. Include 1 std soln between each 5 sample solns, and insert 3 std solns at beginning and end of each 10–30 samples. Draw line between baseline at beginning and end of run. Subtract av. baseline $A$ from max. $A$ to obtain net $A$ ($\Delta A$) for each peak. Calc. av. $\Delta A'$ for std solns, disregarding first and last 2 std peaks.

$$\text{mg Methenamine/unit dose} = (\Delta A/\Delta A') \times C \times D$$

where $C$ = mg methenamine/mL std soln and $D$ = diln factor.

Ref.: JAOAC **56,** 647, 1295(1973).

CAS-100-97-0 (methenamine)
CAS-587-23-5 (methenamine mandelate)

## 923.11 Methylene Blue in Drugs

### Titrimetric Method

**Final Action**

### *A. Preparation of Solution*

(**a**) *Foreign material absent.*—Into 50 mL beaker weigh 0.1–0.14 g powd sample, **927.09A**, and transfer to 200 mL vol. flask with 100–140 mL $H_2O$. Dissolve completely by heating 30 min on steam bath with frequent shaking.

(**b**) *Oils or water-insoluble material present.*—(*Caution:* See safety notes on toxic solvents, and carbon tetrachloride.) To 50 mL beaker transfer weighed amt of prepd sample, **927.09A**, contg 0.1–0.14 g methylene blue. Add 15 mL $CCl_4$, warm on steam bath few min, and stir with glass rod to dissolve oils. Transfer to 100 mL separator, using ca 50 mL hot $H_2O$ and little $CCl_4$, if necessary. Cool, shake, and let sep. Transfer $CCl_4$ with undissolved material to second separator for further treatment. (Clear aq. soln of dye should now remain in first separator. If not clear, ext with another 15 mL portion $CCl_4$, transferring any remaining insol. material in similar manner to second separator.) Add ca 10 mL $CCl_4$ to second separator and remove methylene blue by shaking vigorously with 20–40 mL portions $H_2O$ until practically no more dye is extd. (Few drops

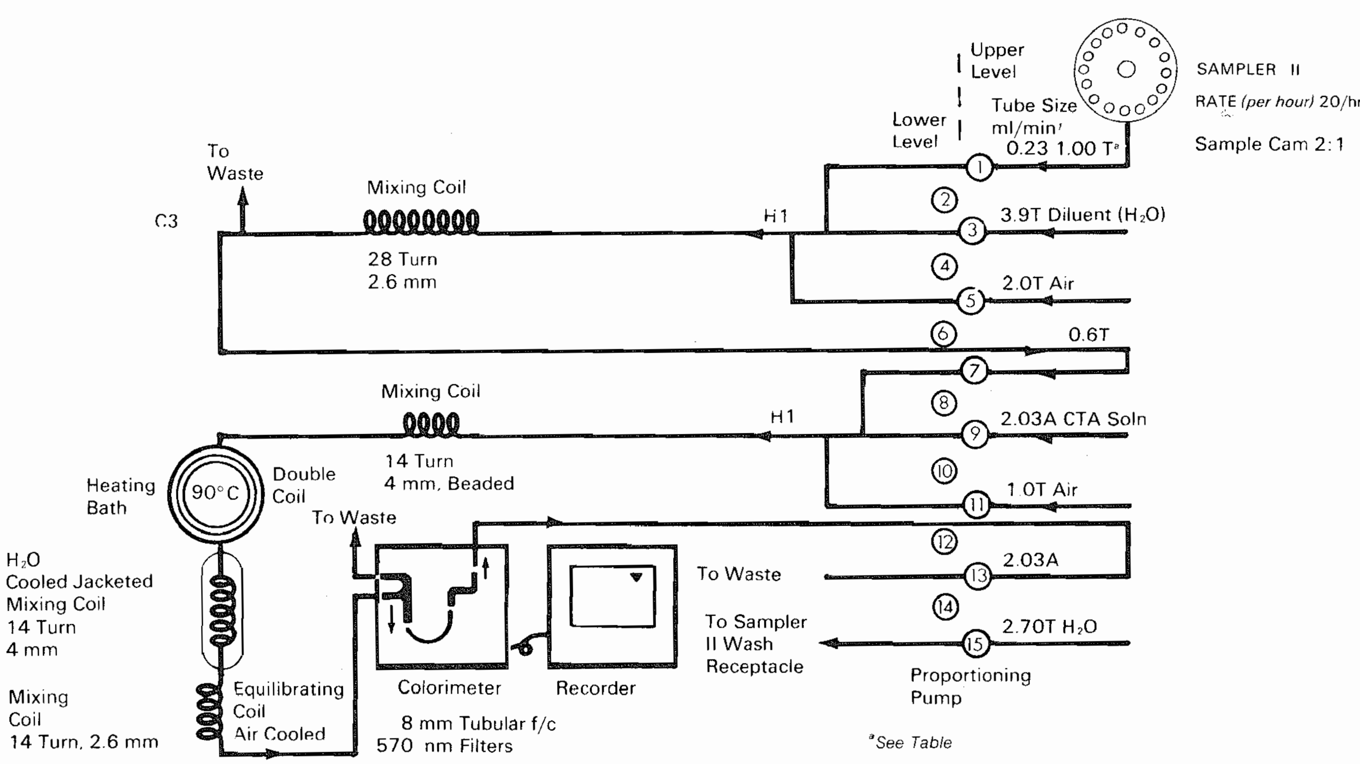



**FIG. 973.71—Flow diagram for automated analysis of methenamine and methenamine mandelate**

of HOAc hasten this extn.) To aq. exts in 400 mL beaker, add main soln from first separator, cover with inverted watch glass on glass rods, and evap. to ca 50 mL. Proceed as in (**c**). $CCl_4$ soln may be reserved for qual. tests for oils.

(**c**) *Water-soluble material present.*—Either use aq. soln from (**b**), or weigh portion of sample contg 0.1–0.14 g methylene blue into 150 mL beaker, add ca 50 mL $H_2O$, and heat 30 min on steam bath with occasional shaking. Transfer to 100 mL separator, keeping vol. as small as possible. Ext with *α-dichlorohydrin,* using 10, 5, 3, and 2 mL portions. Combine dichlorohydrin exts in 200–300 mL separator, add 3 or 4 times their vol. $CCl_4$, and ext dye with $H_2O$ by repeated vigorous shaking with 30–50 mL portions. (Few drops of HOAc hasten removal.) From combined aq. exts, remove any traces of dichlorohydrin by shaking once with ca 15 mL $CCl_4$ and draining after settling 5–10 min. Evap. aq. exts to ca 50 mL over flame, covering beaker as in (**b**) with inverted watch glass. Transfer to 200 mL vol. flask. Dissolve completely by heating 30 min on steam bath with frequent shaking.

### *B. Determination*

Conduct blank as in detn, including filtration. Cool soln, **923.11A**(**a**) or (**c**), add 50 mL HOAc, shake thoroly, and let stand ≥25 min. Add 30 mL 0.2*N* I, **939.13A**, from buret, adding first 10 mL by fast drops with const rotating of flask and remaining 20 mL at full speed, and continue shaking. Stopper flask and let stand 50 min, shaking thoroly 5 or 6 times during interval. Dil. to vol. with $H_2O$, shake, and let stand 10 min longer. Filter rapidly thru dry, folded, 12 cm paper. Titr. 100 mL aliquot with 0.1*N* $Na_2S_2O_3$, with or without starch indicator as desired. Correct for blank titrn. 1 mL 0.2*N* I = 0.01496 g methylene blue, $C_{16}H_{18}N_3ClS.3H_2O$; or 0.01279 g anhyd. methylene blue, $C_{16}H_{18}N_3ClS$.

Ref.: JAOAC **7,** 20(1923).

CAS-61-73-4 (methylene blue)

## 969.50 Fluorescein Sodium in Drugs

### Fluorometric Method

**First Action 1969**
**Final Action 1971**

(Applicable to solns. *Caution:* See safety notes on photofluorometers and hazardous radiations.)

### *A. Apparatus*

(**a**) *Fluorometric apparatus.*—Spectrophotofluorometer or fluorocolorimeter. With cell path ≥1 cm, excitation wavelength 460 nm, emission wavelength 515 nm, and sensitivity to yield ≥85% *T* for most concd std soln. Warm lamp ≥20 min before making measurements.

(**b**) *Thin layer sheets.*—Silica gel (100 μm) with fluorescent indicator (Eastman Kodak Co. Chromagram sheets for TLC, No. 13188, or equiv.).

### *B. Reagents*

(**a**) *Acriflavine hydrochloride soln.*—Dissolve 5 mg salt (J. T. Baker Inc., No. A381, or equiv.) in 0.5 mL $H_2O$ and dil. to 5 mL with alcohol.

(**b**) *Fluorescein diacetate.*—Mp 206–208° (Eastman Kodak Co. No. 1688, or equiv.). If material is impure, indicated by low mp or other evidence, recrystallize from alcohol.

(**c**) *Fluorescein sodium std solns.*—(*1*) *Stock soln.*—903.6 μg fluorescein Na/mL. Accurately weigh 100 mg fluorescein diacetate (equiv. to 90.36 mg fluorescein Na), dried 1 hr at 100°, and transfer to 100 mL vol. flask with ca 10 mL alcohol. Add 2 mL 10% NaOH and heat on steam bath at ca boiling temp. 20 min. Swirl frequently. After hydrolysis, cool flask, dil. to vol. with $H_2O$, and mix. (*2*) *Intermediate soln.*—0.9036 μg fluorescein Na/mL. Dil. 1 mL clear stock soln to 1 L with $H_2O$ and mix. (*3*) *Working solns.*—0.000, 0.009, 0.018, 0.027, 0.036, and 0.045 μg fluorescein Na/mL. Transfer 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 mL intermediate soln to sep. 100 mL vol. flasks, add 20 mL pH 9 buffer to each, and dil. to vol. with $H_2O$.

(**d**) *Boric acid buffer*.—pH 9. Prep. ca 200 mL soln 0.05*M* in boric acid and 0.05*M* in KCl. Adjust to pH 9 with 0.2*M* NaOH.

### C. Preparation of Sample

Quant. dil. sample with $H_2O$ to obtain ca 1 μg fluorescein Na/mL and transfer 3.0 mL aliquot to 100 mL vol. flask contg 20 mL pH 9 buffer. Dil. to vol. with $H_2O$ and mix.

### D. Determination

Measure fluorescent intensity ($I$) of all working std solns and plot std curve (μg fluorescein Na against $I$). Det. $I$ of sample soln and calc. concn of sample.

### E. Purity and Identification

Dil. concd sample and hydrolyzed stock solns with alcohol to contain ca 1 mg fluorescein Na/mL.

Spot 10 μL each of above solns and acriflavine.HCl soln on fluorescent silica gel sheets and develop with *n*-BuOH-alcohol-$H_2O$ (2 + 1 + 1). Dry sheet and view under longwave UV light. Sample and std should exhibit only one spot, which has similar but different $R_f$ from spot obtained with acriflavine.HCl.

Ref.: JAOAC **52,** 110(1969).

CAS-518-47-8 (sodium fluorescein)

## 961.17 Piperazine in Drugs

**Gravimetric Method**

**First Action 1961**
**Final Action 1971**

(Applicable to aq. solns)

### A. Apparatus

*Chromatographic tube*.—40 × 300 mm, with stopcock and fritted glass disk or plug of glass wool as support.

### B. Determination

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Prep. layered column with tamped layer of 5 g diat. earth, **960.53B**, on bottom; add layer of 5 g diat. earth thoroly mixed with 5 mL $H_2O$, and tamp. Thoroly mix 25 g diat. earth and 5 g $NaHCO_3$ in 600 mL beaker, add 25 mL aliquot of piperazine soln contg ca 100 mg piperazine, and again mix thoroly. Add 2 mL $Ac_2O$ and mix 5 min, transferring to another beaker to ensure thoro mixing. Add mixt. to column, using large funnel to prevent loss, and tamp. Dry-wash the 2 beakers with 5 g diat. earth, add to column, and tamp. Place pad of glass wool on top.

Pass 200 mL $CHCl_3$ thru column, adjusting flow to ca 7 mL/min, and collect eluate in 250 mL beaker, previously dried at 80°, cooled in desiccator, and weighed. Evap. $CHCl_3$ on steam bath with air current, and dry to const wt in convection oven at 80° (ca 3 hr). Piperazine = diacetylpiperazine × 0.5061.

Check for complete extn by passing another 100 mL portion $CHCl_3$ thru column, evapg to dryness, and noting if residue is present.

Det. mp of diacetylpiperazine, which should be ca 140°.

Ref.: JAOAC **44,** 312(1961).

CAS-110-85-0 (piperazine)

## 966.25 Piperazine in Drugs

**Near-Infrared Method**

**First Action 1966**
**Final Action 1971**

### A. Apparatus and Reagents

(**a**) *Near-infrared spectrophotometer*.—With 5 cm Si cells.

(**b**) *Drying tube*.—Approx. 3.5 cm diam. × 9 cm long. Pack with glass wool and ca 6 cm granular anhyd. $Na_2SO_4$ prewetted with ca 30 mL reagent grade $CHCl_3$.

(**c**) *Piperazine dihydrochloride*.—Anal. std. (Available from Pfaltz & Bauer Inc., 375 Fairfield Ave, Stamford CT 06902.) Store above Si gel.

### B. Preparation of Standard

Accurately weigh std piperazine equiv. to ca 3.5–3.8 g anhyd. base, transfer to 100 mL vol. flask with $H_2O$, dil. to vol., and mix. Transfer 10.0 mL of this soln and exactly 5 mL $H_2O$ to separator. Add 25 mL *NaOH soln* (1 + 1) and swirl. *Final concn of NaOH must be >30%*. Cool separator under tap, add 30 mL $CHCl_3$, and shake carefully ca 2 min. Drain $CHCl_3$ layer thru drying tube, (**b**), into 100 mL vol. flask. Ext with three 20 mL portions $CHCl_3$, draining thru drying tube into vol. flask. Rinse tube with $CHCl_3$ and dil. to vol. Prep. blank as above, using 15 mL $H_2O$.

### C. Preparation of Sample

(**a**) *Powders*.—Transfer sample contg ca 250–300 mg piperazine base thru small funnel to separator contg exactly 5 mL $H_2O$. Rinse funnel with exactly 10 mL $H_2O$ from pipet. Mix, and proceed as in **966.25B**, beginning, "Add 25 mL *NaOH soln* . . ." Dil. $CHCl_3$ exts to 100 mL.

(**b**) *Sirups*.—Transfer sample contg ca 500 mg piperazine base to separator. Add $H_2O$ to total vol. of exactly 15 mL. Proceed as in **966.25B**, beginning "Add 25 mL *NaOH soln* . . ." *except* use 200 mL vol. flask to collect $CHCl_3$ exts, and ext with three 50 mL portions $CHCl_3$, finally rinsing inside of separator with several 10–15 mL portions $CHCl_3$ before dilg to 200 mL.

### D. Determination

Using 5 cm cells, scan from 1600 to 1450 nm against blank. (Max. is ca 1520 nm.) Draw baseline between min. at ca 1460 and 1565 nm and det. net $A$.

$$(A/A') \times (\text{mg base in std/mL } CHCl_3) = (\text{mg base in sample})/(\text{mL final } CHCl_3 \text{ soln})$$

where $A$ and $A'$ refer to sample and std, resp. Convert sample from base to known salt formula, if desired.

Ref.: JAOAC **48,** 590(1965).

CAS-110-85-0 (piperazine)

# MICROCHEMICAL TESTS

## 930.40 Alkaloids and Related Amines in Drugs

**Microchemical Tests**

**Final Action**

### A. Reagents

(**a**) *Ammoniacal silver nitrate soln*.—Mix 2.5 mL 4% $AgNO_3$ soln with 2.5 mL $NH_4OH$ (1 + 5). Prep. fresh.

(**b**) *Ammonium hydroxide soln*.—10% $NH_3$ (2 + 3).

(**c**) *Ammonium thiocyanate soln*.—Dissolve 5 g $NH_4SCN$ in 100 mL $H_2O$.

(**d**) *Bismuth iodide soln*.—(*1*) Prep. stock concd $Bi(NO_3)_3$

**Table 930.40 Characteristics of Microchemical Tests for Alkaloids and Related Amines**

| Alkaloid | Reagent | Description of Crystals |
|---|---|---|
| Aconitine (*1*) | Sodium carbonate | In 1:3000 soln heated to 50° in test tube. Small, transparent, hexagonal plates; also rods in contact. |
| Amylocaine (*2*) | 1 drop HCl and 1 drop gold chloride | 1:50. Dendritic crystals. |
| Apomorphine (*3*) | Potassium iodide | 1:50. Small crystals that have sharp, clear-cut angles like those of diamond. |
| | Gold chloride | Red-brown, fine needles, in dense masses in all solns to 1:10,000. |
| | Hydrochloric acid | 1:50. Small rods singly and in clusters. |
| Arecoline (*1*) | Bismuth iodide | Red, rhombic crystals. |
| Atropine (*4*) | Iodine potassium iodide | Small, dark rods and triangular plates form in great numbers, singly and in groups. |
| Benzylmorphine (*5*) (Peronine) | Potassium iodide | 1:200. Dense rosettes of needles. Crystals are formed readily in dil. solns (1:1000) in form of sheaves of needles. |
| | Ammonium thiocyanate | 1:200. Rosettes and sheaves of needles in acid or neut. soln. |
| | Hydrochloric acid | 1:100. Rods, usually notched at ends and often in rosettes, are formed on stirring. |
| Berberine (*6*) | Hydrochloric acid | Satd soln; fine yellow needles. (Avoid excess reagent.) |
| Brucine (*7*) | Potassium iodide | Long masses of transparent, rectangular plates; also rosettes of thin plates. |
| | Mercuric chloride | Small, dense rosettes. |
| Choline (*8*) | Reinecke salt | Add 1 drop acetone to 1 drop $H_2O$ soln of base. Stir, add 1 drop reagent, and stir again. 1:100. Thin, hexgonal plates and star-shaped forms. 1:1000–1:10,000. Six-sided, more coffin-shaped plates; sometimes rosette aggregates of plates on edge, resembling needles. |
| | Platinic chloride and sodium iodide | 1:100 in $H_2O$. Add 1 drop $H_2PtCl_6$ soln, stir, and add small drop NaI soln without stirring. Small black rectangular prisms and slender black rods. |
| Cinchonidine (*9*) | Sodium benzoate | Rosettes and sheaves of needles spreading to large size. |
| | Platinic chloride | Rosettes of transparent plates. |
| | Sodium carbonate | Spherical crystals, but not needles as in cinchonine. |
| Cinchonine (*9*) | Sodium carbonate | Dark rosettes, composed of radiating needles, form immediately. |
| | Disodium phosphate | Similar to crystals formed by $Na_2CO_3$, but more burr-shaped. |
| Cocaine (*10*) | Platinic chloride | Delicate, feathery crystals, later becoming heavier in structure. |
| Codeine (*10*) | Potassium cadmium iodide | Silvery, circular masses, crystg into dark rosettes of irregular outline. |
| | Iodine potassium iodide | Heavy, red-brown ppt; crystallizes very slowly in yellow blades extending in branches (never red). |
| Cotarnine (*6*) | Platinic chloride | 1:200. Hair-like crystals, yellow and curving. |
| | Mercuric chloride | Colorless, long, branching needles. |
| | Potassium ferrocyanide | Acidify with 1 drop 5% HCl; globules that develop into dense, burr-shaped crystals; also amber-brown plates. |
| Diacetylmorphine (*11*) (Heroin) | Platinic chloride | Spherical clusters of golden yellow needles form slowly around nucleus; cluster disintegrates on standing. |
| Ephedrine (*12*) | Bismuth iodide in dild sulfuric acid | 1:200. Long, brownish orange, radiating and interlacing needles and branching rods. |
| Ethylhydrocupreine (*13*) | Ammonium thiocyanate | 1:100 in 0.1*N* HCl. Long, straight needles. |
| Ethylmorphine (*5*) | Iodine potassium iodide | 1:200. Groups of yellow needles, branching later. |
| | Mercuric chloride | Transparent plates, often with notched ends; singly and in groups. Stir to start crystn. |
| Homatropine (*14*) | Gold chloride | 1:200. Green-gold blades, often with pointed ends and united in pairs; surfaces appear etched on long standing. |
| Hydrastine (*3*) | 1 drop 5% HCl and 1 drop potassium ferrocyanide | 1:100. Spheres of radiating crystals. Shake slide to start crystn. Avoid excess reagent. |
| Hydrastinine (*5*) | Potassium permanganate | 1:500. Immediate red plates, often with serrated edges. In concd soln, great number of large red or brown plates with deeply cut edges. |
| | Mercuric chloride | 1:500. Transparent needles forming branches rapidly in neut. and acidified solns. |
| | 1 drop 5% HCl and 1 drop potassium ferrocyanide | 1:200. Yellow rhombic plates and tree-like crystals. |
| Hydromorphone (*15*) | Sodium nitroprusside | To minute amt (<1 mg) in 2 drops $H_2O$ add minute fragment of reagent. Elongated 6-sided prisms; also in aggregates. |
| Hyoscyamine (*14*) | Gold chloride | Thin, transparent, nearly colorless irregular plates, often curved. Crystals form slowly in 1:100 to 1:200 soln. Shaking slide aids crystn. |
| Morphine (*10*) | Potassium cadmium iodide | Silvery, gelatinous ppt, crystg in dense masses of fine needles. |
| | Iodine potassium iodide | Small drop of reagent produces heavy, red-brown ppt, slowly crystg in shining, red, overlapping plates extending in branches. |
| Narceine (*6*) | Iodine potassium iodide or zinc potassium iodide | 1:400. Blue, radiating needles, sometimes with yellow dichroism. |
| | Platinic chloride | Beautiful feathery rosettes develop in all solns. |
| Nicotine (*16*) | Mercuric chloride | Radiating, transparent blades form in presence of slight excess of $H_2SO_4$; feather-like blades form in presence of HCl. |
| | Mercuric chloride-sodium chloride | Radiating, transparent blades. |

**Table 930.40 Characteristics of Microchemical Tests for Alkaloids and Related Amines—*Continued***

| Alkaloid | Reagent | Description of Crystals |
|---|---|---|
| Noscapine (*6*) (*l*-Narcotine) | Potassium hydroxide or ammonium hydroxide | 1:200. White, amorphous ppt that crystallizes slowly; dense rosettes of needles. |
| Papaverine (*17*) | Zinc chloride | Thin, rectangular plates in excess HCl. |
| Physostigmine (*18*) | Lead iodide | 1:100. Radiating, serrated plates. |
| | Gold bromide in HCl | 1 mg in 1 drop $H_2O$. Brown, dendritic aggregates. |
| Pilocarpine (*4*) | Platinic chloride | Crystals form slowly; layers of thin, yellow, triangular plates of delicate structure. |
| Procaine (*17*) | Platinic chloride | Spherical crystals of radiating branches. |
| | Gold chloride and HCl | Irregular, radiating branches. |
| Quinidine (*9*) | Potassium iodide | Small, triangular crystals in great numbers; best in 1:1000 diln; sol. in excess reagent. |
| Quinine (*9*) | Disodium phosphate | Silvery, sheaf-like crystals. |
| Racephedrine (*19*) (*dl*-Ephedrine) | Bismuth iodide in dild sulfuric acid | 1:200. Large orange plates and red prisms and grains. |
| Scopolamine (*14*) (Hyoscine) | Gold chloride | Clusters of pale yellow, transparent blades, with coarse, saw-toothed edges form immediately on shaking slide. Crystals grow to large size in 1:200 soln. |
| Sparteine (*16*) | Gold chloride | Large numbers of blade-like crystals varying in size according to concn. |
| Strychnine (*20*) | Platinic chloride | Crystals form immediately in clusters and singly in small, wedge-shaped needles that move about field. |
| | Potassium cadmium iodide | Silvery masses, slowly forming rosettes. |
| Yohimbine (*1*) | Sodium carbonate | In 1:1000 soln heated to 50°. Fine needles in sheaf-like bundles and rosettes. |

soln by dissolving 50 g Bi subnitrate in 70 mL $HNO_3$ (1 + 1) and dilg to 100 mL with $H_2O$. (2) Dissolve 1.25 g KI in 4.5 mL $H_2O$ and add 0.5 mL stock concd $Bi(NO_3)_3$ soln. Prep. fresh when soln darkens appreciably.

(**e**) *Bismuth iodide in diluted sulfuric acid soln.*— Dissolve 1.25 g KI in 2.0 mL $H_2O$, and add 2.5 mL $H_2SO_4$ (1 + 3) and 0.5 mL stock concd $Bi(NO_3)_3$ soln, (**d**)(*1*). Prep. fresh daily.

(**f**) *Disodium phosphate soln.*—Dissolve 5 g $Na_2HPO_4.12H_2O$ in 100 mL $H_2O$.

(**g**) *Gold bromide in hydrochloric acid soln.*—Dissolve 1 g $HAuCl_4.3H_2O$ and 1.5 mL 40% HBr in 18 mL HCl. (Satd aq. NaBr soln may be substituted for the HBr.)

(**h**) *Gold chloride soln.*—Dissolve 1 g $HAuCl_4.3H_2O$ in 20 mL $H_2O$.

(**i**) *Hydrochloric acid.*—5% (1 + 6).

(**j**) *Iodine-potassium iodide soln.*—Dissolve 1.27 g I and 2 g KI in 5 mL $H_2O$, and dil. to 100 mL.

(**k**) *Lead iodide soln.*—To aq. KOAc soln (1 + 3) add 1 drop Me red and HOAc until yellow changes to orange; then, while gently warming, sat. with $PbI_2$, cool, and filter.

(**l**) *Mercuric chloride soln.*—Dissolve 5 g $HgCl_2$ in 100 mL $H_2O$.

(**m**) *Mercuric chloride-sodium chloride soln.*—Dissolve 5 g $HgCl_2$ and 0.75 g NaCl in 100 mL $H_2O$.

(**n**) *Platinic chloride soln.*—Dissolve 5 g $H_2PtCl_6.6H_2O$ in 100 mL $H_2O$.

(**o**) *Potassium cadmium iodide soln.*—Dissolve 3 g $CdI_2$ in 18 mL $H_2O$ contg 6 g KI.

(**p**) *Potassium ferrocyanide soln.*—Dissolve 5 g $K_4Fe(CN)_6.3H_2O$ in 100 mL $H_2O$.

(**q**) *Potassium hydroxide soln.*—Dissolve 5 g KOH in 100 mL $H_2O$.

(**r**) *Potassium iodide soln.*—Dissolve 5 g KI in 100 mL $H_2O$.

(**s**) *Potassium permanganate soln.*—Dissolve 1 g $KMnO_4$ in 100 mL $H_2O$.

(**t**) *Reinecke salt soln.*—Dissolve 0.1 g $NH_4[Cr(NH_3)_2(SCN)_4].H_2O$ and 0.03 g $H_2NOH.HCl$ in 10 mL alcohol. Filter, and store in refrigerator. (Reagent is stable ≥6 months.)

(**u**) *Sodium benzoate soln.*—Dissolve 5 g Na benzoate in 100 mL $H_2O$.

(**v**) *Sodium carbonate soln.*—Dissolve 5 g $Na_2CO_3.H_2O$ in 100 mL $H_2O$.

(**w**) *Sodium iodide soln.*—Dissolve 5 g NaI in 100 mL $H_2O$.

(**x**) *Sodium nitroprusside.*—$Na_2Fe(CN)_5NO.2H_2O$ crystals.

(**y**) *Zinc chloride soln.*—Dissolve 5 g $ZnCl_2$ in 100 mL $H_2O$.

(**z**) *Zinc potassium iodide soln.*—Dissolve 5 g $Zn(OAc)_2.3H_2O$ and 20 g KI in 100 mL $H_2O$.

### B. Preparation of Samples

(**a**) *Usual controls.*—Dissolve 0.4 or 0.2 mg pure alkaloid salt in 0.04 mL $H_2O$ to make ca 1:100 or 1:200 soln.

(**b**) *Alkaloids in compounds.*—Sep. alkaloid in pure form by extg it from ammoniacal soln with suitable immiscible solv., and evap. solv. Dissolve little of residue in min. of 0.1*N* HCl and dil. with $H_2O$, if necessary, to ca alkaloid concn specified in (**a**) or in test.

(**c**) *Hypodermic tablets.*—Dissolve portion of tablet in drop of $H_2O$ to ca same alkaloid concn specified in (**a**) or in test.

### C. Identification

Place drop (ca 0.04 mL) of alkaloid soln on glass slide, add drop of reagent, and without stirring or covering, examine under microscope, using magnification of ca 100–500×. Note kind of crystals formed. Compare their characteristics with descriptions given, Table **930.40**, and with a control. Use polarizing microscope if available, and note characteristics such as birefringence and dichroism.

Refs.: (*1*) JAOAC **15,** 413(1932).
(*2*) JAOAC **23,** 746(1940).
(*3*) JAOAC **20,** 551(1937); **21,** 91(1938).
(*4*) JAOAC **11,** 353(1928); **14,** 316(1931); **18,** 521(1935).
(*5*) JAOAC **21,** 525(1938).
(*6*) JAOAC **22,** 706(1939).
(*7*) JAOAC **13,** 315(1930).
(*8*) JAOAC **26,** 96(1943).
(*9*) JAOAC **12,** 282(1929).
(*10*) JAOAC **10,** 370(1927); **11,** 353(1928).
(*11*) JAOAC **5,** 154(1921); **10,** 370(1927).
(*12*) JAOAC **14,** 316(1931).
(*13*) JAOAC **20,** 553(1937).

(*14*) JAOAC **18,** 521(1935).
(*15*) JAOAC **24,** 830(1941).
(*16*) JAOAC **16,** 345(1933).
(*17*) JAOAC **17,** 433(1934).
(*18*) JAOAC **23,** 746(1940); **24,** 830(1941).
(*19*) JAOAC **43,** 262(1960); **61,** 1435(1978).
(*20*) JAOAC **11,** 353(1928).

## 962.21 Barbiturates in Drugs

### Microchemical Tests

**First Action 1962**
**Final Action 1972**

#### A. Reagent

*Iodine-potassium iodide soln.*—Dissolve 5 g I and 80 g KI in enough $H_2O$ (ca 78 mL) to make 100 mL. Dil. with 2 parts by vol. of $H_3PO_4$. Prep. dild reagent every 2–3 weeks.

#### B. Identification

Dissolve little barbiturate in drop $H_2O$ on slide. If present as Na salt, it dissolves readily; if present as acid, add little droplet 1% NaOH on stirring rod and mix. Add 1 full drop reagent and let stand until crystn occurs (immediate with some compds, 0.5–1 hr with secobarbital). Free acid may ppt or crystallize. However, I reaction crystals are easily distinguished by their color, often coupled with strong dichroism. Det. birefringence with polarizing microscope. Cover glass is usually not needed but may be used for observation at high magnification and when slide stands >1 hr; on standing, KI may crystallize as sq, colorless, isotropic crystals.

Note crystals formed and compare characteristics with descriptions, Tables **962.21A** and **B**.

Ref.: JAOAC **45,** 600(1962).

## 985.44 Phenothiazine Drugs

### Microcrystalline Identification

**First Action 1985**
**Final Action 1988**

(Applicable to perphenazine, promethazine, thiethylperazine, and triflupromazine pure drug substances)

#### A. Reagents

(**a**) *Gold bromide soln.*—Dissolve 1 g $HAuCl_4.3H_2O$ and 1 g NaBr in 30 mL $H_2SO_4$ (2 + 3). Before use, mix 3 vols of this soln with 1 vol. of glacial HOAc. Store in dark bottle.

(**b**) *Iodine-potassium iodide acidic soln.*—Dissolve 5 g I and 30 g KI in 100 mL $H_2O$. Mix 1 vol. of this soln with 1.5 vols of HCl and 1.5 vols of $H_3PO_4$. Store in dark bottle.

#### B. Standard Solutions

Prep. individual solns of perphenazine, promethazine, thiethylperazine, and triflupromazine by dissolving in HOAc (2 + 1) to final concn of ca 2 μg/μL. If necessary, dil. further with HOAc (2 + 1) for optimum results. Exact concn depends on compd being tested.

#### C. Sample Solutions

Dissolve sample in HOAc (2 + 1) to final concn of ca 2 μg/μL. If necessary, dil. further with HOAc (2 + 1) to obtain solns with strength equiv. to std solns.

#### D. Procedure

Place small drop (ca 10 μL) of sample soln on each of 2 clean microscope slides. Place small drop (ca 10 μL) of reagent soln (**a**) or (**b**) on 2 cover slips, resp.; invert cover slips and place over sample solns on slides. Let stand until crystn occurs (immediately with some compds; 1–20 min with others). Examine slide under polarizing microscope at ca 100–300×. If microcrystn product is formed, note shape and rate of formation of crystals. See Table **985.44**. Repeat tests with corresponding std soln of comparable strength, and compare product with that obtained with sample soln.

Ref.: JAOAC **68,** 527(1985).

CAS-58-39-9 (perphenazine)
CAS-60-87-7 (promethazine)
CAS-1420-55-9 (thiethylperazine)
CAS-146-54-3 (triflupromazine)

## 960.55 Sympathomimetic Drugs

### Microchemical Tests

**Final Action 1970**

#### A. Reagents

(**a**) *Bismuth iodide in diluted sulfuric acid soln.*— *See* **930.40A(e)**.

(**b**) *Gold chloride in diluted phosphoric acid soln.*— Dissolve 1 g $HAuCl_4.3H_2O$ in 20 mL $H_3PO_4$ (1 + 2).

(**c**) *Platinic chloride in diluted phosphoric acid soln.*—Dissolve 1 g $H_2PtCl_6.6H_2O$ in 20 mL $H_3PO_4$ (1 + 3).

(**d**) *Sodium tetraphenylboron soln.*—Aq. soln (1 + 20).

#### B. Identification

(**a**) *Direct test.*—Add drop of reagent to little of powd solid or crushed tablet and spread out on slide with little stirring. Do not stir to homogeneity as local concns and dilns will assist crystn. Let stand to evap. to higher acid concn if necessary for crystal formation.

(**b**) *Volatility test.*—Place small amt of substance or crushed tablet in depression of cavity slide, add drop 5% NaOH soln, and stir briefly. Place very small drop of reagent on thin slide, invert over cavity slide, and let stand. As crystals appear, examine with inverted slide in place. After observing crystals or after ≥1 hr exposure, if only few or no crystals form, reinvert thin slide with hanging drop, and let stand for gradual evapn of $H_2O$ from reagent drop. Examine for crystals. Compare with descriptions, Table **960.55**.

Refs.: JAOAC **43,** 262(1960); **61,** 1435(1978).

**Table 985.44 Characteristics of Microchemical Tests for Synthetics**

| Synthetic | Solvent | Concn of Synthetic | Reagent | Description of Test and Crystals |
|---|---|---|---|---|
| Perphenazine | HOAc (2 + 1) | 1:500 | Iodine-potassium iodide acidic | X-shape serrated blades. Pos. elongation |
| Thiethylperazine | HOAc (2 + 1) | 1:500 | Iodine-potassium iodide acidic | Branching clusters. Neg. elongation |
| Promethazine | HOAc (2 + 1) | 1:500 | Gold bromide in $H_2SO_4$/HOAc | Small rods; clusters of rods |
| Triflupromazine | HOAc (2 + 1) | 1:500 | Gold bromide in $H_2SO_4$/HOAc | Large X-shape segmented plates; small swirling tufts |

**Table 962.21A Characteristics of Microchemical Tests for Barbiturates**

| Barbiturate | Crystal Form | Dichroism or Pleochroism | Remarks |
|---|---|---|---|
| Allobarbital (Diallylbarbituric acid, 5,5-Diallylbarbituric acid) | Crystallizes quickly in branching twigs, splinters, and blades. | Extreme black to "white" dichroism by polarized light. | Golden-beetle iridescence by reflected light. |
| 5-Allyl-5-(2-cyclopenten-1-yl) barbituric acid (cyclopal) | Gradual crystn in dichroic straight-edged blades, brown-yellow to brown-orange. | — | Very bright birefringence; free acid as colorless rods, splinters, needles. |
| Amobarbital (5-Ethyl-5-isoamylbarbituric acid) | Dil. soln: fairly large brown blades. Concd soln: multitudes of little pale-colored flakes. | — | Examine at 200×; sensitive test. |
| Aprobarbital (5-Allyl-5-isopropylbarbituric acid) | Light orange-brown rod-blades, birefringent. | Yellow to brown-orange dichroism. | — |
| Barbital (5,5-Diethylbarbituric acid) | Form very soon; fairly large, rectangular or splinter blades. | Extreme pleochroism by transmitted polarized light. | Beetle-green iridescence by reflected light. |
| * Bemegride (4-Ethyl-4-methyl-2,6-piperidinedione) | Small light-colored dichroic rods or blades and flakes or plates, orangish brown to colorless or yellowish. | — | Birefringence is bright and plates that are sq or nearly so extinguish diagonally. |
| Butabarbital Sodium (Sodium 5-sec-butyl-5-ethylbarbiturate) | Dil. soln: red-brown irregular plates. Concd soln: brown blades in clusters. | Slightly dichroic; dichroism yellow to red-brown. | Free acid: colorless blades. |
| Butalbital (Allylbarbital, 5-Allyl-5-isobutylbarbituric acid, "Itobarbital") | Immediate crystn in rods, splinters, and leaflike crystals with pointed ends. | Red to black dichroism. | Free acid may crystallize out. |
| Butethal (5-Butyl-5-ethylbarbituric acid) | Small plate crystals basically rhomboids. "Propeller-type" of elongate pointed blades. | Strong dichroism, light yellow to black. | — |
| Cyclobarbital (5-(1-Cyclohexenyl)-5-ethylbarbituric acid) | Dil. soln: rosettes of little pointed crystals; larger are red-brown plates. | Red-brown plates of variable dichroism frequently four-bladed. | Sensitive test. |
| Heptabarbital (5-(1-Cyclohepten-1-yl)-5-ethylbarbituric acid) | Little red-brown plates in great numbers, often 4-parted; good birefringence. | — | Sensitive. |
| Hexobarbital Sodium (Sodium 5-(1-cyclohexen-1-yl)-1,5-dimethylbarbiturate | Dichroic blades and broad splinters in groups, varying to curving threads and needles in rosettes. | Very strong dichroism; black to light brownish yellow. | Sensitive for I reaction crystals as well as for free acid. |
| Metharbital (5,5-Diethyl-1-methylbarbituric acid) | Dark needles, small to large, and splintery narrow blades. | Dichroism black to brown. | Good birefringence with crossed nicols. |
| Pentobarbital Sodium (Sodium 5-ethyl-5-(1-methylbutyl) barbiturate) | Crystallizes quickly in great numbers of small red-brown plates. | Minute light-colored flakes exhibit dichroism; dark brown or black to yellow. | — |
| Phenobarbital (5-Ethyl-5-phenylbarbituric acid) | Soon crystallizes in little dark grains; also a few larger red blades and dark splinter-rods in clusters. | — | Free acid may also crystallize out. |
| Phenylmethylbarbituric acid (5-Methyl-5-phenylbarbituric acid) | Red-brown irregular platy forms appear after free acid is pptd. | Gradually strongly dichroic rods or blades | Test fairly sensitive for dil. soln. |
| Probarbital (5-Ethyl-5-isopropylbarbituric acid) | Scattered iodine-reaction crystals form in various jagged shapes, color dark brown to black dichrosim, or red-black with but little dichroism. | | Free acid thrown out, forming long rods with pointed ends. |
| Secobarbital (5-Allyl-5-(1-methylbutyl)barbituric acid) | Crystallizes in plates or elongate and rectangular but mostly distorted into any shape after 1 hr. | Light yellow to orange or red dichroism by polarized light. | Distinctly birefringent. |
| Talbutal (5-Allyl-5-sec-butylbarbituric acid) | Amorphous ppt crystallizes in large needles and dichroic blades, lighter to deeper brown, in dendrites; then gray-black curled sheaves of threads. | — | Excellent test. Both types of crystals have good birefringence |
| Vinbarbital (5-Ethyl-5-(1-methyl-1-butenyl) barbituric acid) | Multitudes of small dark crystals, tiny grains and rods with dichroism brown to black. In quite dil. soln possible to get good small crystals, little dark rods with dichroism red to black, and small plates tending to be sq, generally appearing red but with same red to black dichroism, and with sq extinction (not diagonal). | | Very sensitive. |

* This drug has barbiturate-type formula (although there is only one N) but is central nervous stimulant instead of depressant.

**Table 962.21B Characteristics of Microchemical Tests for Synthetics**

| Synthetic | Solvent | Concentration of Synthetic | Reagent | Description of Tests and Crystals |
|---|---|---|---|---|
| Acetanilid (*1*) | HCl (1 + 3) | 1:100 | Phosphotungstic acid | Rosettes of prisms. |
| | HCl (1 + 3) | 1:100 | Bromide-bromate soln | Small prisms. |
| Allobarbital (Diallybarbituric acid) (*2*) | — | Dry powder | Lead triethanolamine | Stir small amt of synthetic into 1 drop reagent. Rods singly and in clusters. |
| | — | Dry powder | Barium hydroxide | Stir small amt of synthetic into 1 drop reagent. Rods singly and in groups. |
| Aminopyrine (*3*) | $H_2O$ | 1:100 | Mercuric chloride | Long, slender, radiating crystals, often curved. |
| | | | Potassium cadmium iodide | Groups of spiny branches. |
| Amobarbital (*4*) | $NH_4OH$ (1 + 9) | 1:50 | Acetic acid | Long, branching needles; some hexagonal plates in groups. |
| | $NH_4OH$ (1 + 9) | 1:25 | Acetic acid | Groups of rectangular plates. |
| Antipyrine (*5*) | $H_2O$ | 1:100 | Potassium ferrocyanide | Add 1 drop HCl (1 + 39). Acicular and prismatic crystals form. |
| Aspirin (Acetylsalicylic acid) (*6*) | 2% triethanolamine | 1:50 | Silver nitrate | Fine, curling, hair-like crystals form first near edge of drop. |
| Barbital (*4*) | — | Approx. 1 mg powder | Ammoniacal silver nitrate | Stir to aid soln and crystn. Very small, twined crystals and larger tufts. |
| | $NH_4OH$ (1 + 9) | 1:50 | Acetic acid | Dark burrs (stirring hastens crystn). |
| Benzocaine (Ethyl aminobenzoate) (*7*) | 0.1*N* HCl | 1:100 | Potassium ferrocyanide | Colorless, irregular plates and rods. |
| Benzoic acid (*6*) | — | Dry powder | Lead triethanolamine | Stir small amt of synthetic into 1 drop reagent. Stir thoroly to induce crystn. 4-sided plates, singly and in groups. |
| | — | Dry powder | Zinc pyridine | Stir small amt of synthetic into 1 drop reagent. Stir thoroly to induce crystn. Hexagonal crystals. |
| | 2% triethanolamine | 1:100 to 1:200 | Silver nitrate | Rods or curving blades with irregular ends. |
| Cinchophen (*7*) | 0.1*N* NaOH. Add $H_2O$ and make slightly acid with HCl | 1:1000 | Gold chloride | Dark clusters of needles. Few short, rhombic crystals. |
| Dinitrophenol (*3*) | Small amt of 0.1*N* NaOH | 1:100 | HCl | Plates with 4 branches. In more dil. soln, single rectangular plates. |
| Diphenhydramine hydrochloride (*8*) | Glycerol-alcohol (1 + 1) or $H_2O$ | Approx. 0.2 mg powder or tablet material or 1:100 | Platinic chloride | Aggregates of platy crystals form readily in glycerol-alcohol, gradually in $H_2O$. Plates with jagged edges, tendency to twin, forming X-shaped aggregates, hour-glass forms, and dendritic structures. First order gray polarization colors; symmetrical or parallel extinction. Plates show pos. elongation. |
| Hydrochlorothiazide (*9*) | 5% NaOH | — | Iodine-potassium iodide, **962.21A** | Burrs, with iodine-colored centers and highly birefringent peripheral blades or dichroic rods, iodine-colored to colorless[a] |
| 8-Hydroxyquinoline sulfate (*7*) | Dissolve salt in $H_2O$. Dissolve free base in HCl (1 + 3), avoiding excess | 1:500 | Magnesia mixt. | Small, elliptical grains. Few burr-shaped crystals on standing. |
| Mandelic acid (*2*) | $H_2O$ | 1:100 | Lead acetate | Rosettes of thin, curving plates. |
| | $H_2O$ | 1:100 | Mercurous nitrate | Burr-shaped groups of needles. |
| Methenamine (*5*) | $H_2O$ | 1:500 | Silicotungstic acid | Thin, transparent, rectangular crystals. |
| Neocinchophen (*1*) | HCl (1 + 3) | Satd soln | Ammonium thiocyanate | Rosettes of needles. (Gentle agitation by tipping slide back and forth hastens crystn.) |
| | HCl (1 + 3) | Satd soln | Platinic chloride | Needles in clusters. |
| Pentylenetetrazol (*10*) | $H_2O$ | — | Mercuric chloride (1:10) | Rods, many almost needle-like; frequency in groups; also in radiating aggregates. |
| | $H_2O$ | 1:100 | Silicotungstic acid | Amorphous, changes to elongated prisms; also long needles. |
| Phenacetin (Acetophenetidin (*1*) | — | Approx. 1 mg powd material | $HNO_3$ | Add 1 drop $HNO_3$, let stand few sec, then add 1 drop $H_2O$. Bright yellow, curving, branched crystals. |
| | HCl (1 + 3) | Satd soln | Iodine-potassium iodide | Large, irregular plates. |
| Phenazopyridine.HCl (*7*) | Dissolve salt in $H_2O$. Dissolve free base in HCl (1 + 3), avoiding excess | 1:1000 | Ammonium thiocyanate | Small, red-brown, dense sheaves. |

**Table 962.21B Characteristics of Microchemical Tests for Synthetics—*Continued***

| Synthetic | Solvent | Concentration of Synthetic | Reagent | Description of Tests and Crystals |
|---|---|---|---|---|
| Phenobarbital (*4*) | — | Approx. 1 mg powder | Ammoniacal nickel acetate | Stir to aid soln and crystn. Single rectangular crystals. |
| Pyrilamine maleate (*8*) | Glycerol-alcohol (1 + 1) or $H_2O$ | 1:1000 or ca 0.1 mg powder | Platinic chloride | Needles in rosette aggregates, sheaves, and singly. Needles show second order blue and green, and first order red and yellow polarization colors; parallel extinction and neg. elongation. |
| Salicylic acid (*6*) | HCl (1 + 3) | Dry powder | Bromide-bromate soln | Stir few crystals into 1 drop of the HCl. Add 1 drop reagent. Fine needles appear to grow from the crystals of salicylic acid. |
| | — | Dry powder | Lead triethanolamine | Stir few crystals into 1 drop reagent. Rods or needles grow from the crystals of salicylic acid. |
| | 2% triethanolamine | 1:100 to 1:200 | Silver nitrate | Small, irregular plates; few short rods. |
| Sulfadiazine (*11*) | $H_2O$ | — | Gold bromide in HCl | Red, circular masses composed of fine needles. |
| Sulfanilamide (*2*) | — | Dry powder | Benzaldehyde | Thoroly stir small amt into 1 drop reagent. 4-sided plates. |
| | 0.1*N* HCl | Satd soln | Sodium nitrite | Yellow needles. |
| Sulfapyridine (*12*) | Acetone + $H_2O$ | — | Gold chloride | Yellow rods or blades; also X-shaped aggregates. |
| Sulfapyridine sodium monohydrate (*12*) | $H_2O$ | 1:100 | Gold chloride | Yellow rods in X-shaped aggregates. |
| Sulfathiazole (*10*) | 50% alcohol | — | Picric acid | Long, fine, yellow needles, many curved, occur in dense rosettes; also short, stout rods in groups or singly. |
| | 50% alcohol (or no solv.) | — | Picrolonic acid | Distinct rosettes of very fine needles; also single needles. |
| Trolamine (Triethanolamine) (*5*) | $H_2O$ | 1:100 | Bismuth iodide | Oily globules changing to large, red, hexagonal plates and prismatic crystals. |
| Tripelennamine hydrochloride (*8*) | Glycerol-alcohol (1 + 1) or $H_2O$ | 1:1000 or ca 0.1 mg powder or tablet material | Platinic chloride | Small needles and bladed crystals in dense rosette aggregates and singly. Needles show first order white and yellow polarization colors, parallel extinction, and pos. elongation. |

[a] Official First Action.

Refs.: (*1*) JAOAC **19,** 514(1936).
(*2*) JAOAC **22,** 709(1939).
(*3*) JAOAC **18,** 523(1935).
(*4*) JAOAC **20,** 553(1937).
(*5*) JAOAC **17,** 435(1934).
(*6*) JAOAC **21,** 528(1938).
(*7*) JAOAC **16,** 391(1933).
(*8*) JAOAC **35,** 576(1952).
(*9*) JAOAC **61,** 1435(1978).
(*10*) JAOAC **25,** 830(1942).
(*11*) JAOAC **26,** 96(1943).
(*12*) JAOAC **24,** 830(1941).

**Table 960.55 Characteristics of Microchemical Tests for Sympathomimetics**

| Sympathomimetic | Reagent | Test | Description of Crystals |
|---|---|---|---|
| | | Volatile Substances | |
| *dl*-Amphetamine | Gold chloride in dild phosphoric acid | direct or volatility | Very irregular plates, with irregular blade-arms especially after evapn; sq if perfect. |
| | Platinic chloride in dild phosphoric acid | volatility | Irregular blades and needles, very low birefringence; after evapn, characteristic plates with narrow irregular arms of blades. |
| Dextroamphetamine (*d*-Amphetamine) | Gold chloride in dild phosphoric acid | direct or volatility | Long yellow rods and blades; with evapn, some crystals as with *dl* may form. |
| | Platinic chloride in dild phosphoric acid | volatility | Long needles, often bent, very little birefringence; after some evapn, long rectangular blades. (*l*-Ephedrine in direct test gives similar crystals which are more sol.; it is less volatile and does not normally form crystals in hanging drop.) |
| (*dl*-Methamphetamine) *dl*-Desoxyephedrine | Bismuth iodide in dild sulfuric acid | volatility | Drops, crystg in orange-red prisms with conspicuously slanting ends; inclined extinction ca 20°; also "mossy" formation of grains and some large deep red grains. |
| | Freshly prepd gold chloride in dild phosphoric acid | volatility | Right-angled crossed blades with serrated and/or lobed edges. |
| | Aged (≥4 months) gold chloride in dild phosphoric acid | volatility | X blade formation with highly birefringent ribs visible in thin crystals or thickened X blades. Right-angled crossed blades are not present. |
| Ephedrine ((-)-Ephedrine) | Gold chloride in dild phosphoric acid | direct or volatility | Long needles or splinters and long jointed forms; strong birefringence. |
| | Bismuth iodide in dild sulfuric acid | volatility | Long brownish orange needles, often branching or in sheaves; also, especially with evapn, orange irregular blades. |
| Epinephrine | Sodium tetraphenylboron | volatility | $MeNH_2$ liberated; birefringent X's or 4-arm crystals; also thick blades with central rib, pointed ends, pos. elongation. |
| Isoproterenol | Sodium tetraphenylboron | volatility | Isopropylamine liberated; plates tending to nonregular hexagons; no birefringence where plates lie flat but there are rods which are birefringent. |
| Methamphetamine and *dl*-Methamphetamine (*d*- and *dl*-Desoxyephedrine) | Gold chloride in dild phosphoric acid | direct or volatility | Long blades and jointed crystals, fairly high birefringence. |
| | Platinic chloride in dild phosphoric acid | volatility | Grains with sharp edges which aggregate in chains and short prisms. Birefringent. |
| Methamphetamine (*d*-Desoxyephedrine) | Bismuth iodide in dild sulfuric acid | volatility | Drops, long orange splinters, blades, needles; also deep red angular grains (red prisms only after evapn). |
| | Freshly prepd gold chloride in dild phosphoric acid | volatility | Numerous multiple "V"-shaped blades, few single "V"-shaped blades. |
| | Aged (≥4 months) gold chloride in dild phosphoric acid | volatility | Numerous single "V"-shaped blades. |
| Pseudoephedrine | Gold chloride in dild phosphoric acid | direct or volatility (2 hr) | Thin branching sticks, many like combs; some broaden to blades or spear-head plates; very high birefringence. |
| | | Slightly Volatile Substances | |
| Racephedrine (*dl*-Ephedrine) | Gold chloride in dild phosphoric acid | direct or volatility | Irregular plates based on the sq, growing along diagonals in 4 arms; some birefringent, some not. |
| | Bismuth iodide in dild sulfuric acid | volatility | Orange rods or sticks, short and stubby, some plates; more irregular plates on evapn. |
| Phenylpropanolamine | Gold chloride in dild phosphoric acid | direct | Plates and blades of extremely high birefringence, elongate hexagonal or diamonds, very bright colors. Branch into 4 or 6 irregular arms. |
| | | volatility (2 hr) | After definite drying, pyramidal grains to blades and plates with irregular arms, very birefringent. |
| Phenmetrazine | Gold chloride in dild phosphoric acid | direct or volatility | Rectangular plates joined in jagged arms of strongly birefringent crystals, often in X forms, very characteristic. |
| | Bismuth iodide in dild sulfuric acid | volatility | Orange-red blades, usually pointed ends, often in rosettes; also with needles in branching aggregates; also red prisms. |

## 935.68 Synthetic Drugs
### Microchemical Tests
### Final Action

(*See* Table **962.21B**.)

(**a**) *Acetic acid*.—Dil. 6 mL HOAc to 100 mL with $H_2O$.
(**b**) *Ammoniacal nickel acetate soln*.—Mix 1 vol. 5% $Ni(OAc)_2.4H_2O$ soln with 1 vol. $NH_4OH$ (2 + 3). Use clear supernate.
(**c**) *Ammoniacal silver nitrate soln*.—*See* **930.40A(a)**.
(**d**) *Ammonium thiocyanate soln*.—*See* **930.40A(c)**.
(**e**) *Barium hydroxide soln*.—Satd aq. soln.
(**f**) *Benzaldehyde*.—USP quality.
(**g**) *Bismuth iodide soln*.—*See* **930.40A(d)**.

(**h**) *Bromide-bromate soln.*—Dissolve 0.3 g $KBrO_3$ and 1.2 g KBr in $H_2O$, and dil. to 100 mL.

(**i**) *Glycerol-alcohol mixture.*—(1 + 1).

(**j**) *Gold bromide in hydrochloric acid soln.—See* **930.40A(g)**.

(**k**) *Gold chloride soln.—See* **930.40A(h)**.

(**l**) *Iodine-potassium iodide soln.—See* **930.40A(j)**.

(**m**) *Lead acetate soln.*—Dissolve 5 g $Pb(OAc)_2.3H_2O$ in $H_2O$ and dil. to 100 mL.

(**n**) *Lead triethanolamine soln.*—Add 1 mL triethanolamine (tech. 90% is satisfactory) to soln of 1 g $Pb(OAc)_2.3H_2O$ in 20 mL $H_2O$. Slight turbidity does not interfere.

(**o**) *Magnesia mixture.*—Dissolve 5.5 g $MgCl_2.6H_2O$ and 14.0 g $NH_4Cl$ in $H_2O$. Add 13.05 mL $NH_4OH$ and dil. to 100 mL with $H_2O$.

(**p**) *Mercuric chloride soln.—See* **930.40A(l)**.

(**q**) *Mercurous nitrate soln.*—Dissolve 15 g $HgNO_3.H_2O$ in mixt. of 90 mL $H_2O$ and 10 mL $HNO_3$ (1 + 9). Store in dark, amber bottle contg small globule of Hg.

(**r**) *Nitric acid.*—(1 + 1).

(**s**) *Phosphotungstic acid soln.*—Dissolve 5 g $P_2O_5.24WO_3.xH_2O$ in 100 mL $H_2O$.

(**t**) *Picric acid.*—Crystals.

(**u**) *Picrolonic acid soln.*—Dissolve 250 mg 1-(*p*-nitrophenyl)-3-methyl-4-nitropyrazolone in 25 mL alcohol.

(**v**) *Platinic chloride soln.—See* **930.40A(n)**.

(**w**) *Potassium cadmium iodide soln.—See* **930.40A(o)**.

(**x**) *Potassium ferrocyanide soln.—See* **930.40A(p)**.

(**y**) *Silicotungstic acid soln.*—Dissolve 5 g $4H_2O.SiO_2.12WO_3.22H_2O$ in 100 mL ca 6*N* $H_2SO_4$.

(**z**) *Silver nitrate soln.*—Dissolve 1 g $AgNO_3$ in 20 mL $H_2O$.

(**aa**) *Sodium nitrite soln.*—Dissolve 10 g $NaNO_2$ in $H_2O$ and dil. to 100 mL.

(**bb**) *Zinc pyridine soln.*—Add 1 mL pyridine to soln of 1 g $Zn(OAc)_2.2H_2O$ in 20 mL $H_2O$.

## 960.56 Xanthine Group Alkaloid Drugs

### Microchemical Tests

### First Action

### A. Reagents

(**a**) *Bismuth iodide soln.—See* **930.40A(d)**(*2*).

(**b**) *Gold bromide in dilute hydrochloric acid.*—Dissolve 1 g $HAuCl_4.3H_2O$ in 1.5 mL 40% HBr and add HCl (1 + 3) to make 45 mL.

(**c**) *Iodine-potassium iodide soln* (*5–14*).—Dissolve 5 g I and 14 g KI in $H_2O$ and dil. to 100 mL with $H_2O$.

### B. General Test

(Murexide reaction)

To small amt of substance in small porcelain crucible add very small crystal $KClO_3$ and 1 drop HCl (1 + 1). Set on hot plate at ca 100°, or hot enough to boil off $H_2O$ in short time. Soon after drying, residue becomes orange to red. Add 1 drop $NH_4OH$. Purple color is produced in presence of caffeine, theobromine, theophylline, and related xanthine derivatives.

### C. Identification

(**a**) *Bismuth iodide soln.*—Add 1 drop reagent to little dry material on slide and cover.

(**b**) *Gold bromide in dilute hydrochloric acid.*—Place 1 drop reagent beside very small amt of dry substance on slide and apply cover glass so that reagent flows over substance.

(**c**) *Iodine-potassium iodide soln* (*5–14*).—In depression of cavity slide dissolve little of substance in small drop 1% NaOH soln and stir in excess $NaHCO_3$ (some undissolved). Add large drop reagent and stir slightly. Add several crystals KCl. Examine center and edge as soln evaps.

*See* Table **960.56**.

Refs.: JAOAC **43**, 262(1960); **61**, 1435(1978).

# MICROSCOPY

## 960.57 Crystalline Substances

### Optical-Crystallographic Examination

### Final Action 1970

(General knowledge of microscopy and crystallography is necessary for application of this technic. Some of std works on this subject are listed in References. Optical-crystallographic properties of antihistamines, alkaloids, antibiotics, barbiturates, hallucinogens, steroids, sulfonamides, sympathomimetic amines and tranquilizers are given in Tables **955.57** and **955.58**.)

### A. Apparatus

(**a**) *Polarizing microscope.*—Fitted with polarizing prisms below and above rotating, graduated circular stage and with accessories (Bertrand lens or pinhole eyepiece, first order red

**Table 960.56 Characteristics of Microchemical Tests for Xanthine Alkaloids**

| Alkaloid | Reagent | Description of Crystals |
|---|---|---|
| Caffeine | Gold bromide in dil. HCl | Outer part: brownish needles with bright white birefringence. Inner part: small rods to sticks, little grains and plates with weak yellow birefringence. |
| | Bismuth iodide | Small brownish orange rods or blades growing from sample or nearby in rosettes; also some orange grains. |
| | Iodine-potassium iodide (5–14) | Grains, dark red to black, sometimes yellow or orange-brown; generally sq or cubical; birefringent with fairly strong light; some irregular dichroic blades. |
| Dyphylline | Gold bromide in dil. HCl | Needles, scattered and in rosettes; fairly bright birefringence. |
| | Bismuth iodide | Very small grains, flakes, blades in multitudes, birefringent. |
| | Iodine-potassium iodide (5–14) | Fuzzy brown dense rosettes thruout drop, birefringent around rims; excess reagent must be used; 5 min required to form crystals. |
| Theobromine | Gold bromide in dil. HCl | Grains or plates in dense groups; bright birefringence at edge of cluster. |
| | Bismuth iodide | Brown needles in rosettes. |
| | Iodine-potassium iodide (5–14) | Orange-brown chips; also rectangular plates with opposite sides incised; smaller crystals: grains, often lens shaped or diamonds; birefringent, somewhat dichroic. |
| Theophylline | Gold bromide in dil. HCl | Long needles in sheaves; fairly bright birefringence. |
| | Bismuth iodide | Grains and short prisms, often rectangular; brightly birefringent. |
| | Iodine-potassium iodide (5–14) | Black needles in rosettes around edge; birefringent; when larger, blades or rods, dichroic black vertically to yellow horizontally. |

or quartz wedge compensators) for observation of interference figures, optic sign, and sign of elongation.

(**b**) *Refractometer*.—For measuring refractive indices of liqs at 20° or 25° from 1.300 to 1.840 with precision of ±0.0005.

### *B. Reagents*

*Immersion media*.—Ideally immersion media for refractive index detn should have same color and intensity of color as substance being examined and be chemically stable. Refractive indices should not vary perceptibly with ordinary changes of temp. with exception of special liqs used in index-variation methods. Permanent set of liqs covering range 1.430–1.790 in 0.005 intervals made with following mixts is useful for both inorg. and org. substances:

| Mixture | $n_D$ (20°) |
|---|---|
| Kerosene and mineral oil | 1.435–1.480 |
| Mineral oil and α-monochloronaphthalene | 1.485–1.640 |
| α-Monochloronaphthalene and methylene iodide | 1.645–1.740 |
| Methylene iodide and sulfur | 1.740–1.790 |

Substances sol. in these liqs require prepg special set of liqs.

### *C. Determinations*

*Refractive indices*.—Det. refractive indices by mounting cryst. material in suitable immersion liqs and observing Becke line. Successively suspend crystals or crystal fragments of substance in immersion liqs of known refractive indices. Greater the difference between refractive indices of crystal and liq., the more prominently one stands out in bold relief from other. By repeatedly mounting such crystals in oils of successively lower or higher index, ultimately zone of contact of crystal and liq. becomes practically invisible, demonstrating that refractive indices of liq. and solid have been matched.

In case of substances crystg in isometric (cubic) system, there is only 1 refractive index, designated by $n$. Such substances are not doubly refractive when examined with crossed nicols. Substances crystg in other systems, hexagonal, tetragonal, monoclinic, triclinic, and orthorhombic, in ideal cases, have >1 measurable refractive index. With uniaxial substances such as those crystg in hexagonal and tetragonal systems, 2 significant indices can be detd, designated as $n_\epsilon$ and $n_\omega$. Substances crystg in monoclinic, triclinic, and ortho-rhombic systems, in ideal cases, have 3 refractive indices, designated as $n_\alpha$, $n_\beta$, and $n_\gamma$.

*Extinction and extinction angle of anisotropic substances*.—Anisotropic crystals, when rotated through 360° on stage, become dark 4 times. Positions of darkness are known as extinction positions and correspond to positions in which vibrations of birefringent rays produced by crystal are mutually parallel to vibration directions of polarizer and analyzer indicated by cross hairs in eyepiece. If crystal extinguishes when crystal edge or face is parallel to one of cross hairs, extinction is *parallel*. If bisector of silhouette angle is parallel to one of cross hairs, extinction is *symmetrical*. Crystals showing extinction differing from these 2 have *inclined* extinction. Measure extinction angles on those crystals showing inclined extinction by rotating crystal so that crystal edge or face is parallel to 1 of cross hairs. Rotate stage until crystal extinguishes. Read on stage vernier extinction angle between face or edge at extinction and nearest cross hair. Express extinction angles with relationship to principal vibration directions of light and crystallographic axes.

*Elongation*.—Many crystals are frequently elongated in 1 direction. Relationship between direction of elongation and vibration directions of slow and fast rays of anisotropic crystal is sometimes of determinative value. If substance is length slow, *i.e.*, slow ray or higher refractive index is parallel to direction of elongation, sign of elongation is pos.; if substance is length fast, sign is neg.

Sign of elongation (+ or −) is detd with gypsum plate and crossed nicols. A long and narrow crystal, showing very little color with crossed nicols, is so oriented that its long dimension is parallel to direction "z" of plate (slow ray) which is inserted in slit of microscope tube. (Direction "z" is indicated by arrow on plate.) If crystal appears blue or other color of higher order than red-violet due to plate, elongation is +; if crystal appears yellow, white, or gray, *i.e.*, of lower order color than red-violet field, elongation is −.

*Optic character and optic sign*.—Det. optic character (uniaxial or biaxial) and optic sign (+ or −), using first order red or quartz wedge compensators in conjunction with interference figures. Obtain interference figures from conoscopic images of crystals suitably oriented. In absence of interference figures, det. these properties from relationship of principal refractive indices. When $(n_\beta - n_\alpha)$ is $<(n_\gamma - n_\beta)$, optic sign is +. When $(n_\beta - n_\alpha)$ is $>(n_\gamma - n_\beta)$, optic sign is −.

*Optic axial angle (2V)*.—Calc. axial angle (2V) from values of 3 refractive indices (here designated α, β, and γ) according to formulas:

$$\mathrm{Cos}^2\, V_\alpha = \frac{\gamma^2(\beta^2 - \alpha^2)}{\beta^2(\gamma^2 - \alpha^2)} \text{ (for − optic sign), or}$$

$$\mathrm{Cos}^2\, V_\gamma = \frac{\alpha^2(\gamma^2 - \beta^2)}{\beta^2(\gamma^2 - \alpha^2)} \text{ (for + optic sign)}$$

where $2V_\alpha$ is axial angle about α, and $2V_\gamma$ is axial angle about γ. Alternatively, est. approx. value of 2V from curvature of isogyre referring to diagrams of substances with known angles. Angle ranges from small (0–25°, sharply curved) to medium (26–60°, moderately curved) to large (61–90°, nearly straight isogyre).

Refs.: Stewart and Stolman, editors, "Toxicology, Mechanisms and Analytical Methods," Vol. 1, pp. 660–713(1960). Hartshorne and Stuart, "Crystals and the Polarizing Microscope," 3rd ed., 1960. Chamot and Mason, "Handbook of Chemical Microscopy," Vol. **1**, 1983. Bloss, "Introduction to the Methods of Optical Crystallography," 1961. Wahlstrom, "Optical Crystallography," 1979. McCrone and Delly, "The Particle Altas," Vols IV and V, 1973. Sunshine, editor, "Handbook of Analytical Toxicology," pp. 289–330, 1969.

## MISCELLANEOUS

## 985.45 Trimethobenzamide Hydrochloride in Drugs

**Ion-Pair Column Chromatographic Method**

**First Action 1985**
**Final Action 1988**

(Applicable to capsules and injections)

### *A. Principle*

Sample is dild in $H_2O$, acidified, mixed with Celite, and packed in chromatgc column. Breakdown products and preservatives are removed with ether. Trimethobenzamide HCl is eluted with $CH_2Cl_2$, and detd by UV spectrophotometry at 261 nm.

### B. Apparatus

(a) *Chromatographic column and tamping rod.*—See **968.42B**.

(b) *Filter paper.*—Schleicher & Schuell, Inc., no. 589 White ribbon, or equiv.

(c) *Ultraviolet spectrophotometer.*—With matched 1 cm cells.

### C. Reagents

(a) *Celite 545.*—See **960.53B**.

(b) *Hydrochloric acid soln.*—1M. Dil. 8.3 mL HCl to 100 mL with $H_2O$.

(c) *Water-saturated ether.*—Shake equal vols ether and $H_2O$ in separator 1 min. Discard lower phase.

(d) *Methylene chloride.*—Distilled-in-glass grade.

(e) *Water-saturated methylene chloride.*—Shake equal vols $CH_2Cl_2$ and $H_2O$ in separator. Discard $H_2O$ phase.

(f) *Trimethobenzamide HCl (TMBH) std soln.*—0.02 mg/mL. Accurately weigh 10 mg USP TMBH, previously dried 4 h at 105°, and transfer to 100 mL vol. flask. Add 70 mL $CH_2Cl_2$ and sonicate until dissolved. Dil. to vol. with $CH_2Cl_2$. Transfer 10.0 mL of this soln to 50 mL vol. flask and dil. to vol. with $CH_2Cl_2$.

### D. Preparation of Sample

(a) *Capsules.*—Det. av. wt of contents per capsule and reduce to powder passing No. 60 sieve. Accurately weigh portion of powder contg ca 200 mg TMBH into 50 mL vol. flask. Add 30 mL $H_2O$, mech. shake 10 min, and dil. to vol. with $H_2O$. Mix and filter, discarding first 10 mL filtrate.

(b) *Injections.*—Transfer accurately measured vol. soln, contg ca 200 mg TMBH, into 50 mL vol. flask. Dil. to vol. with $H_2O$.

### E. Preparation of Column

Pack pledget of fine glass wool in base of chromatgc tube. Mix 1 g Celite 545 with 0.5 mL 1M HCl in 50 mL beaker, transfer to column, and tamp. Transfer 2.0 mL prepd sample soln to 100 mL beaker, add 4 drops concd HCl, and swirl gently. Add 3 g Celite 545, mix well, and transfer to column. Scrub beaker with 1 g Celite 545, transfer Celite to column, wipe beaker and spatula with pledget of glass wool, and tamp glass wool on column.

### F. Determination

Elute column with 50 mL $H_2O$-satd ether. Rinse column tip with ether, and discard eluate and rinses. Place 200 mL vol. flask under column, and elute column with four 50 mL portions of $H_2O$-satd $CH_2Cl_2$. Rinse column tip with $CH_2Cl_2$, add rinse to flask, and dil. to vol. with $CH_2Cl_2$. Transfer 25.0 mL of this soln to 50 mL vol. flask and dil. to vol. with $CH_2Cl_2$. Det. $A$ of sample and std solns at 261 nm, using $CH_2Cl_2$ as blank. If recording spectrophtr is used, scan from 340 to 235 nm.

$$\text{mg TMBH/capsule} = (A/A') \times (C) \times (10\,000) \times (K/W)$$

$$\text{mg TMBH/mL injection} = (A/A') \times (C) \times (10\,000/V)$$

where $A$ and $A'$ refer to sample and std solns, resp.; $C$ = concn std soln, mg/mL; $K$ = av. capsule contents wt, g; $W$ = sample wt, g; and $V$ = vol. injection, mL.

Ref.: JAOAC **68,** 1055(1985).

CAS-138-56-7 (trimethobenzamide)

## ANTIFUNGAL

### 988.21 Flucytosine in Drug Capsules
**Liquid Chromatographic Method**
**First Action 1988**

### A. Principle

Flucytosine content of capsules is detd by liq. chromatgy on $C_{18}$ reverse phase column, using $H_2O$-MeOH-HOAc mobile phase contg 1-octanesulfonic acid Na salt, *p*-aminobenzoic acid as internal std, and UV detection at 285 nm.

### B. Apparatus

(a) *Liquid chromatograph.*—Model 8800 solv. pump with variable wavelength detector capable of monitoring elution at 285 nm (Du Pont Instrument Products Div.), injection valve with 20 µL sample loop (Valco Instruments Co., Inc., PO Box 55603, Houston, TX 77055), and suitable strip chart recorder. Equiv. LC system may be used. Operating conditions: flow rate 1.5 mL/min; detector 285 nm, 0.32 AUFS; chart speed 0.5 cm/min; temp. ambient; injection vol. 20 µL. To detect fluorouracil, main degradation/precursor product of flucytosine, monitor sepn at 266 nm to maximize sensitivity.

(b) *Chromatographic column.*—Stainless steel, 300 mm × 3.9 mm id, packed with 10 µm µBondapak $C_{18}$ (Waters Associates, Inc.), or equiv. column that meets LC system suitability requirements.

### C. Reagents

(a) *1-Octanesulfonic acid NA salt.*—(Eastman Kodak Co.).

(b) *p-Aminobenzoic acid.*—Certified ACS Grade (Fisher Scientific Co.), or equiv.

(c) *Mobile phase.*—$H_2O$-MeOH-HOAc (785 + 200 + 15 v/v/v) contg 2 g/L of 1-octanesulfonic acid Na salt.

(d) *Internal std soln.*—Accurately weigh ca 160 mg *p*-aminobenzoic acid and transfer to 200 mL vol. flask. Add 100 mL mobile phase, sonicate 5 min, shake mech. 25 min, dil. to vol. with mobile phase, and mix well.

(e) *Flucytosine std soln.*—Accurately weigh ca 30 mg USP Flucytosine Ref. Std and transfer to 50 mL vol. flask. Add 25 mL mobile phase, sonicate 5 min with gentle swirling, shake mech. 25 min, dil. to vol. with mobile phase, and mix. Transfer 10.0 mL of this soln to 100 mL vol. flask, add 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix. Prep. this soln fresh daily.

### D. Sample Preparation

Accurately weigh contents of ≥20 flucytosine capsules and det. av. wt/capsule. Accurately weigh portion of powder equiv. to ca 100 mg flucytosine and transfer to 100 mL vol. flask. Add 50 mL mobile phase, sonicate 5 min with gentle swirling, shake mech. 25 min, dil. to vol. with mobile phase, and mix well. Filter portion of soln thru suitable paper or 0.45 µm membrane filter, discarding first 10 mL filtrate. Transfer 6.0 mL filtrate to 100 mL vol. flask, add 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix well. From this point, complete detn on same day.

### E. Suitability Test and Determination

Inject 20 µL each of sample and std solns into LC system by using sampling valve or high pressure microsyringe. Operate system as described in *Apparatus*. Adjust detector sensitivity so peak response for flucytosine is between 40 and 75% full scale. In suitable chromatogram, CV of peak ht (or area) ratios for 6 replicate injections of std soln should be ≤2.0%, and resolution factor, $R$, for flucytosine peak and internal std peak should be ≥2.

Calc. resolution factor as follows:

$$R = [2(t_2 - t_1)]/(W_2 + W_1)$$

where $t_2$ and $t_1$ = retention times of 2 components, and $W_2$ and $W_1$ = corresponding widths of peaks, measured by extrapolating sides of peaks to baseline.

### F. Calculation

Calc. amt flucytosine in dosage form, using response ratios based on either peak hts or peak areas, according to following equation:

$$\text{Flucytosine, mg/capsule} = 1.667C \times (R/R') \times (T/W)$$

where $C$ = concn, μg/mL, of flucytosine in std soln; $R$ and $R'$ = response ratios for flucytosine peak to internal std peak for sample and std, resp.; $T$ = av. capsule wt, mg; $W$ = wt sample taken for assay, mg.

Ref.: JAOAC **71,** 33(1988).

CAS-2022-85-7 (flucytosine)

## ANTIPARKINSONIAN

## 988.22 Levodopa and Levodopa-Carbidopa in Solid Dosage Forms
### Liquid Chromatographic Method
### First Action 1988

### A. Principle

Levodopa in tablets or capsules and levodopa-carbidopa in tablets are detd by reverse phase liq. chromatgy on $C_{18}$ column with 3% HOAc as mobile phase, and UV detection at 280 nm. Methyldopa is internal std for levodopa tablets or capsules; acetaminophen is internal std for levodopa-carbidopa tablets.

### B. Apparatus

(**a**) *Liquid chromatograph.*—Isocratic system equipped with detector capable of monitoring $A$ at 280 nm, suitable strip chart recorder, and injection valve with 20 μL sample loop.

(**b**) *Chromatographic column.*—300 × 3.9 mm id, μBondapak $C_{18}$, 10 μm particle size (Waters Associates, Inc), or equiv. column that meets suitability requirements.

(**c**) *Membrane filters.*—0.45 μm porosity (Millipore, or equiv.).

### C. Reagents

(**a**) *Mobile phase.*—3% aq. HOAc.

(**b**) *Methyldopa internal std soln.*—2 mg/mL. Accurately weigh ca 200 mg USP Methyldopa Ref. Std into 100 mL vol. flask, add 50 mL 0.1N HCl, and sonicate to dissolve std. Dil. to vol. with mobile phase, and mix.

(**c**) *Acetaminophen internal std soln.*—0.5 mg/mL. Accurately weigh ca 125 mg USP Acetaminophen Ref. Std into 250 mL vol. flask, add 75 mL MeOH, and sonicate to dissolve std. Dil. to vol. with mobile phase, and mix.

(**d**) *Levodopa std soln.*—Just prior to use, dry USP Levodopa Ref. Std 4 h at 105°. Store in tightly covered, light-resistant container. Accurately weigh ca 100 mg dried std into 50 mL vol. flask. Add 30 mL 0.1N HCl, and sonicate to dissolve. Dil. to vol. with 0.1N HCl, and mix. Filter soln thru 0.45 μm membrane filter, discarding first 5 mL filtrate. Pipet 5 mL filtrate and 10 mL methyldopa internal std soln into 100 mL vol. flask, dil. to vol. with mobile phase, and mix.

(**e**) *Levodopa-carbidopa std soln.*—Dry USP Carbidopa Ref. Std to const wt at 100° under reduced pressure not exceeding 5 mm Hg. Store in tightly covered, light-resistant container. Accurately weigh ca 100 mg dried USP Levodopa Ref. Std (**d**) into 50 mL vol. flask. Add accurately weighed amt of dried carbidopa std so that carbidopa-to-levodopa ratio corresponds to that found in com. levodopa-carbidopa tablet. Add 30 mL 0.1N HCl, sonicate to dissolve, dil. to vol. with 0.1N HCl, and mix. Filter soln thru 0.45 μm membrane filter, discarding first 5 mL filtrate. Pipet 10 mL filtrate into 100 mL vol. flask, and add vol. of acetaminophen internal std soln so that acetaminophen concn is 1.25 times carbidopa concn. Dil. to vol. with mobile phase, and mix.

### D. Sample Preparation

(**a**) *Levodopa tablets or capsules.*—Weigh and finely powder ≥20 tablets or composite contents of 20 capsules. Weigh portion of powder equiv. to ca 100 mg levodopa into 50 mL vol. flask, and proceed as directed under levodopa std soln (**d**), beginning "Add 30 mL 0.1N HCl . . ."

(**b**) *Levodopa-carbidopa tablets.*—Weigh and finely powder ≥20 tablets. Weigh portion of powder equiv. to ca 100 mg levodopa into 50 mL vol. flask, and proceed as directed under levodopa-carbidopa std soln (**e**), beginning "Add 30 mL 0.1N HCl . . ."

(**c**) *Levodopa-carbidopa tablets for content uniformity determination.*—Dissolve 1 tablet in sufficient 0.1N HCl to prep. soln contg 2 mg levodopa/mL. Filter soln thru 0.45 μm membrane filter, discarding first 5 mL filtrate. Pipet 10.0 mL filtrate into 100 mL vol. flask, add acetaminophen internal std soln (15 mL for levodopa-carbidopa 100/25 tablets, 5 mL for all other dosage levels), dil. to vol. with 0.1N HCl, and mix.

### E. System Suitability Test and Assay

Equilibrate LC system with mobile phase at 1.5 mL/min. Inject 20 μL std soln. Approx. retention times are levodopa, 3 min; methyldopa, 4.5 min; carbidopa, 5 min; and acetaminophen, 9 min. Calc. resolution factor, $R$, as follows:

$$R = [2(t_2 - t_1)]/(W_2 + W_1)$$

where $t_2$ and $t_1$ = retention times of the 2 components, and $W_2$ and $W_1$ = corresponding widths of bases of peaks obtained by extrapolating relatively straight sides of peaks to baseline. $R$ between levodopa and carbidopa and between carbidopa and acetaminophen should be >3.5. $R$ between levodopa and methyldopa should be >2.

Change flow rate to improve resolution. For levodopa-carbidopa tablets, change detector sensitivity between levodopa peak (approx. 0.64 AUFS) and carbidopa peak (approx. 0.08 or 0.04 AUFS). Set detector sensitivity to 35–95% AUFS. If necessary, adjust vol. of internal std soln added to sample soln and std soln to obtain satisfactory detector response for std soln. Inject std soln 5 times and compare peak hts. Calc. CV as follows:

$$\text{CV, \%} = \frac{100}{\bar{X}}\left[\frac{\sum_{i=1}^{N}(X_1 - \bar{X})^2}{N-1}\right]^{1/2}$$

where $\bar{X}$ = mean of set of $N$ measurements, and $X_1$ = an individual detn of ratio of peak ht of analyte to peak ht of internal std. In suitable system, CV = ≤2.0%.

Proceed with sample analysis by injecting 20 μL each of sample soln and corresponding std soln.

### F. Calculation

Using peak ht ratios $R$ and $R'$ relative to internal std, calc. mg drug/tablet or capsule as follows:

$$\text{mg/tablet or capsule} = (R/R') \times C \times (D/W) \times T$$

where $R$ and $R'$ = peak ht ratios for sample and std solns, resp.; $C$ = concn of std soln, mg/mL; $W$ = wt of sample taken, mg; $D$ = sample diln; and $T$ = av. tablet or capsule wt, mg.

Ref.: JAOAC **70,** 987(1987).

CAS-59-92-7 (levodopa)
CAS-38821-49-7 (carbidopa)
CAS-28860-95-9 (carbidopa anhydrous)

## ANTIHYPERTENSIVE

### 989.07 Hydralazine Hydrochloride in Drug Tablets

**Spectrophotometric Method**
**First Action 1989**

Method Performance:

Tablets, 10 mg:
$s_r = 0.53$; $s_R = 1.01$; $RSD_r = 0.55\%$; $RSD_R = 1.06\%$
Tablets, 100 mg:
$s_r = 0.26$; $s_R = 0.77$; $RSD_r = 0.26\%$; $RSD_R = 0.77\%$
Synthetic mixt., 10 mg:
$s_r = 0.78$; $s_R = 0.96$; $RSD_r = 0.77\%$; $RSD_R = 0.95\%$

#### A. Principle

Hydralazine HCl is converted to tetrazolo[5,1-α]phthalazine, which is detd by UV detection at 274 nm.

#### B. Apparatus and Reagents

(**a**) *Spectrophotometer*.—UV-vis., recording, with matched 1 cm quartz cells, to measure $A$ at 274 nm.

(**b**) *$NaNO_2$ soln*.—1% w/v $NaNO_2$ (ACS grade or equiv.). Prep. fresh daily.

(**c**) *Hydralazine HCl std soln*.—Accurately weigh ca 25 mg USP Hydralazine Hydrochloride Ref. Std previously dried under vac. over $P_2O_5$ for 8 h, and transfer to 250 mL vol. flask. Dil. to vol. with 0.1N HCl and mix well. Pipet 20.0 mL dild soln into 100 mL vol. flask, add 1.0 mL 1% $NaNO_2$ soln, mix, and heat mixt. ca 1 h on steam bath. Cool soln to room temp., dil. to vol. with $H_2O$, and mix.

#### C. Preparation of Sample

Weigh and finely powder ≥20 tablets. Accurately weigh portion of powder (or crushed tablet) equiv. to ca 25 mg hydralazine HCl, and transfer to 250 mL vol. flask. Add ca 125 mL 0.1N HCl and mech. shake 20 min. Dil. to vol. with 0.1N HCl, mix, and filter, discarding first 20 mL filtrate. Pipet 20.0 mL filtrate into 100 mL vol. flask, add 1.0 mL 1% $NaNO_2$ soln, mix, and heat ca 1 h on steam bath. Cool soln to room temp., dil. to vol. with $H_2O$, and mix.

#### D. Determination

Use suitable spectrophtr (**a**) to concomitantly det. $A$ of sample and std solns contg ca 20 μg hydralazine HCl/mL.

Calc. mg hydralazine HCl/tablet as follows:

$$\text{Hydralazine HCl, mg/tablet} = (A_u/A_s) \times (W_s/W_u) \times T$$

where $A_u$ and $A_s$ = $A$ of sample and std solns, resp.; $W_s$ and $W_u$ = mg ref. std and sample taken for std and sample solns, resp.; and $T$ = av. tablet wt, mg.

Ref.: JAOAC **71,** 1121(1988).

CAS-86-54-4 (hydralazine)
CAS-304-20-1 (hydralazine monohydrochloride)

**Common and Chemical Names of Drugs in this Chapter**

| Common Name | Chemical Name |
|---|---|
| Acetanilide | *N*-Phenylacetamide |
| Aconitine | 16-Ethyl-1,16,19-trimethoxy-4-(methoxymethyl)aconitane-3,8,10,11,18-pentol 8-acetate 10-benzoate |
| Allobarbital | 5,5-Di-2-propenyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Aminopyrine | 4-(Dimethylamino)-1,2-dihydro-1,5-dimethyl-2-phenyl-3*H*-pyrazol-3-one |
| Amitriptyline hydrochloride | 3- (10,11-Dihydro-5*H*-dibenzo(*a,d*)-cyclohepten-5-ylidene)-*N,N*-dimethyl-1-propanamine hydrochloride |
| Amobarbital | 5-Ethyl-5-(3-methylbutyl)-2,4,6-(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Amphetamine sulfate | (±)-α-Methylphenethylamine sulfate |
| Amylocaine hydrochloride | 1-(Dimethylamino)-2-methyl-2-butanol benzoate hydrochloride |
| Antipyrine | 1,2-Dihydro-1,5-dimethyl-2-phenyl-3*H*-pyrazole-3-one |
| Apomorphine | 5,6,6a,7-Tetrahydro-6-methyl-4*H*-dibenzo(*de,g*)quinoline-10,11-diol |
| Aprobarbital | 5-(1-Methylethyl)-5-(2-propenyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Arecoline | 1,2,5,6-Tetrahydro-1-methyl-3-pyridinecarboxylic acid methyl ester |
| Atropine | α-(Hydroxymethyl) 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester benzeneacetic acid |
| Aspirin | 2-(Acetyloxy)benzoic acid |
| Barbital sodium | 5,5-Diethyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione sodium salt |
| Benzocaine | *p*-Aminobenzoic acid ethyl ester |
| Bemegride | 3-Ethyl-3-methylglutarimide |
| Berberine | 5,6-Dihydro-9,10-dimethoxybenzo(*g*)-1,3-benzodioxolo(5,6-*a*)quinolizinium |
| Brucine | 2,3-Dimethoxystrychnidin-10-one |
| Butabarbital sodium | 5-Ethyl-5-(1-methylpropyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione monosodium salt |
| Butacaine sulfate | 4-Aminobenzoate 3-dibutylamino-1-propanol sulfate |
| Butalbital | 5-(2-Methylpropyl)-5-(2-propenyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Butethal | 5-Butyl-5-ethyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Caffeine | 3,7-Dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione |
| Calomel | Mercurous chloride |
| Carbidopa | (−)-L-α-Hydrazino-3,4-dihydroxy-α-methylhydrocinnamic acid monohydrate |
| Chlorobutanol | 1,1,1-Trichloro-2-methyl-2-propanol |
| Chlorpheniramine maleate | γ-(4-Chlorophenyl)-*N,N*-dimethylpyridine propanamine-2-butenedioate |
| Choline | 2-Hydroxy-*N,N,N*-trimethylethanaminium hydroxide |
| Cinchonidine | (8α,9*R*)-Cinchonan-9-ol |
| Cinchonine | (9*S*)-Cinchonan-9-ol |
| Cinchophen | 2-Phenylcinchoninic acid |
| Cocaine | 3-(Benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester |
| Codeine phosphate | (5α, 6α)-7,8-Didehydro-4,5-epoxy-3-methoxy-17-methylmorphinan-6-ol phosphate (salt) hemihydrate |
| Cotarnine | 5,6,7,8-Tetrahydro-4-methoxy-6-methyl-1,3-dioxolo(4,5-*g*)isoquinolin-5-ol |

**Common and Chemical Names of Drugs in this Chapter (*Continued*)**

| Common Name | Chemical Name |
|---|---|
| Cyclobarbital | 5-(1-Cyclohexen-1-yl)-5-ethyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Dextroamphetamine | *d*-α-Methylphenethylamine |
| Dextroamphetamine sulfate | *d*-α-Methylphenethylamine sulfate |
| Dextromethorphan hydrobromide | 3-Methoxy-17-methyl-9α,13α,14α-morphinan hydrobromate monohydrate |
| Dichlorophen | 2,2′-Methylenebis(4-chlorophenol) |
| Diphenhydramine hydrochloride | 2-Diphenylmethoxy-*N,N*-dimethylethanamine hydrochloride |
| Dyphylline | 7-(2,3-Dihydroxypropyl)-3,7-dihydro-1,3-dimethyl-1*H*-purine-2,6-dione |
| Ephedrine | α-[1-(Methylamino)ethyl]benzenemethanol |
| Ephedrine sulfate | α[1-(Methylamino)ethyl]benzenemethanol sulfate |
| Epinephrine bitartrate | (−)-3,4-Dihydroxy-α-[(methylamino)methyl] benzyl alcohol (+)-tartrate (1:1) salt |
| Erythrityl tetranitrate | 1,2,3,4-Butanetetrol, tetranitrate |
| Ethylhydrocupreine | 8α, 9*R*-6′-Ethoxy-10,11-dihydro-cinchonan-9-ol |
| Ethylmorphine | (5α, 6α)-7,8-Didehydro-4,5-epoxy-3-ethoxy-17-methylmorphinan-6-ol |
| Flucytosine | 5-Fluorocytosine |
| Fluorescein sodium | 3′,6′-Dihydroxyspiro[isobenzofuran-1(3*H*),9′-[9*H*]xanthen]-3-one disodium salt |
| Heptabarbital | 5-(1-Cyclohepten-1-yl)-5-ethyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Heroin | (5α, 6α)-7,8-Didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol diacetate (ester) |
| Hexobarbital sodium | 5-(1-Cyclohexen-1-y)-1,5-dimethyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidetrione monosodium salt |
| Homatropine | α-Hydroxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester benzeneacetic acid |
| Hydralazine hydrochloride | 1-Hydrazinophthalazine monohydrochloride |
| Hydrastine | 6,7-Dimethoxy-3-[5,6,7,8-tetrahydro-6-methyl-1,3-dioxolo(4,5-*g*)isoquinolin-5-yl]-1(3*H*)-isobenzofuranone |
| Hydrastinine | 5,6,7,8-Tetrahydro-6-methyl-1,3-dioxolo(4,5-*g*)isoquinolin-5-ol |
| Hydrochlorothiazide | 6-Chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide |
| Hydromorphone | 4,5α-Epoxy-3-hydroxy-17-methylmorphinan-6-one |
| 8-Hydroxyquinoline sulfate | 8-Quinolinol sulfate |
| Hyoscyamine | α-(Hydroxymethyl)-8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester benzeneacetic acid |
| Iodoform | Triiodomethane |
| Isoproterenol | 4-[1-Hydroxy-2-[(1-methylethyl)-amino]ethyl]-1,2-benzenediol |
| Mandelic acid | α-Hydroxybenzeneacetic acid |
| Meperidine hydrochloride | 1-Methyl-4-phenyl-4-piperidinecarboxylic acid, ethyl ester, hydrochloride |
| Mephentermine sulfate | *N*,α,α-Trimethylphenethylamine sulfate |
| Meprobamate | 2-Methyl-2-propyl-1,3-propanediol dicarbamate |
| Merbromin | 2,7-Dibromo-4-hydroxymercurifluoresceine dibromide salt |
| Methamphetamine hydrochloride | (+)-*N*,α-Dimethylphenethylamine hydrochloride |
| Methapyrilene hydrochloride | 2-[[2-(Dimethylamino)ethyl]-2-thenylamino]pyridine monohydrochloride |
| Metharbital | 5,5-Diethyl-1-methyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Methenamine | 1,3,5,7-Tetraazatricyclo[3.3.1.1$^{3,7}$]-decane |
| Methenamine mandelate | α-Hydroxybenzeneacetic acid, compd. with 1,3,5,7-tetraazatricyclo-[3.3.1.1$^{3,7}$] decane (1:1) |
| Methocarbamol | 3-(*o*-Methoxyphenoxy)-1,2-propanediol 1-carbamate |
| Methyldopa | L-3-(3,4-Dihydroxyphenyl)-2-methylalanine sesquihydrate |
| Methylene blue | 3,7-Bis(dimethylamino)phenothiazin-5-ium chloride |
| Morphine | (5α, 6α)-7,8-Didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol |
| Narceine | 6-[[6[2-(Dimethylamino)ethyl]-4-methoxy-1,3-benzodioxol-5-yl]acetyl]-2,3-dimethoxybenzoic acid |
| Neocinchophen | 6-Methyl-2-phenylquinoline-4-carboxylic acid ethyl ester |
| Nicotine | 3-(1-Methyl-2-pyrrolindinyl)pyridine |
| Nitroglycerin | 1,2,3-Propanetriol trinitrate |
| Nitromersol | 5-Methyl-2-nitro-7-oxa-8-mercurabicyclo[4.2.0]octa-1,3,5-triene |
| Norepinephrine | 4-(2-Amino-1-hydroxyethyl)-1,2-benzenediol |
| Noscapine | 6,7-Dimethoxy-3-(5,6,7,8-tetrahydro-4-methoxy-6-methyl-1,3-dioxolo(4,5-g)isoquinolin-5-yl)-1(3*H*)-isobenzofuranone |
| Papaverine | 1-[(3,4-Dimethoxyphenyl)methyl]-6,7-dimethoxyisoquinoline |
| Pentaerythritol tetranitrate | 2,2-Bis[(nitrooxy)-methyl]-1,3-propanediol dinitrate (ester) |
| Pentobarbital sodium | 5-Ethyl-5-(1-methylbutyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione monosodium salt |
| Pentylenetetrazol | 6,7,8,9-Tetrahydro-5*H*-tetrazolo(1,5-*a*)azepine |
| Perphenazine | 4-[3-(2-Chlorophenothiazin-10-yl)propyl]-1-piperazineethanol |
| Phenacetin | *N*-(4-Ethoxyphenyl)acetamide |
| Phenazopyridine hydrochloride | 3-(Phenylazo)-2,6-pyridinediamine monohydrochloride |
| Pheniramine maleate | 2[[α-(2-Dimethylamino)ethyl]benzyl]pyridine bimaleate |
| Phenmetrazine | 3-Methyl-2-phenylmorpoline |
| Phenobarbital | 5-Ethyl-5-phenyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Phenylephrine hydrochloride | 3-Hydroxy-α-[(methyamino)methyl]benzene-methanol hydrochloride |
| Phenylmethybarbituric acid | 5-Methyl-5-phenyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Phenylpropanolamine hydrochloride | α-(1-Aminoethyl)benzenemethanol hydrochloride |
| Phenylpropylmethylamine | *N*,β-Dimethylphenethylamine |
| Physostigmine | 1,2,3,3a,8,8a-Hexahydro-1,3a,8-trimethylpyrrolo(2,3-b)indol-5-ol methylcarbamate (ester) |
| Pilocarpine | 3-Ethyldihydro-4-[(1-methyl-1*H*-imidazol-5-yl)methyl]-2(3*H*)-furanone |
| Potassium guaiacolsulfonate | Potassium hydroxymethoxybenzenesulfonate hemihydrate |
| Probarbital | 5-Ethyl-5-isopropylbarbituric acid |
| Procainamide hydrochloride | 4-Amino-[2-(diethylamino)ethyl]-benzamide monohydrochloride |
| Procaine hydrochloride | 2-(Diethylamino)ethyl ester-4-Aminobenzoic acid, monohydrochloride |
| Promethazine hydrochloride | 10-[2-(Dimethylamino)propyol]phenothiazine monohydrochloride |
| Propoxycaine hydrochloride | 4-Amino-2-propoxybenzoic acid, 2-(diethylamino)ethyl ester monohydrochloride |
| Pyrilamine maleate | 2-[(2-(Dimethylamino)ethyl](*p*-methoxybenzyl)amino pyridine maleate |
| Quinidine | 6′-Methoxycinchonan-9-ol |
| Ringer's Injection | Sodium chloride (compound solution) |
| Ringer's Injection, lactated | Sodium lactate (compound solution) |
| Salicylic acid | 2-Hydroxybenzoic acid |
| Scopolamine | α-(Hydroxymethyl)9-methyl-3-oxa-9-azatricyclo[3.3.1.0$^{2,4}$]-non-7-yl benzeneacetic acid |
| Secobarbital | 5-(1-Methylbutyl)-5-(2-propenyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Sodium cacodylate | Sodium dimethylarsonate |
| Sparteine | Dodecahydro-7,14-methano-2*H*,6*H*-dipyrido(1,2-a:1′, 2′-e)(1.5)diazocine |

**Common and Chemical Names of Drugs in this Chapter**

| Common Name | Chemical Name |
|---|---|
| Strychnine | Strychnidin-10-one |
| Sulfadiazine | 4-Amino-*N*-2-pyrimidinylbenzenesulfonamide |
| Sulfanilamide | 4-Aminobenzenesulfonilamide |
| Sulfapyridine monohydrate | 4-Amino-*N*-2-pyridinylbenzenesulfonamide monohydrate |
| Sulfathiazole | 4-Amino-*N*-2-thiozolybenzenesulfonamide |
| Talbutal | 5-(1-Methylpropyl)-5-(2-propenyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Theobromine | 3,7-Dihydro-3,7-dimethyl-1*H*-purine-2,6-dione |
| Theophylline | 3,7-Dihydro-1,3-dimethyl-1*H*-purine-2,6-dione |
| Thiethylperazine | 2-(Ethylthio)-10-[3-(4-methyl-1-piperazinyl)propyl]phenothiazine |
| Thonzylamine hydrochloride | 2-[(2-Dimethylaminoethyl)(*p*-methoxybenzyl)amino pyrimidine hydrochloride |
| Triflupromazine | 10-[3-(Dimethylamino)propyl]-2-(trifluoromethyl)phenothiazine monohydrochloride |
| Trimethobenzamide hydrochloride | *N*-[*p*-[2-(Dimethylamino)ethoxy]benzyl]-3,4,5-trimethoxybenzamide monohydrochloride |
| Tripelennamine citrate | *N,N*-Dimethyl-*N'*-(phenylnethyl)-*N'*-2-pyridinyl-1,2-ethanediamine-2-hydroxy-1,2,3-propanetricarboxylate citrate (1:1) |
| Tripelennamine hydrochloride | *N,N*-Dimethyl-*N'*-(phenylmethyl)-*N'*-2-pyridinyl-1,2-ethanediamine monohydrochloride |
| Trolamine | 2,2′,2″-Nitrilotriethanol |
| Vinbarbital | 5-Ethyl-5-(1-methyl-1-butenyl)barbituric acid |
| Yohimbine | 17α-Hydroxyyohimban-16α-carboxylic acid methyl ester |

Source: *USAN and the USP Dictionary of Drug Names,* (1983; 1989) U.S. Pharmacopeial Convention, Rockville, MD.

# 19. Drugs: Part II


## Salvatore M. Marchese, *Associate Chapter Editor*
*Food and Drug Administration*


### ACIDS

### 945.98★ Benzoic and Salicylic Acids in Drugs
**Titrimetric Method**
**Final Action**
**Surplus 1972**

(Applicable to ointments. *Caution:* See safety notes on distillation, flammable solvents, toxic solvents, diethyl ether, and chloroform.)

*See* **37.001**, 12th ed.

### 967.31 Benzoic and Salicylic Acids in Drugs
**Chromatographic Method**
**First Action 1967**
**Final Action 1968**

#### *A. Apparatus*

(**a**) *Chromatographic tubes.*—Fuse 6 cm length of 5–6 mm tubing to piece of 25 mm tubing ca 25 cm long (25 × 200 mm test tube may be used). Constrict stem slightly ca 2 cm below seal. Com. tubes with dimensions ±10% are satisfactory. Pack wad of Pyrex glass wool in base as support.

(**b**) *Tamping rod.*—Flatten end of glass rod to circular head with clearance of ca 1 mm in tube (**a**). Or use disk of stainless steel, Al, etc., of diam. ca 1 mm less than id of column, (**a**), attached to 30–45 cm (12–18″) rod.

#### *B. Reagents*

(**a**) *Ferric chloride-urea soln.*—Dissolve, without heating, 18 g reagent grade urea in 2.5 mL 60% $FeCl_3.6H_2O$ (EM Science, No. FX0210) and 12.5 mL 0.05*N* HCl. Prep. fresh daily.

(**b**) *Phosphoric acid.*—30%. Dil. 30 mL 85% $H_3PO_4$ to 85 mL with $H_2O$.

(**c**) *Sodium bicarbonate.*—1*N*. Dissolve 2.5 g $NaHCO_3$ in 30 mL $H_2O$. Use only freshly prepd soln.

(**d**) *Diatomaceous earth.*—*See* **960.53B**.

(**e**) *Benzoic acid std soln.*—Accurately weigh 40–100 mg benzoic acid (depending on concn benzoic acid in sample), dissolve in $CHCl_3$, and dil. to 100 mL with $CHCl_3$. Shortly before use, transfer 5 mL to 50 mL vol. flask; add 4 drops HCl, 1 mL HOAc, 5 mL ether, and 7 mL MeOH; and dil. to vol. with $CHCl_3$.

(**f**) *Salicylic acid std soln.*—Dissolve 50 mg salicylic acid in $CHCl_3$ and dil. to 100 mL. Transfer 5 mL to 100 mL vol. flask; add 2 drops HCl, 2 mL HOAc, 10 mL MeOH, and 20 mL ether; and dil. to vol. with $CHCl_3$.

#### *C. Preparation of Sample*

(**a**) *Ointments.*—Dissolve ca 1 g sample, accurately weighed, in $CHCl_3$ and dil. to 100 mL. If necessary, dil. aliquot to prep. final soln contg 0.15–0.25 mg salicylic acid/mL $CHCl_3$.

(**b**) *Liquids.*—Dil. aliquot of liq. contg 150–250 mg salicylic acid to 100 mL with MeOH. Dil. 10 mL methanolic soln to 100 mL with $CHCl_3$.

#### *D. Preparation of Columns*

*Column I.—Lower stage:* Mix 1 g diat. earth with 0.5 mL 30% $H_3PO_4$ to uniform fluffy mixt. Transfer to tube and tamp to uniform mass with gentle pressure. *Upper stage:* Similarly mix 5 g diat. earth with 3 mL $FeCl_3$ urea reagent. (Mix thoroly, as nonuniform column may cause difficulty in elution of salicylic acid.) Transfer to tube directly above $H_3PO_4$ layer. Cover with glass wool.

*Column II.*—Mix 2 g diat. earth with 1 mL freshly prepd 1*N* $NaHCO_3$ soln.

#### *E. Determination*

(Use $H_2O$-satd solvs.)

Mount *Column I* directly above *Column II*. Pipet 10 mL dild sample into small beaker. Pour onto upper column, washing beaker with 10 mL $CHCl_3$ in small portions. Let sample sink into column and wash column with 75 mL $CHCl_3$. If purple salicylic acid band reaches $H_3PO_4$ layer, repeat with smaller sample. Sep. columns and wash *Column II* with 50 mL ether. Discard wash. Elute *Column I* into 100 mL vol. flask (contg 10 mL MeOH and 2 drops HCl) with 2 mL HOAc in 20 mL ether followed by enough 1% HOAc in $CHCl_3$ to bring to vol. Measure *A* of eluate and salicylic acid std at max., ca 306 nm, and calc. concn salicylic acid.

Elute *Column II* into 50 mL vol. flask (contg 7 mL MeOH and 4 drops HCl) with 0.5 mL HOAc in 5 mL $CHCl_3$ followed by enough 1% HOAc in $CHCl_3$ to bring to vol. Measure *A* of eluate and benzoic acid std at max., ca 275 nm, and calc. concn benzoic acid.

Ref.: JAOAC **50,** 666(1967).

CAS-65-85-0 (benzoic acid)
CAS-69-72-7 (salicylic acid)

### 930.41★ Salicylic Acid in Drugs in Presence of Other Phenols
**Final Action**
**Surplus 1965**

*See* **32.184–32.185**, 10th ed.

### 939.16 Mandelic Acid in Drugs
**Final Action**

#### *A. Qualitative Tests*

(Applicable to free acid)

*See* Microchemical Tests, Table **962.21B**.

### B. Determination

(*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, diethyl ether, and chloroform.)

(**a**) *Tablets.*—Weigh amt of powd sample contg 0.4–0.5 g mandelic acid and transfer to separator contg 10 mL $H_2O$. Acidify with HCl (1 + 3) and add 2 mL excess. Ext with six 20 mL portions $CHCl_3$-ether (2 + 1); wash each portion in second separator with 2 mL $H_2O$, and pass soln thru cotton plug, previously satd with solv., into 250 mL beaker. Wash outer surface of separator stem with few mL solv. and add to main portion. Test for complete extn with 15 mL addnl solv. and evap. in sep. beaker. Wash any residue thus obtained into beaker contg main ext with few mL solv. Evap. to dryness at ≤40° with aid of air current. Dissolve residue in 25 mL $CO_2$-free $H_2O$, **936.16B(a)**, and titr. with 0.1*N* NaOH, using phthln. 1 mL 0.1*N* NaOH = 0.01522 g mandelic acid ($C_6H_5CHOHCOOH$); 0.01692 g $NH_4$ mandelate, 0.01741 g Na mandelate, 0.01712 g Ca mandelate, and 0.01633 g Mg mandelate.

After titrn, mandelic acid may be re-extd and ext used for mp detns or qual. tests.

(**b**) *Liquid preparations.*—Measure 1 mL sample, or aliquot of diln contg 0.4–0.5 g mandelic acid, into separator and acidify with HCl (1 + 3). Proceed as in (**a**).

Ref.: JAOAC **22,** 757(1939).

CAS-90-64-2 (mandelic acid)

## PHENOLIC DRUGS

### 958.15 *p*-Aminosalicylic Acid and Isoniazid in Drugs

**Spectrophotometric Method**

**Final Action**

### A. Reagents

(**a**) *Benzaldehyde.*—USP or reagent grade.

(**b**) *Concentrated phosphate buffer.*—pH 7. Dissolve 34 g anhyd. $KH_2PO_4$ in 136 mL 1*N* NaOH and dil. to 1 L with $H_2O$.

### B. Extraction of Tablets

(**a**) Accurately weigh sample of powd tablets contg 35–40 mg INH and transfer to 150 mL beaker. Stir with 20 mL $H_2O$, add 1.5 g $NaHCO_3$, and continue stirring until effervescence stops. Filter with vac. thru medium porosity fritted glass filter (3.5 cm diam. is convenient) precoated with ca 3 mm layer of diat. earth, **960.53B**. Rinse beaker thoroly with 5 mL $H_2O$, break vac., transfer rinsings to funnel, washing down inside wall, and reapply vac. Repeat washing of beaker and funnel with 3 addnl 5 mL portions $H_2O$. Quant. transfer filtrate to 50 mL vol. flask with aid of small portions $H_2O$, dil. to vol., and mix. Proceed immediately with detn of PAS. Det. INH as soon as practicable, preferably ≤4 hr after prepn of $NaHCO_3$ soln.

(**b**) (*Applicable when filtration with vacuum is not feasible.*) —Weigh sample as in (**a**), and transfer quant. to 40–50 mL r-b centrf. tube. Cautiously add, in small portions, freshly prepd soln of 1.5 g $NaHCO_3$ in 20 mL $H_2O$. Agitate well after each addn, avoiding loss from foaming by occasionally adding few drops of ether. After all $NaHCO_3$ soln is added, continue agitation until effervescence stops. Centrf. 5–10 min at ca 2000 rpm and decant supernate into 50 mL vol. flask. Add 10 mL $H_2O$ to tube, using rubber policeman to wash down wall, to disintegrate residual cake, and to secure uniform suspension. Centrf. as before, and combine supernate wash with original ext. Repeat washing with three 5 mL portions $H_2O$, dil. combined aq. phases to vol., mix, and filter thru fluted paper. Proceed as in (**a**).

### C. *p*-Aminosalicylic Acid (PAS)

From aq. $NaHCO_3$ ext, transfer aliquot contg ca 150 mg PAS to 500 mL vol. flask and dil. to vol. with $H_2O$. Transfer 10 mL aliquot to 250 mL vol. flask, add 12.5 mL concd pH 7 buffer, and dil. to vol. with $H_2O$. Measure *A* of this diln in 1 cm cell at 299 (max.), 244 (min.), and 325 nm against 1 + 19 diln of the buffer. (With instruments suitable for *A* readings in range 1.0–1.5, use 2 cm cell thruout method or modify diln so that concn of substance is twice that specified.) Calc. baseline $A_B$:

$$A_B = A_{299} - (0.3210\ A_{244} + 0.6790\ A_{325})$$

Accurately weigh ca 50 mg finely powd pure PAS, dissolve in 2 mL alcohol, add 5 mL 0.1*N* NaOH, and dil. with $H_2O$ to exactly 500 mL. Transfer 25 mL aliquot to 200 mL vol. flask, add 10 mL concd pH 7 buffer, and dil. to vol. with $H_2O$. Det. *A* at 244, 299, and 325 nm as above. Det. $A_B$, and from this value and that obtained from sample soln, calc. amt PAS in sample.

### D. Isoniazid (INH)

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Transfer 20 mL aliquot of the $NaHCO_3$ ext to 125 mL separator, add 0.5 mL benzaldehyde, shake 15 min, and let stand 10 min. Ext with six 20 mL portions $CHCl_3$, filter exts thru compact pledget of absorbent cotton into 150 mL beaker, and evap. filtrate on steam bath in air current until residue has only faint odor of benzaldehyde. Rinse down wall of beaker with little $CHCl_3$ to conc. residue at bottom, and evap. to dryness. Add 1–2 mL $CHCl_3$, evap. again to dryness on steam bath in air current, and heat residue few min. Repeat $CHCl_3$ and heating treatment until hot residue of benzylidine isoniazid (BINH) is odorless, or has at most very faint odor of benzoic acid (there must be no sweet odor or odor of benzaldehyde; take care to avoid loss from spattering).

Dissolve residue in $CHCl_3$ and transfer quant. to separator with addnl solv. Add $CHCl_3$ to vol. of 20–30 mL, shake with 10 mL freshly prepd 5% $NaHCO_3$, and filter $CHCl_3$ layer thru compact pledget of absorbent cotton. Wash aq. soln with three 10 mL portions $CHCl_3$, passing each wash thru filter, and evap. combined $CHCl_3$ exts to dryness on steam bath in air current.

Dissolve residue of BINH in alcohol without heat, and dil. to exactly 100 mL with alcohol. Dil. 5 mL aliquot of this soln to exactly 200 mL with alcohol, and det. *A* of diln (1 cm cell; alcohol blank) at 302 (max.) and 375 nm. Subtract reading at 375 (background *A* from impurities) from that at 302 nm. Difference represents *A* from BINH at 302 nm.

Dissolve ca 20 mg, accurately weighed, of pure BINH in alcohol and dil. to exactly 100 mL. Dil. 10 mL aliquot of this soln to exactly 250 mL with alcohol and det. *A* at 302 nm. Using this value and that due to BINH obtained from sample, calc. equiv. amt of BINH in sample. BINH × 0.6088 = isoniazid.

Ref.: JAOAC **41,** 496(1958).

CAS-65-49-6 (*p*-aminosalicylic acid)
CAS-54-85-3 (isoniazid)

## 935.69★ Dinitrophenol (or Its Sodium Compound) in Drugs

### Bromination Method

**Final Action**
**Surplus 1965**

*See* **32.331–32.332**, 10th ed.

## 974.40 Guaifenesin in Drugs

### Polarographic Method

**Final Action 1974**

(Not applicable in presence of salicylate)

### *A. Apparatus*

(**a**) *Polarograph*.—Any voltammetric or polarographic instrument with necessary accessories (cells, electrodes, Hg, capillaries) capable of scanning up to 3.0 V in either pos. or neg. direction.

(**b**) *Micro or std cell, H-shaped*.—Satd calomel electrode, with 3% KCl-agar plug.

(**c**) *Water bath*.—Maintain at 65 ± 1° in freely circulating $H_2O$ bath.

### *B. Reagents*

(**a**) *Potassium nitrate soln*.—1*M*. Weigh 50.5 g $KNO_3$ into 500 mL vol. flask, dil. to vol. with $H_2O$, and mix.

(**b**) *Dilute sulfuric acid soln*.—10% (v/v). Dil. 20 mL $H_2SO_4$ (1 + 1) to 100 mL with $H_2O$ and mix.

(**c**) *Gelatin maximum suppressor*.—1 mg/mL. Accurately weigh 100 mg gelatin (Difco Laboratories No. 0143; or Kind & Knox Pharmaceutical Grade Gelatin, Kind & Knox, Div. of Knox Gelatin, Inc., Park 80 West, Plaza Two, Saddle Brook, NJ 07662) into 100 mL vol. flask, and dissolve in small amt $H_2O$ on steam bath. Cool, dil. to vol. with $H_2O$, and mix. Prep. fresh daily, as needed.

(**d**) *Supporting electrolyte*.—pH 10.4. Weigh 53.5 g $NH_4Cl$ into 1 L vol. flask, add 400 mL $NH_4OH$, mix to dissolve, and dil. to vol. with $H_2O$.

(**e**) *Guaifenesin std soln*.—1 mg/mL. Accurately weigh 25 mg guaifenesin std into 25 mL vol. flask. Dil. to vol. with $H_2O$ and mix.

### *C. Preparation of Sample*

(**a**) *Sirups*.—Quant. transfer accurately measured portion of sample contg ca 100 mg guaifenesin to 125 mL separator, add 10 mL dil. $H_2SO_4$, and ext with four 20 mL portions $CHCl_3$, and then with 15 mL $CHCl_3$. Collect $CHCl_3$ exts in second separator and wash with 10 mL $H_2O$. Filter $CHCl_3$ layer thru pledget of $CHCl_3$-washed cotton into 100 mL vol. flask. Rinse separator with 2–3 mL $CHCl_3$ and add wash to vol. flask. Dil. to vol. with $CHCl_3$ and mix.

(**b**) *Tablets*.—Det. av. wt/tablet. Grind without loss to pass No. 60 sieve. Accurately weigh powder contg ca 50 mg guaifenesin and transfer to 125 mL separator. Add 10 mL $H_2O$ and shake 2 min. Proceed as in (**a**), beginning ". . . add 10 mL dil. $H_2SO_4$, . . ."

### *D. Derivative Formation*

Pipet duplicate 10 mL aliquots for sirups or 20 mL aliquots for tablets of prepd soln into sep. 100 mL vol. flasks and carefully evap. to dryness with aid of air only. Add 10 mL $H_2O$ to each and shake to dissolve guaifenesin. Label flasks as sample and blank. Pipet 10 mL guaifenesin std soln into third 100 mL vol. flask and label as std.

Pipet 3 mL $H_2SO_4$ (1 + 1) into each flask. Pipet 3 mL 1*M* $KNO_3$ into std and sample flasks and 3.0 mL $H_2O$ into blank flask. Place flasks in 65° const temp. bath. When solns reach 65°, heat addnl 60 min. Remove from bath and cool to room temp. Into each flask pipet 25 mL electrolyte soln and 5 mL gelatin soln, cool to room temp., dil. to vol. with $H_2O$, and mix thoroly.

### *E. Polarography*

Transfer soln to polarographic cell and bubble N thru for 5 min with micro H cell or 10 min with std H cell at moderate rate. Polarograph from −0.2 to −0.9 v against satd calomel ref. electrode. Measure ht of diffusion current ($I_d$) at half-wave potential as follows: Draw line tangent to top of residual current extending to half-wave potential point. Draw line along top of limiting current extending to half-wave potential point. Measure vertical drop at half-wave potential between the 2 lines in convenient units.

Det. guaifenesin concn by comparing wave ht of sample soln with those of std and blank solns.

Subtract diffusion current ($I_d^b$) of blank, if any, from sample only. Perform all detns at same current sensitivity and within same time span.

### *F. Calculations*

(**a**) *Sirup*.—mg Guaifenesin/mL

$$= 100 \times (I_d - I_d^b) \times C/(I_d' \times V)$$

(**b**) *Tablets*.—mg Guaifenesin/tablet

$$= 50 \times (I_d - I_d^b) \times C \times W_t/(I_d' \times W_s)$$

where $I_d$, $I_d^b$, and $I_d'$ = diffusion current of sample, blank, and std solns, resp.; $C$ = mg guaifenesin/mL std soln; $W_t$ and $W_s$ = av. tablet wt and wt sample taken, resp.; and $V$ = mL liq. prepn taken.

Ref.: JAOAC **57**, 756(1974).

CAS-93-14-1 (guaifenesin)

## 938.15 Guaiacol in Drugs

### Titrimetric Method

**Final Action**

### *A. Reagent*

*Hydriodic acid*.—Sp gr 1.7. Boil HI 30 min under reflux with excess of hypophosphorous acid. When cool, transfer to dark, g-s bottle. Do not leave bottle unstoppered more than few min.

### *B. Apparatus*

*Methoxyl apparatus*.—*See* Fig. **956.07B**.

### *C. Determination*

Place aliquot of alk. guaiacol soln (guaiacol dissolved in 1% NaOH) contg 0.03–0.06 g guaiacol in boiling flask and evap. soln just to dryness on steam bath in air current. For solid guaiacol compds, weigh 0.06–0.10 g and transfer directly to flask. Complete detn by method for methoxyl group, **956.07C**, beginning "Add 2.5 mL melted *phenol* from wide-tip pipet . . ." Boil 30 min and titr. with 0.1*N* $Na_2S_2O_3$. 1 mL 0.1*N* I = 0.00207 g guaiacol; 0.00229 g guaiacol carbonate; 0.00404 g K guaiacol sulfonate.

Refs.: JAOAC **21**, 543(1938); **22**, 721(1939).

CAS-90-05-1 (guaiacol)

## 933.10 Hexylresorcinol in Drugs

### Titrimetric Method

### Final Action

#### A. Reagents

(**a**) *Sodium thiosulfate std soln.*—0.1*N*. Prep. as in **942.27A**.

(**b**) *Purified methanol.*—Purify if necessary as follows: Add enough Br to com. MeOH to give bright yellow soln, heat to bp on $H_2O$ bath, and boil 5 min. Cool, and carefully decolorize by adding 10% $NaHSO_3$ soln dropwise until just colorless.

(**c**) *Potassium bromide-bromate soln.*—0.1N. Prep. as in **947.13A**. Stdze as follows: Transfer 30 mL to I flask, and add 25 mL $H_2O$, 5 mL 20% KI soln, and 5 mL HCl. Shake thoroly and titr. with 0.1N $Na_2S_2O_3$, using starch indicator (mix 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring const., and immediately discontinue heating; add ca 1 mL Hg, shake, and let soln stand over the Hg).

#### B. Standardization of Thiosulfate

Add 30 mL 0.1*N* KBr-$KBrO_3$, and 10 mL purified MeOH to 150 mL g-s flask. Wet stopper. Add 5 mL HCl, stopper flask, immediately place under running tap $H_2O$, and swirl until flask cools to room temp.; continue to shake 5 min after adding HCl. Cautiously loosen stopper and add 5 mL 20% KI soln. Swirl gently to liberate I, wash stopper, and titr. with $Na_2S_2O_3$ soln. Add starch paste when soln is pale yellow.

#### C. Determination

Transfer 0.07–0.09 g sample to 150 mL g-s flask. Add 10 mL MeOH, (**b**), and swirl gently to dissolve sample. Add 30 mL 0.1*N* KBr-$KBrO_3$. Moisten stopper, add 5 mL HCl, stopper flask, and immediately hold under running $H_2O$ while swirling vigorously. When cooled to room temp. (ca 1 min), remove from tap and shake vigorously 5 min after adding HCl. Cautiously loosen stopper and add 5 mL 20% KI soln. Swirl gently, wash stopper with little $H_2O$, add 1 mL $CHCl_3$, and titr. with $Na_2S_2O_3$ soln while swirling flask gently. Near end point, stopper flask and shake vigorously to remove halogen from $CHCl_3$. When soln becomes pale yellow, add starch paste and continue titrn. End point is reached when starch-I color does not return during 30 sec of vigorous shaking. 1 mL 0.1*N* KBr-$KBrO_3$ soln = 0.00486 g hexylresorcinol.

Refs.: JAOAC **16,** 384(1933); **20,** 564(1937).

CAS-136-77-6 (hexylresorcinol)

## 945.97 Oxyquinoline Sulfate in Drugs

### Titrimetric Method

### Final Action 1965

### *Method I*

(For amts of oxyquinoline sulfate between 25 and 250 mg. Use this method whenever nature of sample permits.)

#### A. Extraction

(**a**) *Interfering substances absent.*—Dissolve sample in ca 75 mL $H_2O$ and add 5 mL HCl.

(**b**) *Nonoily preparations.*—Ext preferably from soln alk. with $NaHCO_3$ or borax. If extn from such medium is impracticable, or if compds of $NH_3$ or heavy or alk. earth metals are present, add Me red, **936.15D(a)**, and adjust with NaOH and/or HCl to slight acidity. Add $NaOAc.3H_2O$ in proportion of 1 g/100 mL soln. If heavy or alk. earth metals are present, also add 2 mL HOAc/100 mL soln.

Ext adjusted soln with enough 20 mL portions $CHCl_3$. For alk. or slightly acid soln, usually 6 extns suffice; when extra HOAc has been added, 10–12 extns are needed. Test for complete extn by adding little HCl (1 + 9) to last portion, evapg $CHCl_3$ on steam bath, adjusting to 70°, and adding drop of 0.01*N* KBr-$KBrO_3$ and then drop of Me red; Me red should be bleached immediately.

Ext combined $CHCl_3$ exts with five 10 mL portions HCl (1+9). If salicylic acid, volatile oils, etc., are present, wash each acid portion with same 10 mL ether. If sample contains phenol or other volatile interfering substances not completely removed by preceding process, boil acid soln to remove them, keeping vol. ca const by adding more $H_2O$.

(**c**) *Ointments, etc.*—Transfer sample to separator with 50 mL ether, and ext with five 10 mL portions HCl (1 + 9). If salicylic acid, etc., is present, wash each acid portion with same 10 mL ether. Add Me red; make just alk. with 10% NaOH soln, then just acid with dil. HCl, and proceed as in (**b**), beginning "Add $NaOAc.3H_2O$ . . ."

#### B. Determination

Adjust acid soln (**a**), (**b**), or (**c**), to 50° and keep at this temp. during titrn by reheating occasionally. Add drop (or more) Me red **936.15D(a)**, *from buret* and titr. with 0.1*N* KBr-KBr$O_3$, **933.10A(g)**. (Color of liq. gradually changes from brown-orange to yellow; add more indicator whenever soln becomes yellow. At slightly beyond halfway point, dibromohydroxyquinoline may crystallize and adsorb dye. Disregard color of ppt and judge by that of soln. By dilg to a concn ≤0.1 g oxyquinoline/100 mL, formation of ppt can be avoided.) End point is reached when, after waiting 10 sec for absorption of last drop of KBr-$KBrO_3$ soln and adding drop of indicator, it is bleached almost immediately. Timing for addn of drop of indicator at end point is important, as proper conditions prevail only brief period.

Read vols of 2 solns consumed. Measure 10 mL Me red into erlenmeyer, add 2 mL HCl, and titr. with 0.1*N* KBr-$KBrO_3$. Correct main titrn for vol. of Br consumed by measured vol. indicator used in titrn. 1 mL 0.1*N* KBr-$KBrO_3$ = 0.00508 g $(C_9H_7NO)_2.H_2SO_4.H_2O$.

### *Method II*

(For amts between 2 and 10 mg)

#### C. Determination

Ext as in **945.71A**. Start titrn as in **945.71B**, using 0.01*N* KBr-$KBrO_3$, and dild Me red (1 vol. Me red, **936.15D(a)**, 4 vols $H_2O$, and enough NaOH to dissolve dye) instead of stronger reagents. Use as little indicator as possible. When near end point, shown by more rapid consumption of indicator, heat to 70°, and complete titrn at this temp.

Refs.: JAOAC **28,** 699(1945); **29,** 280(1946).

CAS-134-31-6 (oxyquinoline sulfate)

## 960.58 Methyl Salicylate in Drugs

### Spectrophotometric Method

### First Action 1960
### Final Action 1961

#### A. Reagents

(**a**) *Salicylic acid std soln.*—20 μg/mL. Dissolve 0.2500 g reagent grade salicylic acid in 95 mL $CHCl_3$ in 250 mL vol.

flask and dil. to vol. with alcohol. Dil. 2.00 mL to 100 mL with alcohol.

(**b**) *Sodium bicarbonate soln.*—Dissolve 5 g $NaHCO_3$ in 100 mL of $H_2O$ to which 1 drop HCl has been added.

### *B. Determination*

Prep. sample diln, if necessary, to contain ca 5% Me salicylate. Pipet 5 mL sample or diln into 50 mL ether-pet ether mixt. (1 + 1) in separator and wash with two 5 mL portions cold, freshly prepd $NaHCO_3$ soln. Discard unemulsified aq. phases. Ext org. layer with two 5 mL portions 5% NaOH soln followed by two 5 mL portions $H_2O$. Let phases sep. 5 min and drain unemulsified aq. layers into another separator. Wash combined exts with 10 mL pet ether and drain aq. phase into another separator. Acidify cautiously with HCl (litmus paper) and ext with four 20 mL and one 15 mL portions $CHCl_3$. Filter each ext thru $CHCl_3$-moistened plug of cotton into 250 mL vol. flask. Dil. to vol. with alcohol and transfer 2.00 mL aliquot to 100 mL vol. flask.

Dil. to vol. with alcohol and det. *A* at max. (ca 305 nm). Calc. as salicylic acid by comparison with *A* of std soln. Salicylic acid × 1.1016 = Me salicylate.

Refs.: JAOAC **43,** 239(1960); **44,** 152(1961).

CAS-119-36-8 (methyl salicylate)

## 948.29 Phenolphthalein in Chocolate Drug Preparations

**Gravimetric Method**

**Final Action**

### *A. Reagents*

(**a**) *Potassium hydroxide soln.*—5 ± 0.1*N*.

(**b**) *Iodine soln.*—0.5*N*. Dissolve 12.7 g KI in 10 mL $H_2O$, add 6.35 g I, and when dissolved add 12 mL KOH soln, (**a**). Dil. to 100 mL with $H_2O$.

(**c**) *Sodium sulfite soln.*—Dissolve 12.6 g anhyd. $Na_2SO_3$ in $H_2O$ and dil. to 100 mL with $H_2O$.

### *B. Preparation of Alcoholic Extract*

Chill sample until hard; then reduce to granules by grating, shaving, or grinding. Mix thoroly. Accurately weigh amt of prepd sample contg ca 0.1 g phthln into gooch with thin asbestos mat or fritted glass disk. Ext fat with 5, 4, and 3 mL $CCl_4$, using slight suction towards end. Place crucible on bell jar app. Ext phthln from sample with several portions hot alcohol, collecting filtrate in 300 mL tall beaker. Wash underside of crucible free from phthln with hot alcohol (ca 50 mL is enough for extn and washings). Evap. combined alc. exts to dryness on steam bath.

### *C. Determination*

Dissolve residue at room temp. in 1–1.5 mL KOH soln. (Alk. phthln soln is unstable in air, and phthln should be converted to tetraiodo compd within 1 hr.) Add piece of ice (ca 40 g) and 7–8 mL I soln. Add HCl dropwise from buret, using stirring rod, to complete pptn. If ppt and supernate are not brown, add addnl I soln to ensure excess. Again dissolve ppt by adding KOH from buret dropwise, with stirring. Wash down any unreacted phthln adhering to sides of beaker with little $H_2O$. (Soln should now be blue to blue-purple.)

Repeat pptn with acid and resoln with alkali 3 more times, adding small piece of ice if necessary. Then add 1–1.5 mL $Na_2SO_3$ soln to blue alk. soln and filter into 250 mL beaker thru gooch with *thin* asbestos mat or *coarse* fritted glass disk. Wash crucible several times with $H_2O$. Acidify filtrate with HCl, using few mL excess, and heat on steam bath 20–30 min, stirring occasionally. Decant hot supernate thru weighed gooch (with asbestos mat or medium fritted glass disk). Wash white to cream-colored ppt in beaker by decantation with hot $H_2O$ few times. Completely transfer ppt to the gooch and wash with hot $H_2O$ until filtrate is clear and gives neg. test for Cl. When app. has cooled and ppt has been sucked fairly dry, wash ppt several times with pet ether, using suction toward end. Dry tetraiodophenolphthalein to const wt at 110–130°. Wt ppt × 0.3873 = wt phthln.

Refs.: JAOAC **31,** 547(1948); **33,** 203(1950).

CAS-81-90-3 (phenolphthalein)

## 942.29 Phenolphthalein in Drug Emulsions

**Gravimetric Method**

**Final Action**

Shake sample well, preferably in mech. shaker, 10 min. Accurately weigh amt of sample contg ca 0.1 g phthln from weighing buret directly into centrf. bottle. Add 100 mL alcohol-ether (1 + 3), stopper bottle, shake vigorously, and then centrf. until clear. Decant into separator. Wash residue in bottle twice with 10 mL portions solv. mixt., adding these washings to separator. Dissolve residue in bottle in few mL $H_2O$ and reppt gums with 50 mL solv. mixt. Shake and centrf. as before, decanting into separator. Wash residue and bottle with three 10 mL portions solv. mixt. and add these to separator. Dissolve residue in few mL $H_2O$ and test for complete extn with NaOH.

Shake exts in separator repeatedly with 25 mL portions ca 0.1*N* NaOH until phthln is completely removed, as shown by absence of color. Combine alk. exts in another separator and acidify soln with dil. $H_2SO_4$ (1 + 15).

Ext phthln by shaking acid mixt. repeatedly with 10 mL portions ether. Test for complete extn with NaOH soln. Combine ether exts in 150 mL beaker, evap. to dryness, and det. phthln as in **948.29C**, omitting filtration of alk. soln.

Refs.: JAOAC **25,** 843(1942); **26,** 311(1943).

CAS-81-90-3 (phenolphthalein)

## 923.12★ Phenolphthalein in Tablets

**Ether Extraction Method**

**Final Action**
**Surplus 1972**

*See* **37.138**, 12th ed.

## 930.42★ Phenolsulfonates in Drugs

**Bromination Method**

**Final Action**
**Surplus 1965**

*See* **32.342**, 10th ed.

## 929.12 Thymol in Drug Substance

**Titrimetric Method**

**Final Action**

### *A. Preparation of Solution*

Weigh 2 g pulverized thymol, transfer to 500 mL vol. flask, and add 25 mL 25% NaOH soln. Agitate until thymol is dissolved and dil. to vol. at 20° with $H_2O$.

***B. Method I***

Transfer 25 mL aliquot thymol soln to 250 mL g-s erlenmeyer, add 20 mL hot HCl (1 + 1), and immediately add 1–3 mL less than theoretical vol. 0.1*N* KBr-$KBrO_3$, **933.10A(c)**. Warm to 70–80°, add 2 drops 0.1% aq. Me orange, and titr. slowly with KBr-$KBrO_3$ soln, swirling vigorously after each addn. When red of Me orange has been bleached, add 2 drops titrg soln, stopper, shake vigorously 10 sec, add 1 drop Me orange, and again shake vigorously 10 sec. Continue addn of KBr-$KBrO_3$ soln, 2 drops at time, shaking each time until red disappears. Add 1 drop Me orange, shake vigorously, and if red does not disappear, repeat alternate addn of 2 drops KBr-$KBrO_3$ soln and 1 drop Me orange, shaking after each addn as before until red disappears. Calc. mL KBr-$KBrO_3$ soln used to % thymol. 1 mL 0.1*N* KBr-$KBrO_3$ = 0.003756 g thymol. Reserve mixt. in titrg flask for **929.12C**.

***C. Method II***

To cooled mixt. from titrn, **929.12B**, add 3–5 mL addnl KBr-$KBrO_3$ soln. (If sample has not been previously analyzed by **929.12B**, det. approx. vol. of KBr-$KBrO_3$ soln to use by adding 20 mL HCl (1 + 1) to 25 mL soln, **929.12A**, heating to ca 80°, and titrg slowly with KBr-$KBrO_3$ soln, while vigorously swirling flask, to yellow color maintained 1 min.) Stopper, shake, add 1 g solid KI, wash sides of flask and stopper with $H_2O$, and titr. I liberated by excess KBr-$KBrO_3$ soln with 0.1*N* $Na_2S_2O_3$, using starch soln as indicator. Calc. vol. $Na_2S_2O_3$ soln used in terms of KBr-$KBrO_3$ soln, deduct from total vol. KBr-$KBrO_3$ soln added, and calc. to % thymol.

Refs.: JAOAC **12,** 296(1929); **14,** 330(1931).

CAS-89-83-8 (thymol)

## 930.43 Thymol in Antiseptics

**Titrimetric Method**

**Final Action**

(*Caution:* See safety notes on distillation, flammable solvents, and diethyl ether.)

If alc. content is not known, make preliminary alcohol detn.

Transfer 50 mL sample (or aliquot contg 0.05–0.10 g thymol) to Pt or porcelain evapg dish. Add 6–7 mL 50% NaOH soln, mix well, and carefully dealcoholize by placing dish on steam bath before elec. fan. Evap. vol. slightly more than amt of alcohol present. (If >30% alcohol is present, dil. with $H_2O$ to alc. content of 25%. In no case should evapn be carried >70% of original vol.) Transfer soln to 125 mL separator, washing out evapg dish with enough $H_2O$ to bring vol. to ca 75 mL.

Ext alk. soln with two 20 mL portions pet ether. Wash combined exts once with 5–10 mL 5% NaOH soln and add washings to aq. layer. Ext aq. soln contg thymol, together with Na salts of boric, benzoic, and salicylic acids, with ether as in **960.53A**, using 20, 15, 15, 10, and 10 mL. Use 8–10 extns if prepn contains glycerol. Combine ether exts, transfer to 250 mL g-s erlenmeyer, and add 5 mL recently prepd alc. KOH soln, **920.160A**. Evap. most of ether, using steam bath and elec. fan but do not evap. entirely to dryness. Leave 6–8 mL residue and add to it 75 mL hot $H_2O$ (80–90°) and 10 mL HCl.

Immediately add 1–3 mL less than theoretical vol. 0.1*N* KBr-$KBrO_3$, **933.10A(g)**, swirling constantly. Add 2 drops aq. 0.1% Me orange and titr. slowly with KBr-$KBrO_3$ soln as in **929.12B**.

Test for complete extn by shaking aq. layer with two 15–20 mL portions ether and titrg the thymol, if any, in ether exts. Add this titrn to that obtained for main ether ext.

If theoretical amt of thymol present is not known, add 2 drops Me orange and titr. slowly, swirling constantly during addn of KBr-$KBrO_3$ soln until red color is bleached. Continue as in **929.12B**, beginning ". . . add 2 drops titrg soln, stopper, shake vigorously . . ."

*Note:* To avoid loss of thymol by volatilization, both evapn of alcohol and later evapn of ether must be done carefully.

Refs.: JAOAC **13,** 332(1930); **14,** 330(1931); **15,** 418(1932).

CAS-89-83-8 (thymol)

# ANALGESICS AND ANTIPYRETICS

## 970.80 Acetaminophen in Drugs

**Spectrophotometric Method**

**First Action 1970**
**Final Action 1971**

***A. Reagents***

(**a**) *Bicarbonate-carbonate buffer.*—pH 10.1. Weigh 1.0 g $NaHCO_3$ and 4.5 g $Na_2CO_3$ into 100 mL vol. flask and dil. to vol. with $H_2O$.

(**b**) *Acidic methanol.*—1.0 mL 0.1*N* HCl/100 mL MeOH. Prep. enough to ensure same MeOH is used thruout for std and sample.

(**c**) *Diatomaceous earth.*—*See* **960.53B**.

(**d**) *Acetaminophen std soln.*—0.008 mg/mL. Accurately weigh 40 mg acetaminophen std into 100 mL vol. flask. Dil. to vol. with acidic MeOH and mix well. Transfer 2.0 mL to 100 mL vol. flask and dil. to vol. with acidic MeOH.

***B. Preparation of Sample***

(**a**) *Sirup.*—Transfer 15.0 mL 0.1*N* NaOH to 25 mL vol. flask. Dil. to vol. with acetaminophen sirup, avoiding wetting flask neck above graduation mark while adding sirup, and mix. Transfer 10.0 mL of this diln to 100 mL vol. flask, dil. to vol. with $H_2O$, and mix.

(**b**) *Tablets.*—Det. av. wt/tablet and pulverize. Accurately weigh portion of powder contg ca 240 mg acetaminophen and transfer to 250 mL vol. flask. Add 2 mL 1.0*N* NaOH and ca 100 mL $H_2O$. Shake, dil. to vol. with $H_2O$, and mix.

***C. Preparation of Chromatographic Column***

Pack fine glass wool plug in base of chromatgc tube (25 × 250 mm) with aid of tamping rod ca 45 cm long and having disk with diam. ca 1 mm less than tube. To 3.0 g diat. earth, add 2.0 mL buffer soln and mix until fluffy. Transfer mixt. to column and tamp gently to compress to uniform mass. Transfer 2.0 mL sample soln to 100 mL beaker, add 1 drop HCl, and mix. Add 3.0 g diat. earth, mix thoroly, and transfer to column. Scrub beaker with 1 g diat. earth and 2 drops $H_2O$. Transfer to column, tamp, and top with fine glass wool pad.

***D. Determination***

(*Caution:* See safety notes on distillation, flammable solvents, and diethyl ether.)

(Use $H_2O$-washed solvs thruout.)

Pass 100 mL $CHCl_3$ thru column and discard eluate. Elute acetaminophen with 150 mL ether, collecting eluate in 400 mL beaker. Evap. soln to dryness on steam bath under air stream. Dissolve residue in acidic MeOH, transfer quant. to 50 mL vol. flask, and dil. to vol. with same solv. Transfer 10.0 mL of this soln to 50 mL vol. flask, dil. to vol. with acidic MeOH, and mix. Scan spectra of sample and std solns from 350 to 240 nm in 1 cm cells, using acidic MeOH as blank.

mg Acetaminophen in portion of sirup or tablet taken

$$= 31.25 \times C \times (A/A')$$

where $C$ = mg/mL std soln, and $A$ and $A'$ refer to sample and std, resp., at max., ca 249 nm.

Ref.: JAOAC **53,** 591(1970).

CAS-103-90-2 (acetaminophen)

## 987.12 Acetaminophen in Drug Tablets

### Liquid Chromatographic Method

### First Action 1987

(Applicable to single-component tablets and to multi-component tablets contg aspirin and caffeine)

#### *A. Principle*

Acetaminophen is detd by reverse phase liq. chromatgy using MeOH–HOAc mobile phase and UV detention at 280 nm.

#### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Isocratic system equipped with detector capable of monitoring $A$ at 280 nm, and sample injection valve with 20 µL sample loop. Operating conditions: flow rate ca 2 mL/min; temp. ambient; detector sensitivity 1.28 AUFS.

(**b**) *LC column.*—Stainless steel, 300 × 4.6 mm id, packed with octadecylsilane chemically bonded to 10 µm porous silica or ceramic microparticles.

#### *C. Reagents*

(**a**) *Solvents.*—LC grade MeOH; $H_2O$, double distd in glass.

(**b**) *Acetic acid soln.*—Pipet 7.5 mL HOAc (AR grade) into 1 L vol. flask, dil. to vol. with $H_2O$, and mix.

(**c**) *Mobile phase.*—MeOH–HOAc soln (1 + 3). Degas with aid of sonication and vac. pH of this soln should be ca 3.0.

(**d**) *Std preparation.*—Accurately weigh ca 60 mg USP Acetaminophen Ref. Std, previously dried 18 h over silica gel, and transfer to 100 mL vol. flask. Dissolve and dil. to vol. with mobile phase, and mix.

#### *D. Sample Preparation*

Weigh and finely powder ≥20 tablets. Transfer portion of powder, equiv. to ca 60 mg acetaminophen, to 100 mL vol. flask, add ca 50 mL mobile phase, and sonicate or mech. shake 10 min. Dil. to vol. with mobile phase, and mix. Filter soln thru microfilter into g-s flask. Discard first portion of filtrate (ca 10 mL).

#### *E. System Suitability*

Make 5 replicate injections of std prepn and record peak responses (hts or areas). Coeff. of var. (CV) should be ≤2.0%, tailing factor should be ≤2.0, and column efficiency should be ≥1000 theoretical plates, all calcd as shown in **985.49D** and **985.50D**.

#### *F. Determination*

Make duplicate 20 µL injections of std prepn and sample prepn into liq. chromatograph, and measure peak responses (hts or areas). From av. values, calc. amt acetaminophen, in mg, in portion of tablets taken for assay as follows:

$$\text{Acetaminophen, mg in sample weighed} = 0.1C \times (R/R')$$

where $C$ = concn of std prepn, µg/mL, and $R$ and $R'$ = av. peak responses for sample prepn and std prepn, resp. To verify that no appreciable change in chromatgc conditions has occurred during course of chromatgc run, at end of series of detns make dup. injections of std prepn, and evaluate reproducibility.

Ref.: JAOAC **70,** 212(1987).

CAS-103-90-2 (acetaminophen)

## 972.49 Acetaminophen and Salicylamide in Drugs

### Spectrophotometric Method

### First Action 1972
### Final Action 1973

(Applicable in presence of antihistamines, barbiturates, caffeine, ascorbic acid, prednisone, and belladonna alkaloids. Aspirin interferes in salicylamide detn.)

#### *A. Apparatus*

(**a**) *Chromatographic tube.*—Plain, 250 × 25 mm i.d.

(**b**) *Tamping rod.*—*See* **967.31A(b)**; use for packing columns.

(**c**) *UV spectrophotometer.*—Preferably recording, with matched 1.0 cm cells.

(**d**) *Infrared spectrophotometer.*—With equipment suitable for prepg KBr disks.

#### *B. Reagents*

(**a**) *Tripotassium phosphate.*—20%. Dissolve 10.0 g $K_3PO_4$ in $H_2O$ and dil. to 50 mL.

(**b**) *Water-washed chloroform.*—Shake equal vols $CHCl_3$ and $H_2O$ in separator 1 min. Use within 1 day.

(**c**) *Water-washed ethyl acetate.*—Shake equal vols EtOAc and $H_2O$ in separator 1 min. Discard lower phase.

(**d**) *Acetic acid in chloroform.*—(*1*) 10%.—Dil. 10.0 mL HOAc to 100 mL with $CHCl_3$. (*2*) 1%.—Dil. 10.0 mL 10% soln to 100 mL with $CHCl_3$. Prep. ca 375 mL for each sample and 225 mL for std solns.

(**e**) *Diatomaceous earth.*—*See* **960.53B**.

(**f**) *Acetaminophen std soln.*—0.6 mg/100 mL. Accurately weigh ca 60 mg USP Ref. Std Acetaminophen; dissolve and dil. with alcohol in 100 mL vol. flask. Dil. 10.0 mL of this soln to 100 mL with alcohol. Dil. 10.0 mL of second diln to 100 mL with alcohol.

(**g**) *Salicylamide std soln.*—2 mg/100 mL. Accurately weigh ca 50 mg USP Ref. Std Salicylamide; dissolve and dil. with 1% HOAc-$CHCl_3$ in 100 mL vol. flask. Dil. 4.0 mL of this soln to 100 mL with 1% HOAc-$CHCl_3$.

#### *C. Preparation of Sample*

Det. av. wt of tablets or capsules, reduce to powder passing No. 60 sieve.

#### *D. Preparation of Columns*

*Column I.*—Place small piece of glass wool in chromatgc tube. Mix 1.0 g diat. earth, (**e**), and 0.5 mL HCl (1 + 1) in beaker until fluffy, and pack in tube. Accurately weigh portion powd sample contg ca 50 mg acetaminophen into 100 mL beaker and thoroly wet with 2.0 mL HCl (1 + 1). Add 3.0 g diat. earth, mix until fluffy, transfer quant. to column, and pack. Scrub sample beaker with 1.0 g diat. earth and 0.5 mL HCl (1 + 1), and pack on column. Wipe all utensils and sample beaker with glass wool and pack on column.

*Column II.*—Place small piece of glass wool in chromatgc tube. Mix 3.0 mL 20% $K_3PO_4$ and 5.0 g diat. earth until fluffy, and pack on column. Cover diat. earth with small piece of glass wool.

### *E. Determination*

Mount columns so that *I* elutes into *II*. Place waste beaker under column *II*. Add 100 mL $H_2O$-washed $CHCl_3$ to column *I*. After all $CHCl_3$ has passed thru both columns, sep. columns, rinsing stem of top column with 5 mL $H_2O$-washed $CHCl_3$ into bottom column. Elute column *II* with addnl 25 mL $H_2O$-washed $CHCl_3$. Rinse stem of column *II* with $CHCl_3$ after all $CHCl_3$ has passed thru. Discard wash $CHCl_3$.

Place 250 mL vol. flasks under each column. Elute acetaminophen from column *I* with 100 mL $H_2O$-washed EtOAc. Do not let column run dry until entire 100 mL has passed into column. Rinse column stem with EtOAc (not $H_2O$-washed) into flask. Add 5 mL alcohol, mix, and dil. to vol. with EtOAc (not $H_2O$-washed). Pipet 15 mL into 50 mL vol. flask and dil. to vol. with EtOAc (not $H_2O$-washed). Pipet 10 mL of this soln into 100 mL beaker and evap. to dryness with gentle air current. Do not use heat. Dissolve residue in alcohol and transfer quant. to 100 mL vol. flask with several portions alcohol; dil. to vol. with alcohol. Det. *A* of sample and std, (**f**), solns at 248 nm in 1.0 cm cell against alcohol as ref. If recording spectrophtr is available, scan soln from 320 to ca 210 nm in 1.0 cm cells against alcohol.

$$\text{mg Acetaminophen in portion sample taken} = 83.33(A/A') \times C$$

where $A$ and $A'$ refer to sample and std solns, resp., at 248 nm; and $C$ = exact concn of ref. std soln in mg/100 mL final soln.

Elute salicylamide from column *II* by passing 10.0 mL 10% HOAc-$CHCl_3$ thru column, followed by 100 mL 1% HOAc-$CHCl_3$. After complete elution, rinse stem of column into flask with 1% HOAc-$CHCl_3$ and dil. to vol. with same solv. Pipet aliquot contg ca 2 mg salicylamide into 100 mL vol. flask and dil. to vol. with 1% HOAc-$CHCl_3$. Det. *A* of sample and std, (**g**), solns at 308 nm in 1.0 cm cells against 1% HOAc-$CHCl_3$ as ref. If recording spectrophtr is used, scan solns from 370 to ca 260 nm in 1.0 cm cells against 1% HOAc-$CHCl_3$.

$$\text{mg Salicylamide in portion sample taken} = (250/B) \times (A/A') \times C$$

where $B$ = vol. (mL) aliquot taken to contain 2 mg salicylamide; $A$ and $A'$ refer to sample and ref. std solns, resp., at 308 nm; and $C$ = exact concn of ref. std soln in mg/100 mL final soln.

### *F. Identification*

Prep. KBr disks of each ingredient from eluates by evapg, with gentle air current only, aliquot contg ca 1 mg each ingredient. Be sure no detectable odor of HOAc is present from salicylamide aliquot before prepg KBr disk.

Compare IR spectrum of each with its respective std from 2.0 μm (5000 $cm^{-1}$) to 15 μm (660 $cm^{-1}$).

Ref.: JAOAC **54,** 895(1971).

CAS-103-90-2 (acetaminophen)
CAS-65-45-2 (salicylamide)

## 916.02★ Acetanilid and Phenacetin in Drugs

**Surplus 1965**

### *A. Qualitative Test for Phenacetin—Procedure*

*See* **32.129**, 10th ed.

### *B. Quantitative Methods—Final Action*

(**a**) *Phenacetin*.— (*1*) *Volumetric*. *See* **32.131**(**a**)(1), 10th ed. (*2*) *Gravimetric*. *See* **32.131**(**a**)(2), 10th ed.
(**b**) *Acetanilid*.—*See* **32.131**(**b**), 10th ed.

## 916.03★ Acetanilid and Caffeine in Drugs

**Final Action**
**Surplus 1965**

*See* **32.132–32.134**, 10th ed.

## 916.04★ Acetanilid, Caffeine, and Codeine in Drugs

**Final Action**
**Surplus 1965**

*See* **32.135–32.136**, 10th ed.

## 916.05★ Acetanilid, Caffeine, and Quinine in Drugs

**Final Action**
**Surplus 1965**

*See* **32.137–32.139**, 10th ed.

## 916.06★ Acetanilid, Caffeine, Quinine, and Morphine in Drugs

**Final Action**
**Surplus 1965**

*See* **32.140–32.141**, 10th ed.

## 916.07★ Acetanilid and Sodium Salicylate in Drugs

**Final Action**
**Surplus 1965**

*See* **32.142–32.143**, 10th ed.

## 960.59 Aspirin, Phenacetin, and Caffeine (APC) in Drugs

**First Action 1960**
**Final Action 1961**

### *A. Method I (For Aspirin Only)★ —Surplus 1974*

*See* **36.190**, 11th ed.

### *B. Method II★ —Surplus 1974*

*See* **36.191–36.192**, 11th ed.

#### *Chromatographic Method*

### *C. Reagents*

(**a**) *Sodium bicarbonate soln*.—1*M*. Dissolve 4.2 g $NaHCO_3$ in 48 mL $H_2O$.
(**b**) *Washed ether*.—Wash USP ether with equal vol. $H_2O$ in separator. Filter thru paper, rejecting first 15 mL. Use within 3 days. Approx. 70 mL required for each sample.

(c) *Chloroform.*—USP. *A* against $H_2O$ at 276 nm ≤0.050. Use same lot thruout.

(d) *Washed chloroform.*—Wash and filter $CHCl_3$ as in (b). Use within 3 days. Use same lot thruout. Approx. 700 mL is required for stds and 170 mL for each sample.

(e) *Isooctane.*—*A* against $H_2O$ at 286 nm ≤0.050. Use same lot thruout.

(f) *Phenacetin std soln.*—7 mg/100 mL. Dissolve 70.0 mg pure phenacetin in $CHCl_3$ and dil. to 100 mL with $CHCl_3$. Dil. 10 mL aliquot to 100 mL with isooctane.

(g) *Caffeine std soln.*—1.4 mg/100 mL. Dissolve 140.0 mg caffeine in washed $CHCl_3$ and dil. to 100 mL. Dil. 10 mL aliquot to 100 mL; dil. 10 mL aliquot of this soln to 100 mL with washed $CHCl_3$.

(h) *Aspirin std soln.*—5 mg/100 mL. Dissolve 100.0 mg aspirin in washed $CHCl_3$ and dil. to 100 mL. To 5 mL aliquot add 1.0 mL HOAc and dil. to 100 mL with washed $CHCl_3$. Prep. fresh daily.

(i) *Salicylic acid std soln.*—2.5 mg/100 mL. Dissolve 100.0 mg salicylic acid in washed $CHCl_3$ and dil. to 100 mL. Dil. 25 mL aliquot to 100 mL; to 10 mL aliquot of this soln, add 1.0 mL HOAc and dil. to 100 mL with washed $CHCl_3$.

### D. Apparatus

*See* **967.31A**.

### E. Preparation of Sample

Weigh powd sample contg ca 100 mg aspirin and transfer to 100 mL vol. flask. Add 60 mL $CHCl_3$ and shake well. Add 0.2 mL HOAc and dil. to vol. with $CHCl_3$.

### F. Preparation of Chromatographic Column

Loosely pack small amt of fine glass wool in base of chromatgc tube so as to support diat. earth, but not cause irregularity in thickness of diat. earth layer.

To 2.0 g diat. earth, **960.53B**, in 100 mL beaker, or glass mortar, add 2.0 mL $H_2SO_4$ (1 + 9). Mix well with metal spatula. Transfer to chromatgc tube, and with packing rod, compress lightly to uniform mass. Mix 2.0 g diat. earth with 2.0 mL 1*M* $NaHCO_3$ and place in column above acid layer. Wash column with 15–20 mL washed ether and discard washings.

### G. Separation

(Use washed ether and washed $CHCl_3$ thruout, except for dissolving phenacetin residue.)

(a) *Phenacetin.*—Dil. 5 mL aliquot prepd sample soln with 20 mL ether and pass thru column, receiving eluate in 100 or 150 mL beaker. After soln has passed into adsorbent, wash with five 5 mL portions ether, letting each portion pass into adsorbent before adding next. Wash tip of outlet with $CHCl_3$ and evap. total eluate to dryness by gentle heating on steam bath with air current. Dissolve phenacetin residue in 5 mL USP $CHCl_3$ and dil. with isooctane to 50 mL.

(b) *Caffeine.*—Immediately after passage of last portion of ether thru column, replace beaker with 50 mL vol. flask. Pass 48 mL $CHCl_3$ thru column, wash tip with $CHCl_3$ and dil. eluate to vol.

(c) *Aspirin and salicylic acid.*—Immediately replace receiver with 100 mL vol. flask. Pass soln of 0.5 mL HOAc in 5 mL $CHCl_3$ thru column, followed by 90–92 mL 1% soln of HOAc in $CHCl_3$. Wash tip with $CHCl_3$ and dil. eluate to vol.

### H. Determination

Immediately det. *A* of acid fraction and of aspirin and salicylic acid std solns at 280 and 310 nm against 1% HOAc in $CHCl_3$. Det *A* of phenacetin fraction and std at 286 nm against isooctane-USP $CHCl_3$ (9 + 1) and that of caffeine fraction and std at 276 nm against washed $CHCl_3$ blank.

Calc. amt of each ingredient in sample. Aspirin and salicylic acid may be calcd by successive approximations as follows: Attributing entire *A* at 310 nm to salicylic acid, use ratio of salicylic acid std readings at the 2 wavelengths to calc. *A* due to salicylic acid at 280 nm, and deduct from total *A* at 280 nm. Attributing remainder to aspirin, use ratio of aspirin std readings to calc. *A* due to aspirin at 310 nm. Deduct this *A* from total at 310 nm. Use remainder to calc. amt of salicylic acid in sample and also to recalc. *A* due to salicylic acid at 280 nm. Deduct latter from total *A* at 280 nm and use remainder to calc. amt of aspirin in sample. Alternatively, calc. these two ingredients by simultaneous equations. Amt of aspirin hydrolyzed may be calcd by multiplying amt of salicylic acid by 1.3044.

Ref.: JAOAC **43**, 241(1960).

CAS-50-78-2 (aspirin)
CAS-58-08-2 (caffeine)
CAS-62-44-2 (phenacetin)

## 940.38★ Aspirin, Phenacetin, and Phenyl Salicylate in Drugs

**Final Action**
**Surplus 1965**

*See* **32.153**, 10th ed.

## 941.22★ Aminopyrine, Phenacetin, and Caffeine in Drugs

**Final Action**
**Surplus 1965**

*See* **32.154**, 10th ed.

## 942.30★ Aminopyrine, Phenacetin, Caffeine, and Phenobarbital in Drugs

**Final Action**
**Surplus 1965**

*See* **32.155**, 10th ed.

## 916.08★ Phenacetin and Caffeine in Drugs

**Final Action**
**Surplus 1970**

*See* **36.202–36.203**, 11th ed.

## 916.09★ Phenacetin and Phenyl Salicylate in Drugs

**Final Action**
**Surplus 1965**

### A. Acid Hydrolysis Method

*See* **32.158**, 10th ed.

### B. Alkaline Hydrolysis Method

*See* **32.159**, 10th ed.

## 922.13★ Aspirin

**Final Action**
**Surplus 1970**

### A. Melting Point

*See* **36.206**, 11th ed.

### B. Free Salicylic Acid

*See* **36.207**, 11th ed.

### C. Total Salicylate

*See* **36.209(a)** and **(b)**, 11th ed.

### D. Combined Acetic Acid

*See* **36.210**, 11th ed.

### E. Double Titration Method

*See* **37.046–37.047**, 13th ed.

## 955.52 Aspirin and Phenobarbital in Drugs

**Spectrophotometric Method**
**Final Action 1965**

### A. Apparatus

(**a**) *Spectrophotometer*.—Capable of isolating spectrum of $\leq$2 nm in region 230–300 nm.

(**b**) *Chromatographic tube and tamping rod*.—*See* **967.31A**.

### B. Reagents

(**a**) *Dibasic potassium phosphate soln*.—Approx. 2*M*. Dissolve 35 g $K_2HPO_4$ in $H_2O$, cool to room temp., and dil. to 100 mL.

(**b**) *Diatomaceous earth*.—*See* **960.53B**.

(**c**) *Washed chloroform*.—Wash USP $CHCl_3$ with $^1/_2$ vol. $H_2O$ in separator.

(**d**) *Aspirin std soln*.—5 mg/100 mL. Dissolve 100 mg aspirin in $CHCl_3$ and dil. to 100 mL with $CHCl_3$. Dil. 5 mL aliquot to 100 mL with $CHCl_3$.

(**e**) *Phenobarbital std soln*.—1 mg/100 mL. Dissolve 100 mg phenobarbital in $NH_4OH$ (1 + 27) and dil. to 500 mL with $NH_4OH$ (1 + 27). Dil. 5 mL aliquot to 100 mL with $NH_4OH$ (1 + 27).

### C. Preparation of Sample Solution

Transfer accurately weighed portion of finely ground tablets contg 60–120 mg phenobarbital to 100 mL vol. flask. Dissolve in $CHCl_3$ by shaking vigorously and dil. to vol. with $CHCl_3$.

### D. Preparation of Chromatographic Column

Pack small pledget of glass wool in constricted portion of stem of tube and place pad of glass wool ca 5 mm thick in bottom of large portion of tube. Fasten piece of rubber tubing with attached screw clamp to outlet to limit flow during packing. Clamp tube in vertical position.

To 10 g diat. earth in mortar add 50 mL $CHCl_3$, and mix with pestle to form slurry. Distribute 10 mL 2*M* $K_2HPO_4$ soln, (**a**), over surface of slurry and mix thoroly until homogeneous, adding more $CHCl_3$ if necessary. Add this slurry to tube, ca $^1/_5$ at time, alternately packing and forming flocculent suspension by working packing rod up and down. Diat. earth must be covered with $CHCl_3$ at all times.

After column is packed, remove rubber tube from stem and rinse stem with $CHCl_3$. Check flow rate of column with $CHCl_3$ level ca 5 cm above surface of column. Adjust rate of flow to 2–5 mL/min by tightening or loosening glass wool pledget in constricted portion of stem. When level of solv. just reaches surface of column, place 100 mL vol. flask under stem.

### E. Determination of Phenobarbital

Add 5 mL prepd sample soln, **955.52C**, from pipet to side of tube near diat. earth surface. When level of sample soln reaches surface of column, add 5 mL washed $CHCl_3$, let sink into column, and repeat with another 5 mL washed $CHCl_3$. After last rinse enters column, add washed $CHCl_3$ to tube and keep level of 4–8 cm $CHCl_3$ above column during elution.

Collect 95 mL eluate in the 100 mL vol. flask. Dil. to vol. with $CHCl_3$, mix, and transfer 20 mL aliquot to 100 mL beaker. Evap. to dryness on steam bath under air current. Dissolve residue in $NH_4OH$ (1 + 27) and transfer to 100 mL vol. flask. Rinse, and dil. to vol. with $NH_4OH$ (1 + 27). Det. *A* at max., ca 240.5 nm, against blank prepd by passing 5 mL $CHCl_3$ thru column as with sample soln.

$$\text{g Phenobarbital in sample} = 10A/a$$

where *a* is absorptivity of phenobarbital at 240.5 nm obtained by dividing *A* of std phenobarbital soln in 1 cm cell at 240.5 nm by its concn (0.01 g/L).

### F. Determination of Aspirin

Dil. 5 mL original sample soln, **955.52C**, to 100 mL with $CHCl_3$ in vol. flask. Dil. 10 mL aliquot of this soln to 100 mL with $CHCl_3$. Det. *A* of final diln on spectrophtr at 278 nm against $CHCl_3$ blank.

$$\text{g Aspirin in sample} = 20A_{278}/a_{278}$$

where $a_{278}$ is absorptivity of aspirin at 278 nm obtained by dividing *A* of std aspirin soln in 1 cm cell at 278 nm by its concn (0.05 g/L).

Ref.: JAOAC **38**, 635(1955).

CAS-50-78-2 (aspirin)
CAS-50-06-6 (phenobarbital)

## 938.16★ Aspirin and Phenolphthalein in Tablets

**Gravimetric Method**
**Final Action**
**Surplus 1977**

*See* **37.054**, 13th ed.

## 923.13 Aminopyrine

**Final Action**

### A. Qualitative Tests

(**a**) Dissolve 0.01 g sample in 2 mL $H_2O$ and add few drops of yellow $HNO_3$ (contg $HNO_2$). Purplish blue soln is produced.

(**b**) Dissolve 0.01 g sample in 2 mL $H_2O$ and add 1 mL 10% $FeCl_3$ soln. Purple to violet color develops, which becomes red on addn of $H_2SO_4$ (1 + 9).

(**c**) Dissolve 0.1 g sample in 2 mL $H_2O$ and add few drops of 5% $AgNO_3$ soln. After few sec, purple to violet color is produced, and on standing, deposit of metallic Ag results (useful for detecting aminopyrine in antipyrine).

(**d**) Dissolve 0.1–0.2 g sample in 2 mL $H_2O$, add 1 or 2 drops 0.2% $NaNO_2$ soln and few drops of $H_2SO_4$ (1 + 9), and shake few sec. Purplish blue color develops and then gradually fades, leaving colorless soln. Excess $NaNO_2$ destroys aminopyrine color. On addn of few more drops of $NaNO_2$ soln and dil. $H_2SO_4$, yellowish green color remains after purple disappears if antipyrine is present. (Useful for detecting antipyrine in presence of aminopyrine.)

Refs.: JAOAC **7,** 29(1923); **8,** 40(1924); **8,** 544(1925).

### *B. Quantitative Method*

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Place 1 g powd sample in 100 mL vol. flask, add 60 mL $1N$ $H_2SO_4$, and shake several min to ensure complete soln of aminopyrine. Dil. to vol. with $1N$ $H_2SO_4$. Filter, if not clear, thru dry filter, rejecting first part of filtrate. Pipet 20 mL aliquot of soln or filtrate into separator; make distinctly alk. with either $NH_4OH$ or 5% NaOH; and ext with 20, 15, 10, 10, and 5 mL portions $CHCl_3$. Combine $CHCl_3$ exts in second separator and wash with 2 mL $H_2O$. Filter $CHCl_3$ soln into weighed beaker thru cotton pledget satd with $CHCl_3$. Ext wash $H_2O$ with 5 mL $CHCl_3$ and add this to combined $CHCl_3$ exts. Evap. combined $CHCl_3$ exts just to dryness on $H_2O$ bath with aid of air current and dry residue 10 min at 100°. Cool in desiccator, and weigh as aminopyrine. Identify aminopyrine by its mp, 106.5–109°, and qual. tests, **923.13A**, or microchem. tests, Table **962.21B**.

Refs.: JAOAC **11,** 51, 350(1928); **23,** 60, 742(1940).

CAS-58-15-1 (aminopyrine)

## HYPNOTICS AND SEDATIVES

## 968.43 Acetylcarbromal and Bromisovalum in Drugs

**Infrared Spectrophotometric Method**

**First Action 1968**
**Final Action 1970**

### *A. Principle*

Acetylcarbromal is eluted with heptane-$CCl_4$ and bromisovalum with $H_2O$-satd $CHCl_3$ from diat. earth column. Eluates are dried; acetylcarbromal residue is dissolved in $CCl_4$ and bromisovalum residue in $CHCl_3$. Concns are detd by IR spectrometry at 5.8 μm. Identification is made from residues in KBr disks.

### *B. Apparatus*

(**a**) *Infrared spectrophotometer.*—Beckman IR-5, (replacement model Varian Instruments Carg 2000 Series), or equiv., with 1 mm liq. cells.
(**b**) *Chromatographic tube.—See* **967.31A**.

### *C. Reagents*

(**a**) *Diatomaceous earth.—See* **960.53B**.
(**b**) *Reference std solns.*—1.00 mg/mL. Completely dissolve 50.0 mg acetylcarbromal (mp 109°) in $CCl_4$ in 50 mL vol. flask and dil. to vol. with $CCl_4$. Completely dissolve 50.0 mg bromisovalum (mp 148–149°) in $CHCl_3$ in 50 mL vol. flask and dil. to vol. with $CHCl_3$.

### *D. Column Chromatography*

(*Caution:* See safety notes on distillation, toxic solvents, carbon tetrachloride, and chloroform.)

Pack small wad of fine glass wool into bottom of tube. Thoroly mix 4 g diat. earth with 5 mL HCl (2 + 1), transfer to tube, and tamp to uniform mass with tamping rod.

Det. av. wt/tablet and pulverize. Accurately weigh portion of powder contg 10–30 mg acetylcarbromal, and mix in beaker with 1 g diat. earth and 1 mL $H_2O$. Transfer to tube, dry-rinse beaker with small portion diat. earth, and tamp to uniform mass. Wash beaker with few portions $H_2O$-satd $CCl_4$-heptane (1 + 1) and pour thru column. Elute acetylcarbromal with $CCl_4$-heptane mixt., collecting 50 mL eluate in beaker. Immediately, without letting column go dry, elute bromisovalum with 100 mL $H_2O$-satd $CHCl_3$, collecting eluate in beaker. Evap. both eluates to complete dryness on steam bath with air current.

Dissolve acetylcarbromal residue with several portions of $CCl_4$ (dried with anhyd. $Na_2SO_4$) and dil. to concn of 1 mg/mL. Dissolve bromisovalum residue in several portions of $CHCl_3$ (dried with anhyd. $Na_2SO_4$) and dil. to concn of 1 mg/mL. Using their resp. solvs as ref. solns, det. IR spectrum at 5–7 μm. For calcn, use $A$ of max. at ca 5.8 μm, using baseline technic:

$$\% \text{ Compd} = (100 \times A \times C \times V)/(A' \times W)$$

where $A$ and $A'$ refer to sample and std solns, resp., $C$ = concn of std soln (mg/mL), $V$ = final vol. sample soln, and $W$ = mg sample.

### *E. Identification*

Evap. 1 mL each of std and sample soln, and prep. KBr disk from each of residues, using ca 200 mg KBr. Scan IR spectra for identification.

Ref.: JAOAC **51,** 621(1968).

CAS-77-66-7 (acetylcarbromal)
CAS-496-67-3 (bromisovalum)

## 943.07 Carbromal in Drugs

**Titrimetric Method**

**Final Action**

### *A. Apparatus*

See Fig. **943.07**. Consists of 100 mL r-b flask with 24/40 ₸ inner joint; condenser with jacket ca 130 mm long; and absorption flask with 2 bulbs. Condenser is equipped with 12/30 and 24/40 ₸ inner joints. Small dropping funnel is fused to tube above jacket. Absorption flask has outer 12/30 ₸ joint. Small springs (not shown) are attached to hooks on joints to keep app. tightly connected during use.

### *B. Determination*

Place sample calcd to contain 40–60 mg Br in oxidn flask and dissolve in 2 mL 10% NaOH soln and 8 mL $H_2O$. Lubricate joints of app. with $H_3PO_4$ and connect flask to condenser. Place ca 15 mL *1% $N_2H_4.H_2SO_4$ soln* and 5 mL 10% NaOH soln in absorption flask and connect to app. (Use <20 mL absorbing soln if app. has smaller absorption bulbs than indicated.)

Add 5 mL *$CrO_3$ soln (1 + 1)* thru addn tube, wash down with 2–3 mL $H_2O$, and then slowly add 10 mL $H_2SO_4$. If vigorous reaction begins, let it subside before heating flask; if reaction does not begin as acid is added, heat gently with small flame, but remove flame before reaction becomes too vigorous, otherwise reaction mixt. may foam up into condenser. When reaction subsides, heat mixt. to boiling. When foaming subsides, add 5 mL $H_2SO_4$ thru dropping funnel, boil 10 min, add another 5 mL $H_2SO_4$, and boil again 10 min. Drain $H_2O$ from condenser and boil reaction mixt. until 2–3 drops $H_2O$ distil into absorber.

Disconnect absorption flask, wash contents into 500 mL I flask, and dil. to ca 100 mL with $H_2O$. Add ca 12 mL $H_3PO_4$, 5 mL *3% KCN soln,* and 15 mL *3% $KMnO_4$ soln,* wetting

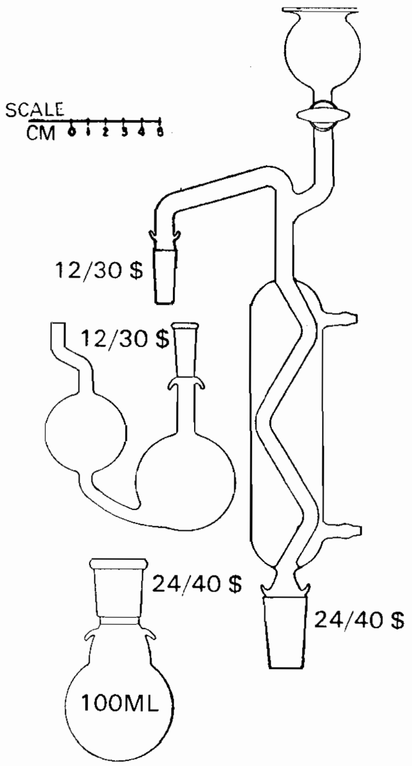



**FIG. 943.07—Bromine apparatus**

sides of flask with each reagent as it is added. Stopper flasks and mix by gentle swirling, wetting entire inside surface. Let stand ≥7 min; then add ca 2 g solid $FeSO_4.(NH_4)_2SO_4.6H_2O$. Wash down sides of flask and mix. (Clear, nearly colorless soln should result.) If any $KMnO_4$ or $MnO_2$ remains, add more $Fe(NH_4)_2(SO_4)_2.6H_2O$ (2 g excess does no harm).

Add ca 2 g KI and immediately titr. liberated I with 0.05*N* $Na_2S_2O_3$, using starch indicator. (End point is disappearance of starch-I color; avoid over-titrn as color of soln remains light blue.) 1 mL 0.05*N* $Na_2S_2O_3$ = 0.001998 g Br or 0.00593 g carbromal.

Refs.: JAOAC **26,** 433(1943); **28,** 757(1945); **34,** 570(1951).

CAS-77-65-6 (carbromal)

## 943.08 Carbromal and Pentobarbital in Drugs

### Gravimetric Method

### Final Action

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Transfer 0.5–0.7 g sample to separator, and add 15 mL $H_2O$ and 0.5 mL 1*N* NaOH from pipet. Ext carbromal with at least five 25 mL portions $CHCl_3$, washing each portion in second separator contg 10 mL $H_2O$ and 2 drops 0.1*N* NaOH. Filter $CHCl_3$ thru cotton and transfer to tared flask or beaker. Test for complete extn. Evap. $CHCl_3$ soln of carbromal nearly but not quite to dryness on steam bath in air current. Remove container and let stand in air to const wt.

Combine aq. solns and proceed as in **925.59A**, beginning "Acidify to litmus paper . . ." Wt pentobarbital × 1.097 = wt Na pentobarbital in portion taken for assay. Det. mp of dried exts. Carbromal melts at 116–119° and pentobarbital at 126–130°.

Refs.: JAOAC **26,** 433(1943); **28,** 757(1945); **34,** 570(1951).

CAS-77-65-6 (carbromal)
CAS-76-74-4 (pentobarbital)

## 925.59 Barbiturates in Drugs

### Gravimetric Method

### Final Action

(*See also* **974.39** for barbital and phenobarbital and **958.14** for mannitol hexanitrate and phenobarbital.)

#### *A. Method I*

(Applicable in absence of stearic acid.)

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Accurately weigh 0.3–0.5 g sample into separator, add 10 mL $H_2O$, and shake well. Add 5 mL 0.5*N* NaOH and shake again. Acidify to litmus paper with HCl (1 + 3), added dropwise, and add ca 1 mL excess. Ext with successive 40, 30, 20, 20, and 10 mL portions $CHCl_3$. Test for complete extn by shaking with addnl 10 mL solv. and evapg in sep. beaker.

Combine solv. in second separator and wash with 2 mL $H_2O$ acidified with 1 drop HCl. Filter solv. thru cotton pledget into small weighed beaker. Evap. on steam bath with aid of air current, heat 10 min at 80–90°, cool in desiccator, and weigh. Add 2 or 3 mL anhyd. ether and evap. solv. (Usually 2 treatments with 2 mL each of anhyd. ether are enough to remove last traces of $CHCl_3$ and to produce cryst. residue.) Dry at 80–90°, cool, and weigh. Repeat treatment with anhyd. ether and evapn to const wt. Det. mp to check purity of residue.

Refs.: JAOAC **8,** 47, 510(1925); **25,** 799(1942); **26,** 101(1943).

#### *B. Method II*

(Applicable in presence of stearic acid)

Dissolve residue obtained in **925.59A** in 10 mL alcohol, add 20 mL *satd* $Ba(OH)_2$ *soln,* and stir well. Filter into separator, and wash residue and filter with two or three 10 mL portions of the $Ba(OH)_2$ soln. Acidify filtrate with HCl (1 + 3) and proceed as in **925.59A**, beginning "Ext with successive . . ."

Ref.: JAOAC **19,** 508(1936).

## Barbiturate Drugs

### Microscopic Tests

### Final Action

*See* **962.21**, Tables **962.21A** and **B**, and **960.57**.

## 955.53★ Amobarbital Sodium and Secobarbital Sodium in Drugs

### Final Action

### Surplus 1977

*See* **37.068–37.072**, 13th ed.

## 955.54 Phenobarbital and Aminophylline in Drugs

### Spectrophotometric Method

### Final Action

#### *A. Reagents*

(**a**) *Dilute ammonium hydroxide soln.*—0.1% $NH_3$. Dil. 4 mL $NH_4OH$ to 1 L with $H_2O$.

(**b**) *Phenobarbital std soln.*—10 µg/mL. Dissolve 100.0 mg phenobarbital in the dil. $NH_4OH$ soln in 500 mL vol. flask, dil. to vol. with the dil. $NH_4OH$, and mix. Transfer 5 mL aliquot to 100 mL vol. flask, dil. to vol. with the dil. $NH_4OH$, and mix.

(**c**) *Theophylline std soln.*—10 µg/mL. Dissolve 100.0 mg theophylline in HCl (1 + 18) in 500 mL vol. flask. Dil. to vol. with HCl (1 + 18) and mix. Transfer 5 mL aliquot to 100 mL vol. flask, dil. to vol. with $H_2O$, and mix.

#### *B. Separation of Aminophylline and Phenobarbital*

Transfer weighed portion of powd sample contg ca 15 mg phenobarbital to separator contg 25 mL HCl (1 + 1). Add 60 mL ether, shake, and let stand to clear. Pass aq. soln successively thru 2 other separators, each contg 50 mL ether, shake, and let stand to clear. Transfer ether-washed aq. soln to 500 mL vol. flask. Wash the 3 ether solns successively with three 10 mL portions HCl (1 + 1) and one 10 mL portion $H_2O$, and add these washes to the vol. flask. Reserve for detn of theophylline.

#### *C. Determination of Phenobarbital*

(*Caution:* See safety notes on distillation, flammable solvents, and diethyl ether.)

Combine ether solns and evap. to dryness. Dissolve residue in ca 100 mL of the dil. $NH_4OH$ and transfer to 200 mL vol. flask. Dil. to vol. with the dil. $NH_4OH$ and mix. Filter, if necessary, transfer 10 mL aliquot to 100 mL vol. flask, dil. to vol. with the dil. $NH_4OH$, and mix. Det. $A_P$ at max., ca 240.5 nm, against dil. $NH_4OH$. Read this soln same day it is prepd.

Det. $A'_P$ of std phenobarbital soln, (**b**), at same wavelength, using the dil. $NH_4OH$ as blank. Calc. $a_p = A'_P/cb$, where $c$ = 0.01 g/L, and $b$ = cell length in cm. Phenobarbital (g/L sample soln) = $A_P/a_P$.

If stearates are present, proceed as above, dissolving residue in ca 100 mL of the dil. $NH_4OH$ and dilg to ca 190 mL with the dil. $NH_4OH$. Acidify with HCl, testing with litmus paper. Dil. to vol. with $H_2O$, mix, and filter. Transfer 10 mL aliquot to 100 mL vol. flask, add 1 drop $NH_4OH$ (1 + 1), dil. to vol. with the dil. $NH_4OH$, and det. $A_P$ at 240.5 nm.

#### *D. Determination of Theophylline*

Dil. aq. soln in vol. flask to vol. with $H_2O$ and mix. Transfer aliquot contg 0.5–1.0 mg theophylline to 100 mL vol. flask, dil. to vol. with $H_2O$, and mix. Det. $A_T$ at 271 nm against blank soln contg same vol. HCl. Det. $A'_T$ of std theophylline soln, (**c**), and calc. $a_T$ as in **955.54C**. Theophylline (g/L sample soln) = $A_T/a_T$. Aminophylline, $C_{16}H_{24}N_{10}O_4.2H_2O$ = 1.267 × theophylline.

Ref.: JAOAC **38**, 624(1955).

CAS-317-34-0 (aminophylline)
CAS-50-06-6 (phenobarbital)

## 965.43 Phenobarbital and Phenytoin in Drugs

### Spectrophotometric Method

### First Action 1965
### Final Action 1967

#### *A. Reagents*

(**a**) *Water-saturated soln of 15% n-amyl alcohol in $CHCl_3$.*—Sat. 500 mL 15% n-amyl alcohol in $CHCl_3$ with 25 mL $H_2O$ and let stand 30 min. Det. suitability of reagent by passing 80 mL thru prepd column followed by 25 mL $H_2O$-satd $CHCl_3$, evapg to dryness on steam bath with air current, dilg to 25.0 mL with alcohol, and reading $A$ in 1 cm cell on recording spectrophtr from 320 to 250 nm. If $(A_{258} - A_{263}) \leq 0.190$, make blank correction. Higher $\Delta A$ indicates better grade reagent must be used.

(**b**) *Acetic acid-chloroform soln.*—1% HOAc in $H_2O$-satd $CHCl_3$.

#### *B. Preparation of Standards*

(**a**) *Phenobarbital std soln.*—Weigh and transfer 20 mg phenobarbital, USP, to 50 mL vol. flask; dissolve and dil. to vol. with alcohol. Pipet 10 mL into 125 mL g-s erlenmeyer and evap. to dryness on steam bath with air current (4 mg/100 mL 0.1$N$ NaOH has $A$ of ca 1.30 at max., ca 253 nm, in 1 cm cell).

(**b**) *Phenytoin (5,5-Diphenylhydantoin) std soln.*—Weigh and transfer 90 mg diphenylhydantoin (Eastman), to 50 mL vol. flask; dissolve and dil. to vol. with alcohol. Pipet 10 mL into 125 mL g-s erlenmeyer and evap. to dryness on steam bath with air current (18 mg/25 mL alcohol has $A$ of ca 1.90 at max., ca 258 nm, in 1 cm cell).

#### *C. Preparation of Sample*

Accurately weigh powd sample contg ca 90 mg phenytoin and transfer to 50 mL vol. flask. Add 1.5 mL alcohol, 0.1 mL HOAc, and ca 25 mL reagent, **965.43A(a)**. Heat on steam bath with swirling until $CHCl_3$ boils. Remove from heat and swirl 5 min. Heat to boiling as before, remove, and let stand 15 min with frequent agitation. Let cool and dil. to vol. with reagent (**a**). (Phenytoin dissolves with difficulty. Turbidity of soln may persist because of insol. excipients.)

#### *D. Preparation of Column*

Use glass tube 25 mm diam. × 15–30 cm long, with stem plugged with glass wool, and glass tamping rod weighing ca 32 g and having 20–22 mm ram head.

(**a**) *Column packing.*—(*1*) *Bottom layer.*—Mix 2 g diat. earth, **960.53B**, and 1 mL *12% $BaCl_2$ soln.* (*2*) *Top layer.*—Mix 4 g diat. earth, **960.53B**, and 3 mL *satd $Na_3PO_4$* soln.

(**b**) *Packing technic.*—*Column must be packed exactly as follows:* Transfer sep. bottom and top packing layers to tube in 1–2 g portions and tamp 10–15 times with tamping rod after addn of each portion by dropping rod from 2.5 cm above packing surface.

Place glass wool pad over diat.earth mixt. and pass 25 mL reagent (**a**) thru column at 5–10 mL/min, discarding eluate.

#### *E. Determination*

(*Caution:* See safety notes on distillation, acetic acid, toxic solvents, and chloroform.)

Place 125 mL g-s erlenmeyer under column and pipet 10 mL sample soln directly over glass wool pad. Let drain into column, and wash column with three 10 mL portions reagent (**a**), letting each drain into column. Add addnl 40 mL reagent (**a**). Pass 25 mL $H_2O$-satd $CHCl_3$ thru column, wash stem with

$CHCl_3$, and evap. eluate to dryness on steam bath with air current. (Odor of *n*-amyl alcohol must be absent.) Residue is phenytoin.

Place 125 mL g-s erlenmeyer under column and add to column 5 mL HOAc in $CHCl_3$ (1 + 4). Let drain into column. Add 20 mL reagent, **965.43A(b)**, let drain into column, and add 70 mL more. Wash stem with $CHCl_3$ and evap. eluate to dryness on steam bath with air current. Residue is phenobarbital.

Add 25 mL alcohol by pipet to both sample and std phenytoin residues. Stopper and warm with swirling. Let stand, swirling occasionally, until solid matter is completely dissolved. Det *A* of sample and std solns against reagent, **965.43A(a)**, on recording spectrophtr from 320 to 250 nm in 1 cm cell.

$$\text{mg Phenytoin/capsule} = C \times (K/W) \times (A_{258} - A_{263})/(A'_{258} - A'_{263})$$

where $A_{258}$ and $A_{263}$ = max. and min. *A* of sample soln at ca 258 and 263 nm, resp.; $A'_{258}$ and $A'_{263}$ = max. and min. *A* of std soln at 258 and 263 nm, resp.; *C* = mg std phenytoin; *K* = av. capsule content wt (mg); and *W* = mg sample. Phenytoin × 1.087 = Na phenytoin.

Add 100 mL 0.1*N* NaOH by pipet to both sample and std phenobarbital residues. Stopper and shake vigorously 2 min. *Immediately* read *A* of solns on recording spectrophtr from 350 to 230 nm in 1 cm cell.

$$\text{mg Phenobarbital/capsule} = C \times (K/W) \times (A_{253}/A'_{253})$$

where $A_{253}$ and $A'_{253}$ = *A* of sample and std solns, resp., at max., ca 253 nm; *C* = mg std phenobarbital; *K* = av. capsule content wt (mg); *W* = mg sample.

Ref.: JAOAC **48,** 582(1965).

CAS-57-41-0 (diphenylhydantoin)
CAS-50-06-6 (phenobarbital)

## 949.16 Phenobarbital and Theobromine in Drugs

### Spectrophotometric Method

### Final Action

#### *A. Reagents*

(**a**) *Theobromine std soln.*—1.00 mg/100 mL. Dissolve 100 mg theobromine in $H_2SO_4$ (1 + 4), and dil. to 100 mL with this acid. Transfer 5.0 mL aliquot to 500 mL vol. flask, add 200 mL 5% NaOH soln, and cool to room temp. Dil. to vol. with $H_2O$ and mix thoroly.

(**b**) *Phenobarbital std soln.*—1.50 mg/100 mL. Dissolve 75.0 mg phenobarbital in $CHCl_3$ and dil. to 100 mL with $CHCl_3$. Dil. 10 mL aliquot to 50 mL with $CHCl_3$. Transfer 10 mL aliquot of latter soln to 100 mL vol. flask, dil. to vol. with $CHCl_3$, and mix.

#### *B. Separation of Theobromine and Phenobarbital*

(*Caution:* See safety notes on distillation, flammable solvents, and diethyl ether.)

Transfer portion of well mixed sample contg ≥15 mg phenobarbital to 125 mL separator, add 15 mL 5% NaOH soln, and ext with three 30 mL portions $CHCl_3$. Wash each $CHCl_3$ ext with 10 mL 5% NaOH soln in second separator. Discard $CHCl_3$.

Add 30 mL $H_2SO_4$ (1 + 4) to alk. mixt. in first separator, cool thoroly, and shake with 50 mL ether. Transfer aq. layer contg dissolved theobromine to second separator, cool, and shake with 40 mL ether. Remove lower phase to third separator and wash with another 40 mL portion ether. Repeat extn thru the 3 separators, using two 40 mL portions $H_2SO_4$ (1 + 4) and two 20 mL portions $H_2O$. Collect aq. exts in 250 mL vol. flask, dil. to vol. with $H_2O$, and mix. Reserve for theobromine detn.

Filter ether solns thru cotton pledget into beaker, washing the 3 separators and filter successively with three 5 mL portions ether. Evap. carefully to dryness, and dissolve residue in $CHCl_3$.

#### *C. Spectrophotometric Determinations*

(**a**) *Theobromine.*—Pipet aliquot contg 4–8 mg theobromine into 500 mL vol. flask, add 200 mL 5% NaOH soln, and cool to room temp. Dil. to vol. with $H_2O$ and mix. Det. *A* at 274 nm of this soln and of std theobromine soln, (**a**), *A'*, relative to soln prepd by dilg 10 mL 5% NaOH soln to 25 mL. Calc. theobromine content of sample.

$$\text{mg Theobromine in aliquot} = 5.0\ A/A'$$

(**b**) *Phenobarbital.*—Transfer $CHCl_3$ soln to vol. flask and dil. with $CHCl_3$ to obtain soln contg 20–40 mg phenobarbital/100 mL. Place 5.0 mL in 100 mL vol. flask, dil. to vol. with $CHCl_3$, and mix. Transfer 20 mL aliquot of latter soln to separator contg 25 mL $NH_4OH$ (1 + 24). Similarly treat 20 mL aliquot std phenobarbital soln, (**b**), and 20 mL portion $CHCl_3$ as blank. Shake vigorously ≥1 min, sep., and discard $CHCl_3$. Let aq. ext stand 30 min. Det. *A* at 241 nm of clear aq. solns of sample, and of std, *A'*, relative to blank, using same cell for std and sample. Calc. phenobarbital content of sample.

$$\text{mg Phenobarbital in final aliquot} = 0.30A/A'$$

In presence of salicylates, proceed as in (**c**):

(**c**) *Phenobarbital in presence of salicylates.*—Prep. chromatgc column as in **955.52D**, and adjust flow to 2–4 mL/min.

When $CHCl_3$ just stops flowing from tube, pipet 5 mL original $CHCl_3$ soln, (**b**), (equiv. to 1–2 mg phenobarbital) into tube, and collect eluate in 100 mL vol. flask. As level of $CHCl_3$ soln reaches top of column, add ca 5 mL $CHCl_3$, and repeat with second $CHCl_3$ wash. Add enough $CHCl_3$ to keep column of solv. 2–5 cm high, and collect ca 95 mL eluate. Wash outside surface of stem with stream of $CHCl_3$ and collect washings in vol. flask. Dil. to vol. with $CHCl_3$ and mix thoroly. Det. phenobarbital in eluates as in (**b**), beginning "Transfer 20 mL aliquot of latter soln . . ."

Refs.: JAOAC **34,** 566(1951).

CAS-50-06-6 (phenobarbital)
CAS-83-67-0 (theobromine)

## 972.50 Butabarbital Sodium in Drugs

### Spectrophotometric Method

### First Action 1972
### Final Action 1973

#### *A. Reagents*

(**a**) *Sodium carbonate soln.*—1*M*. Dissolve 10.6 g $Na_2CO_3$ and dil. to 100 mL with $H_2O$.

(**b**) *Mixed solvent.*—Isooctane-ether (4 + 1). Wash mixed solv. with equal vol. $H_2O$.

(**c**) *Ether.*—$H_2O$-satd. Use thruout.

(**d**) *Dilute ammonium hydroxide.*—Dil. 30 mL $NH_4OH$ to 1 L with $H_2O$.

(**e**) *Diatomaceous earth.*—*See* **960.53B**.

(**f**) *Dimethylsulfoxide* (*DMSO*).—Spectral grade (Fisher

Scientific Co. D-136; EM Science, OmniSolv, No. MX1456).

(**g**) *Butabarbital std solns.*—(*1*) *Stock soln.*—0.1 mg/mL. Dissolve 20 mg USP Ref. Std in ≤1 mL MeOH and dil. to 200 mL with dil. $NH_4OH$. (*2*) *Working soln.*—0.01 mg/mL. Dil. 10 mL stock soln to 100 mL with dil. $NH_4OH$.

### B. Preparation of Sample

Det. av. wt/tablet and pulverize. Transfer accurately weighed portion contg ca 20 mg butabarbital to 100 mL beaker. Add 1 mL *DMSO* and 2 drops HCl, and swirl to dissolve active ingredient. Add 1 mL $H_2O$ and 3 g diat. earth, and mix thoroly until uniform.

### C. Preparation of Column

Place small glass wool plug at base of 250 × 25 mm chromatgc tube. Transfer uniform mixt. of 4 g diat. earth and 3 mL $Na_2CO_3$ soln to tube and tamp to uniform mass. Transfer sample prepn to column, drywash beaker with 1 g diat. earth, transfer wash to column, and tamp.

### D. Determination

Pass 75 mL mixed solv. thru column and discard. Elute butabarbital with 100 mL ether, collecting eluate in 250 mL separator. Rinse tip of column with ether. Ext eluate with three 50 mL portions dil. $NH_4OH$, collect extns in 200 mL vol. flask, and adjust to vol. with dil. $NH_4OH$. Dil. 10.0 mL of this soln to 100.0 mL with dil. $NH_4OH$. Read *A* of sample and std solns at max. ca 239 nm against dil. $NH_4OH$ as ref. and calc. Na butabarbital.

Ref.: JAOAC **55,** 152(1972).

CAS-143-81-7 (sodium butabarbital)

## 972.51 Phenytoin Sodium in Drug Capsules

**Spectrophotometric Method**

**First Action 1972**
**Final Action 1973**

### A. Reagents

(**a**) *Sodium carbonate.*—0.5*M*. Dissolve 5.3 g $Na_2CO_3$ and dil. to 100 mL.

(**b**) *Mixed solvent.*—Isooctane-$CHCl_3$ (7 + 3). Wash mixed solv. with equal vol. $H_2O$.

(**c**) *Chloroform.*—$H_2O$-satd. (*Caution:* See safety notes on chloroform.)

(**d**) *Acid-alcohol.*—Dil. 1 mL HCl (1 + 99) with 50 mL alcohol.

(**e**) *Diatomaceous earth.—See* **960.53B**.

(**f**) *Phenytoin std soln.*—0.25 mg/mL. Dissolve 25 mg USP Ref. Std in 100 mL acid-alcohol.

### B. Preparation of Sample

Remove, as completely as possible, contents of capsules and weigh. Det. av. net contents. Mix, and transfer accurately weighed portion contg ca 100 mg Na phenytoin to 25 mL vol. flask. Add 2 mL *DMSO*, **972.50A(f)**, and swirl mixt. ca 3 min. Add 4 drops HCl, swirl to mix, and immediately but cautiously add 0.5*M* $Na_2CO_3$ to vol. Mix, and filter thru rapid paper.

### C. Preparation of Column

Place small glass wool plug at base of 250 × 25 mm chromatgc tube. Mix 3 g diat. earth and 2 mL 0.5*M* $Na_2CO_3$, and transfer to tube; tamp. Mix 3 g diat. earth and 2.0 mL prepd sample soln, and transfer to column. Dry-wash beaker with ca 1 g diat. earth, and transfer wash to column. Place pad of glass wool on top of column.

### D. Determination

Pass 75 mL mixed solv. thru column, and discard. Elute phenytoin with 75 mL $CHCl_3$, collecting eluate in 125 mL g-s erlenmeyer. Rinse tip of column with $CHCl_3$. Evap. solv. to dryness on steam bath under air current. Dissolve residue in 25.0 mL acid-alcohol. Det. *A* of sample and std solns at max., ca 258 nm, against acid-alcohol as ref. Calc. phenytoin Na content. Phenytoin × 1.087 = phenytoin Na.

Ref.: JAOAC **55,** 170(1972).

CAS-57-41-0 (phenytoin)

## 968.44 Chloral Hydrate in Drugs

**Spectrophotometric Method**

**First Action 1968**
**Final Action 1969**

### A. Principle

Quinaldine ethyl iodide reacts with chloral hydrate to produce stable blue cyanine dye with *A* max. at ca 605 nm. Other polychlorinated compds do not interfere.

### B. Reagents

(**a**) *Quinaldine ethyl iodide soln.*—1.5%. Dissolve 1.5 g quinaldine ethyl iodide in $H_2O$ and dil. to 100 mL. Filter if necessary.

(**b**) *2-Aminoethanol soln.*—0.1*N*. Dissolve 6.1 g 2-aminoethanol in $H_2O$ and dil. to 1 L.

(**c**) *Chloral hydrate std soln.*—100 μg/mL. Dissolve 0.2500 g chloral hydrate USP in $H_2O$ and dil. to 250 mL. Dil. 10 mL aliquot to 100 mL with $H_2O$.

### C. Apparatus

*Recording spectrophotometer.*—400–800 nm range with matched 1 cm cells.

### D. Preparation of Sample

(**a**) *Capsules.*—Place counted number of capsules contg ca 2.5 g chloral hydrate in g-s 250 mL flask, add 25 mL $H_2O$, stopper, and heat on steam bath with frequent swirling until dissolved. Cool, and transfer quant. to 250 mL vol. flask with $H_2O$. Dil. to vol., mix, and dil. stepwise to ca 100 μg/mL with $H_2O$.

(**b**) *Solns.*—Prep. soln contg ca 100 μg chloral hydrate/mL by stepwise diln with $H_2O$.

### E. Determination

Pipet 10 mL sample soln contg ca 1 mg chloral hydrate into 100 mL vol. flask and pipet 10 mL std chloral hydrate soln into second 100 mL vol. flask. Pipet 10 mL $H_2O$ into third 100 mL vol. flask as blank. To each flask add 10 mL quinaldine ethyl iodide soln and 60 mL isopropanol, and mix. Add 5 mL 0.1*N* 2-aminoethanol and dil. to vol. with $H_2O$. Place in $H_2O$ bath 1 hr at 60°. Cool, and record spectra of sample and std from 400 to 800 nm against blank. Do not exceed 120 nm/min near max. Det *A* max. at ca 605 nm, using baseline technic with ca 430 and ca 770 nm as base.

$$\text{mg Chloral hydrate in sample aliquot} = (\text{net } A \text{ of sample soln}/\text{net } A \text{ of std soln}) \times \text{mg chloral hydrate in 10 mL std soln}$$

Ref.: JAOAC **51,** 626(1968).

CAS-302-17-0 (chloral hydrate)

## 944.14★ (2-Isopropyl-4-Pentenoyl) Urea in Drugs

**Chloroform Extraction**

**Final Action**
**Surplus 1965**

*See* **32.237**, 10th ed.

## 932.22★ Sulfonmethane or Sulfonethylmethane in Drugs

**Ether Extraction**

**Final Action**
**Surplus 1965**

See **32.238**, 10th ed.

## 970.81 Ethchlorvynol in Drugs

**Gas Chromatographic Method**

**First Action 1970**
**Final Action 1972**

### A. Reagents

(**a**) *Ethchlorvynol*.—(*Caution:* See safety notes on distillation and vacuum.) Purify by vac. distn (62° at ca 10 mm) or assay by titrn as follows: Transfer ca 110 mg ethchlorvynol, accurately weighed, to 250 mL erlenmeyer contg 50 mL 2.5% $AgNO_3$ soln in 70% alcohol. Immediately titr. with 0.05*N* NaOH, using 8–10 drops Me red-methylene blue, (**e**). Perform blank detn and make any necessary correction. 1 mL 0.05*N* NaOH = 7.230 mg ethchlorvynol. (*Caution:* Protect pure ethchlorvynol from excessive exposure to light and air.) Store at <10° in glass containers with polyethylene or Teflon stopper liners.

(**b**) *Ethchlorvynol stock soln*.—10 mg/mL. Accurately weigh ca 0.5 g ethchlorvynol and dissolve in 5 mL alcohol. Transfer quant. to 50 mL vol. flask with 10 mL alcohol. Dil. to vol. with $H_2O$.

(**c**) *Internal std soln*.—2.0%. Dissolve 2.0 g 1,3-dichloro-2-propanol in 10 mL alcohol and dil. to 100 mL with $H_2O$.

(**d**) *Dichlorodimethylsilane soln*.—Dissolve 5 mL dichlorodimethylsilane in 100 mL toluene. (*Caution:* Dichlorodimethylsilane causes severe burns. Vapor is harmful. Avoid contact with skin, eyes, or clothing. Use effective fume removal device.)

(**e**) *Methyl red-methylene blue mixed indicator*.—Dissolve 0.3 g Me red in 60 mL alcohol and dil. to 100 mL with $H_2O$. Dissolve 0.2 g methylene blue in 100 mL 50% alcohol and add to Me red soln.

### B. Apparatus

*Gas chromatograph*.—With 1.2 m (4') × 4 mm glass column, packed with Carbowax 20M on 100–120 mesh Gas-Chrom Q, and H flame ionization detector. *Operating conditions:* temps (°)—column 115, detector 190, injection port 200; flow rates (mL/min)—N 50, H 92, air ca 500. Adjust column temp. to elute ethchlorvynol in 12–15 min (relative retention time of internal std is ca 0.8.). Adjust H and air flow rates to give stable flame and good sensitivity. Adjust electrometer sensitivity so that 12 μg ethchlorvynol gives 50–70% deflection.

### C. Preparation of GC Column

Carefully wash inside of column and small amt of fine glass wool with dichlorodimethylsilane soln, rinse with alcohol, and dry thoroly. Dissolve 5.0 g Carbowax 20M in 100 mL $CHCl_3$. Add Carbowax soln to 10.0 g 100–120 mesh Gas-Chrom Q in 250 mL filter flask fitted with trap and stopper. Slowly apply vac. and maintain 5 min. Swirl slurry rapidly and transfer in small portions to buchner fitted with 9 cm Whatman No. 4 paper. Maintain vac. 5 min after last portion is added; then air dry coated support 1 hr by spreading on smooth surface. Oven dry addnl hr at 100°.

Carefully plug column exit with small pad of glass wool. Apply vac. to exit end and slowly add coated support thru inlet, tapping very gently to pack firmly. Pack to within 1 cm of area heated by injection port. Plug with glass wool and condition overnight at 220° with slow N stream.

### D. Preparation of Sample

(**a**) *Capsules* (*200—500 mg*).—Place counted number of capsules contg ca 2.5 g ethchlorvynol in 250 mL vol. flask; add 75 mL $H_2O$ and 30 mL alcohol, stopper, and heat on steam bath with frequent swirling until dissolved. Cool and dil. to vol. with $H_2O$.

(**b**) *Capsules* (*100 mg*).—Place 10 capsules in 100 mL vol. flask, add 50 mL $H_2O$ and 15 mL alcohol, stopper, and heat on steam bath with frequent swirling until dissolved. Cool and dil. to vol. with $H_2O$.

(**c**) *Solutions*.—Prep. soln contg ca 10 mg ethchlorvynol/mL by stepwise diln with 20% alcohol.

### E. Determination

Pipet 10 mL sample soln contg ca 100 mg ethchlorvynol into 50 mL vol. flask; pipet 10 mL ethchlorvynol stock soln into second 50 mL vol. flask. Pipet 10 mL internal std soln into each flask and dil. to vol. with $H_2O$.

Rinse 10 μL syringe with 50% alcohol and draw up 1 μL 50% alcohol. Draw in 1 μL air followed by 6 μL sample. Draw in 1 μL air and note sample vol. Insert needle thru septum of gas chromatograph, quickly depress plunger, and retract syringe needle. Inject 6 μL of each soln. Run std before and after sample. Calc. amt of ethchlorvynol in 10 mL sample aliquot as follows:

$$C = C' \times (X/X') \times (I'/I)$$

where $C$ and $C'$ = mg ethchlorvynol in 10 mL sample aliquot and std stock soln, resp.; $X$ = area ethchlorvynol peak in sample chromatogram; $X'$ = av. area ethchlorvynol peak in std chromatograms; $I$ = area internal std peak in sample chromatogram; and $I'$ = av. area internal std peak in std chromatograms.

Ref.: JAOAC **53,** 834(1970).

CAS-113-18-8 (ethchlorvynol)

## 972.52 Paraldehyde in Drugs

**Gas Chromatographic Method**

**First Action 1972**
**Final Action 1974**

### A. Reagents

(**a**) *Paraldehyde std*.—USP. Redistil twice and collect only fraction distg 120.5–123°. Store in amber g-s bottle. (*Caution:* Paraldehyde is toxic. Use effective fume removal device.)

(**b**) *Paraldehyde std solns*.—(*1*) *Stock soln*.—0.05 mL/mL. Pipet 5 mL std paraldehyde into 100 mL vol. flask, add 8.0 mL internal std, and dil. to vol. with acetone. (*2*) *Working std soln*.—0.005 mL/mL. Pipet 5 mL stock soln into 50 mL vol. flask and dil. to vol. with acetone.

(**c**) *Isoamyl alcohol*.—(EM Science, No. AX1440). Check purity by injecting 5 μL into gas chromatograph. If any interfering peaks are present, redistil. (*Caution:* See safety notes on distillation.)

(**d**) *Internal std.*—Dil. isoamyl alcohol, (**c**), with equal vol. acetone.

### B. Apparatus

(**a**) *Gas chromatographic column.*—2% cyclohexane dimethanol succinate (HIEFF-8BP) plus 20% Carbowax 20M on 80–100 mesh Diatoport S (Hewlett-Packard Co., Avondale, PA 19311) or Chromosorb W (HP). Prep. as follows: Weigh 500 mg HIEFF-8BP plus 5.0 g Carbowax 20M into 500 mL Morton flask. Add 200 mL $CHCl_3$ and, if necessary, heat on steam bath with swirling to dissolve liq. phase. Add 20 g 80–100 mesh Diatoport S (or Chromosorb W (HP)) and evap. $CHCl_3$ under reduced pressure on rotating evaporator. Remove last traces $CHCl_3$ in 100° oven. Pack 1.8 m (6′) × 4 mm id glass column and condition column 16 hr at 190° with N flow of 60 mL/min before use.

(**b**) *Gas chromatograph.*—Packard Model 7800 (replaced by 5700) with flame ionization detector and Hewlett-Packard Model 3370A (replaced by 3380) electronic integrator, or equivs. GC conditions: temps (°)—column 110, injection port 125, detector 125; flow rates (mL/min)—N carrier gas 60, air 600, H 60; sensitivity $1 \times 10^{-8}$ amp; and chart speed 26″/hr.

### C. Preparation of Sample

Pipet amt elixir contg 2.5 mL paraldehyde into 50 mL vol. flask. Add 4.0 mL internal std and dil. to vol. with acetone. Pipet 5 mL this soln into 50 mL vol. flask and dil. to vol. with acetone.

### D. Determination

Inject ca 4 μL std and sample solns into gas chromatograph operated as in **972.52B(b)**. Make ≥3 injections of sample and std solns and take av.

Calc. amt paraldehyde in sample as follows:

$$\% \text{ Paraldehyde} = (R_x/R_s) \times C \times DF$$

where $R_x$ = ratio of sample to internal std peak area in sample soln, $R_s$ = ratio of std to internal std peak area in std soln, $C$ = concn of std (%, v/v), and $DF$ = sample diln factor.

Since GC peaks for paraldehyde are narrow, symmetrical, and well defined, peak hts may be used in place of peak area.

Ref.: JAOAC **55,** 166(1972).

CAS-123-63-7 (paraldehyde)

## 968.45 Phenaglycodol in Drugs

**Infrared Spectroscopic Method**

**First Action 1968**
**Final Action 1970**

### A. Apparatus

*Recording infrared spectrophotometer.*—With two 1.0 mm liq. cells with NaCl windows (preferably matched) and KBr disk holder.

### B. Reagents

(**a**) *Phenaglycodol std.*
(**b**) *Carbon disulfide.*—Spectral grade.
(**c**) *Cotton.*—Wash thoroly with $CHCl_3$ and dry.

### C. Determination

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Det. av. wt/tablet or capsule, pulverize, and sieve to obtain uniform sample. Accurately weigh sample contg ca 200 mg phenaglycodol and transfer to 125 mL separator with 50 mL $CHCl_3$. Add 15 mL 0.5*N* NaOH, shake 1 min, and filter sepd $CHCl_3$ layer thru cotton into 150 mL beaker. Ext alk. soln with two addnl 25 mL portions $CHCl_3$, filtering each sepd $CHCl_3$ layer into beaker. Evap. combined exts just to dryness, using gentle current of air, at temp. <50°. Dissolve residue in $CS_2$, transfer quant. to 50 mL vol. flask, and dil. to vol. with $CS_2$.

Accurately weigh ca 200 mg std phenaglycodol, transfer to 125 mL separator with 50 mL $CHCl_3$, and ext as above.

Det. baseline *A* of sample and std solns against $CS_2$ at ca 9.85 μm. Draw baseline between minima at ca 9.75 μm and ca 10.0 μm. Calc. phenaglycodol content of sample.

Prep. KBr disk by grinding 2 mg residue and 200 mg spectroscopic grade KBr in Mullite mortar and press in die with hydraulic press. Record spectrum at 2–15 μm and compare with spectrum of extd std residue to det. identity of sample.

Ref.: JAOAC **51,** 631(1968).

CAS-79-93-6 (phenaglycodol)

## 968.46 Meprobamate in Drugs

**Infrared Spectroscopic Method**

**First Action 1968**
**Final Action 1970**

### A. Apparatus

(**a**) *Spectrophotometer.*—Recording IR spectrophtr, effective over 0.75–3.5 μm range, with 1 cm matching near-IR silica cells. Peak at ca 2.91 μm for meprobamate must be resolved.

(**b**) *Chromatographic tubes.*—Glass, 20 × 300 mm.

### B. Reagents

(**a**) *Alcohol-free chloroform.*—Thruout detn use only $CHCl_3$ prepd daily as follows: Ext alcohol by passing $CHCl_3$ successively thru three 500 mL separators, each contg 50–75 mL $H_2O$. Pack 2 chromatgc tubes half-full with alumina (80–200 mesh, Fisher No. A-540, or equiv.) activated by heating 2 hr at 300°. Mount one column above other and pass $CHCl_3$ thru both columns. Pass ≤500 mL $CHCl_3$ at one time. If more $CHCl_3$ is needed, repeat purification with fresh alumina.

(**b**) *Meprobamate std soln.*—0.5 mg/mL. Accurately weigh ca 25 mg USP Meprobamate Ref. Std and transfer to 50 mL vol. flask. Dissolve in and dil. to vol. with $CHCl_3$. Absorptivity should be ca 1.0 if 2.91 μm peak is properly resolved.

### C. Preparation of Sample

Finely pulverize tablets, accurately weigh portion contg ca 50 mg meprobamate, and transfer to dry 100 mL vol. flask. Add 50 mL $CHCl_3$, shake 15–20 min, and dil. to vol. with $CHCl_3$. Filter soln thru dry Whatman No. 1 paper, or equiv. Discard first 20–25 mL and collect remainder in dry g-s erlenmeyer.

### D. Determination

Zero instrument at 2.914 μm with $CHCl_3$ in both cells. Scan sample and std solns against $CHCl_3$ between 3.000 and 2.790 μm. Measure baseline *A* values at max., ca 2.91 μm, from straight line drawn between minima at ca 2.980 and 2.875.

$$\text{mg Meprobamate/tablet} = A_{\text{sample}} \times (C/A_{\text{std}}) \times 100 \times (T/W)$$

where *A* refers to baseline values; *C* = mg meprobamate/mL std soln; *T* and *W* = av. wt/tablet and sample wt, resp., in mg.

Ref.: JAOAC **51**, 616(1968).

CAS-57-53-4 (meprobamate)

## ANTICOAGULANTS

### 973.72 Acenocoumarol, Dicumarol, Phenprocoumon, Warfarin Potassium, and Warfarin Sodium in Drugs

**Automated Spectrophotometric Method**

**First Action 1973**
**Final Action 1975**

#### A. Principle

Basic soln of drug is acidified and extd with $CHCl_3$ or $CHCl_3$-pyrimidine-propylene glycol (for dicumarol), and *A* of extd material is read in flowcell at 308 nm.

#### B. Apparatus

(**a**) *Automatic analyzer*.—AutoAnalyzer with following modules (Technicon Instruments Corp.): Sampler II with 20/hr (2:1) cam; proportioning pump I; manifold (Fig. **973.72**).

(**b**) *Filter*.—Fill 50 × 5 mm id glass tubing completely, but loosely, with glass wool.

(**c**) *Spectrophotometer*.—Double-beam spectrophtr which records *A* at fixed wavelength, with 10 mm flowcell (Thomas Scientific) (2 mm flowcell for dicumarol).

(**d**) *Ultrasonic generator*.—Model II, 150 watt (Heat Systems-Ultrasonic, Inc., 38 E Mall, Plainview, NY 11803).

#### C. Reagents

(**a**) *Chloroform*.—$H_2O$-washed and filtered thru paper. Prep. fresh daily. (Use in all except dicoumarol detn.)

(**b**) *Chloroform-pyridine-propylene glycol soln*.—Mix 50 mL pyridine with 50 mL propylene glycol and dil. to 1 L with $CHCl_3$. Use in dicumarol assay.

(**c**) *Acenocoumarol std soln*.—0.08 mg/mL. Accurately weigh ca 20 mg acenocoumarol std in 250 mL vol. flask. Dissolve in ca 100 mL 0.01*N* NaOH with aid of ultrasonic generator and dil. to vol. with 0.01*N* NaOH.

(**d**) *Dicumarol (bishydroxycoumarin) std soln*.—0.25 mg/mL. Accurately weigh ca 25 mg USP Ref. Std Bishydroxycoumarin in 100 mL vol. flask. Dissolve in ca 50 mL 0.01*N* NaOH with aid of ultrasonic generator and dil. to vol. with same solv. Prep. fresh daily.

(**e**) *Phenprocoumon std soln*.—0.12 mg/mL. Accurately weigh ca 30 mg USP Ref. Std Phenprocoumon in 250 mL vol. flask. Dissolve in ca 100 mL 0.01*N* NaOH with aid of ultrasonic generator and dil. to vol. with 0.01*N* NaOH.

(**f**) *Warfarin std soln*.—0.1 mg/mL. Accurately weigh ca 25 mg USP Ref. Std Warfarin into 250 mL vol. flask, and dissolve and dil. to vol. with 0.01*N* NaOH.

#### D. Preparation of Sample

Disintegrate individual tablet or capsule or disperse weighed composite in accurately measured vol. 0.01*N* NaOH to give drug concn (mg/mL) as follows: acenocoumarol 0.08, dicumarol 0.25, phenprocoumon 0.12, Na or K warfarin 0.10. Use ultrasonic generator ca 10 min to assure tablet disintegration. Let suspension stand 1.5 hr with occasional mixing.

#### E. Analytical System

Sample is withdrawn, segmented with air, and acidified with 0.2*N* $H_2SO_4$. Solv., (**a**) or (**b**), is added, mixed in beaded coil, and phases are sepd in BO fitting. Org. phase contg extd drug is debubbled, and *A* of soln at 308 nm is measured in 10 mm flowcell (2 mm flowcell for dicumarol).

#### F. Start-Up

Pump alcohol thru solv. line 10 min; then pump solv. thru line 5 min. Place remaining tubes in their resp. solns and let system equilibrate 20–30 min. Calibrate spectrophtr at 2 or 3

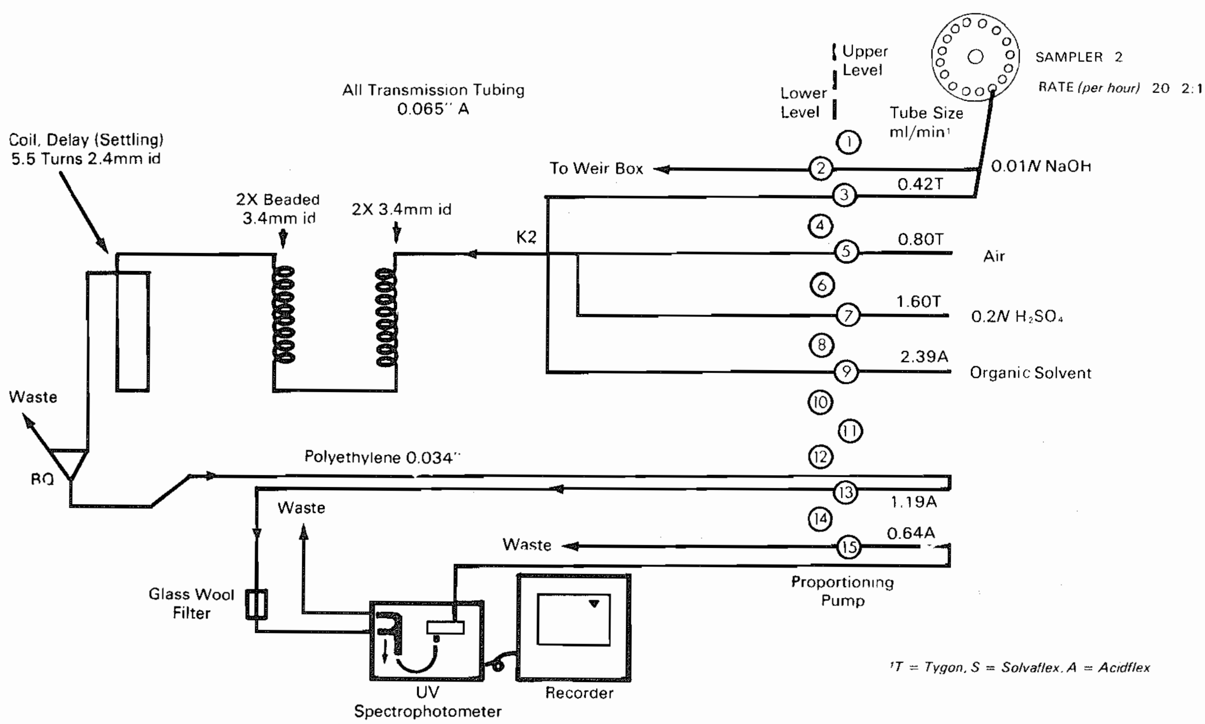



**FIG. 973.72—Flow diagram for automated analysis for acenocoumarol, dicumarol, phenprocoumon, potassium warfarin, and sodium warfarin**

absorbances. Adjust mask in ref. compartment of spectrophtr to set desired baseline.

### G. Shut-Down

Place acid, base, and sampling lines in $H_2O$, leave solv. line in its reservoir, and pump 5 min. Remove acid, base, and sampling lines from $H_2O$ and continue pumping 5 min to purge system of $H_2O$. Place solv. line in alcohol and pump 5 min. Remove line and pump system dry.

### H. Determination

Fill sample cups in following order: 4 cups std soln, 5 cups sample soln, 1 cup std soln, 5 cups sample soln, etc. Place 2 cups std soln at end of each run. (Extra cups of std solns at start and end of sampling pattern are used to overcome carry-over effect in transitions from wash soln to std soln and vice versa. Three extra cups at beginning and 1 extra cup at end should suffice, but det. exact number needed for equilibrium by experiment. System should give uniform response for at least final pair of extra std cups before sample pattern is started.) Start Sampler II. After last cup has been sampled, let system operate until steady baseline is obtained. Draw tangent to initial and final baselines. Subtract baseline to det. net $A$ and $A'$ for each sample and std peak, resp. Discard values for first 3 and last std peaks and calc. av. std $A'$.

$$\text{mg warfarin Na (or K) in portion taken} = 1.071 \text{ (or } 1.124) \times (A/A') \times C \times D$$

where 1.071 and 1.124 = ratios of MW of Na and K warfarin to warfarin, resp.; $C$ = concn of std in mg/mL, and $D$ = diln factor.

$$\text{mg Acenocoumarin, dicumarol, or phenprocoumon in portion taken} = (A/A') \times C \times D.$$

Refs.: JAOAC **56**, 692(1973); **58**, 80(1975).

CAS-152-72-7 (acenocoumarol)
CAS-66-76-2 (dicumarol)
CAS-435-97-2 (phenprocoumon)
CAS-2610-86-8 (potassium warfarin)
CAS-129-06-6 (sodium warfarin)
CAS-81-81-2 (warfarin)

## 988.24 Dicumarol, Phenprocoumon, and Warfarin Sodium in Drug Tablets

**Liquid Chromatographic Method**

**First Action 1988**

### A. Principle

Coumarin anticoagulants dicumarol, phenprocoumon, and warfarin Na are identified and quant. detd in tablets by reverse phase LC on $C_{18}$ column with tetrahydrofuran–MeOH–$H_2O$–HOAc mobile phase, and photometric detection at 311 nm.

### B. Apparatus

(**a**) *Liquid chromatograph.*—Equipped with DuPont Model 8800 solv. pump, variable wavelength detector, and strip chart recorder (E.I. DuPont de Nemours & Co.), or equiv.; and Rheodyne Model 7125 injection valve with 20 μL sample loop (Rheodyne Inc.), or equiv. Operating conditions: column temp. ambient; solv. flow rate 1.5 mL/min; detector wavelength 311 nm; detector attenuation 16 AUFS; recorder range 1 mV; chart speed 0.5 cm/min.

(**b**) *Chromatographic column.*—Stainless steel, 30 cm × 3.9 mm id, packed with 10 μm μBondapak $C_{18}$ (Waters Associates, Inc.), or equiv.

### C. Reagents

(**a**) *Solvents.*—LC grade MeOH and reagent grade glacial HOAc (Fisher Scientific Co.); tetrahydrofuran (Mallinckrodt, Inc.); and $H_2O$ double distd in glass.

(**b**) *Mobile phase.*—Tetrahydrofuran–MeOH–$H_2O$–HOAc (35 + 10 + 65 + 0.1 v/v/v/v). Filter thru 0.45 μm membrane and degas under vac.

(**c**) *Dicumarol std soln.*—0.05 mg/mL. Accurately weigh ca 25 mg USP Ref. Std Dicumarol into 100 mL vol. flask, dissolve in and dil. to vol. with 0.01N NaOH, and mix. Pipet 5 mL of this soln into 25 mL vol. flask, dil. to vol. with mobile phase, and mix.

(**d**) *Warfarin Na std soln.*—0.2 mg/mL. Accurately weigh ca 10 mg USP Ref. Std Warfarin Na into 50 mL vol. flask, and dissolve in mobile phase. Dil. to vol. with mobile phase, and mix.

(**e**) *Phenprocoumon std soln.*—0.12 mg/mL. Accurately weigh ca 3 mg USP Ref. Std Phenprocoumon into 25 mL vol. flask, and dissolve in mobile phase. Dil. to vol. with mobile phase, and mix.

### D. Preparation of Sample

(**a**) *Tablet composites.*—Weigh and finely powder ≥20 tablets. Transfer accurately weighed amt of powder to suitable vol. flask and quant. dissolve in mobile phase with aid of ultrasonic bath. Dil. to vol. with mobile phase to prep. soln contg ca 0.12 mg/mL of phenprocoumon or 0.2 mg/mL of warfarin Na. For dicumarol samples, first dissolve powder in 0.01N NaOH with aid of ultrasonic bath to obtain soln contg 0.25 mg/mL; then quant. dil. 5.0 mL aliquot of soln with mobile phase to final dicumarol concn of ca 0.05 mg/mL. Filter all sample prepns prior to injection into LC system.

(**b**) *Single tablets.*—Place 1 powdered tablet in suitable vol. flask, and proceed as described for tablet composites.

### E. Determination

Equilibrate system with mobile phase at 1.5 mL/min until baseline is steady. Use sampling valve to inject measured vol. of std soln into LC system. Adjust injection vol. and operating parameters so std soln gives peak ht ca 60% full scale. Under these conditions, 3 replicate injections of a std soln should give RSD ≤3% and tailing factor ≤2.0. Make alternate injections of equal vols of std and sample solns. Measure peak responses in sample and std solns.

### F. Calculations

Calc. amt coumarin anticoagulant in sample as follows:
Tablet composite sample:

$$\text{mg/tablet} = (H/H') \times (W'/D') \times (D/W) \times A$$

Single tablet sample:

$$\text{mg/tablet} = (H/H') \times (W'/D') \times D$$

where $H$ and $H'$ = peak responses of sample and std solns, resp.; $W$ and $W'$ = mg sample and std taken, resp.; $D$ and $D'$ = diln factors for sample and std solns, resp.; and $A$ = av. tablet wt, mg. To calc. amt warfarin Na in either tablet composites or individual tablets, use 1.071 as multiplier in above equations (1.071 = ratio of MW of warfarin Na/MW of warfarin).

Ref.: JAOAC **70**, 834(1987).

CAS-66-76-2 (dicumarol)
CAS-435-97-2 (phenprocoumon)
CAS-81-81-2 (warfarin)
CAS-129-06-6 (warfarin Na)

## 971.38 Menadione Sodium Bisulfite in Drugs

**Spectrophotometric Method**

**First Action 1971**
**Final Action 1973**

(Applicable to injections)

### A. Principle

Aq. menadione.$NaHSO_3$ soln is mixed with diat. earth and placed in chromatgc column over lower layer of HCl-diat. earth. Excipients are eluted with $CHCl_3$ and menadione with $NH_3$-$CHCl_3$. Excess $NH_3$ which could decompose menadione is neutzd by acidic lower layer. Menadione is detd by UV spectrophotometry.

### B. Reagents

(**a**) *Chloroform*.—Use $H_2O$-washed $CHCl_3$ thruout.
(**b**) *Ammoniacal chloroform*.—Mix 1 part $NH_4OH$ with 25 parts $CHCl_3$ as needed.
(**c**) *Menadione std soln*.—50 μg/mL. Dissolve 50 mg USP Menadione Ref. Std in $CHCl_3$ and dil. to 100 mL with $CHCl_3$. Dil. 10 mL aliquot to 100 mL with $CHCl_3$.

### C. Determination

Mix 1 mL HCl (1 + 3) with 1.5 g diat. earth, **960.53B**, and pack into chromatgc tube, **967.31A**. Dil. aq. sample soln to contain ca 5 mg menadione.$NaHSO_3.3H_2O$/mL, mix 2.0 mL this soln with 3 g diat. earth in beaker, and pack into tube. Dry-wash beaker with 1 g diat. earth, add to tube, tamp until compressed, and overlay with piece of glass wool used to wipe beaker.

Wash column with 100 mL $CHCl_3$ and discard $CHCl_3$. Wash tip of column with few mL $CHCl_3$. With 100 mL vol. flask as receiver, add 5 mL $NH_4OH$-$CHCl_3$, let sink into diat. earth, and elute menadione with 90 mL $CHCl_3$. Rinse tip of column with $CHCl_3$ and dil. to vol. with $CHCl_3$. Scan soln and std soln from 280 to 400 nm against $CHCl_3$ and det. $A$ at max., ca 334 nm.

$$\text{mg Menadione.NaHSO}_3.3\text{H}_2\text{O/mL dild assay soln} = 0.05 \times 1.918 \times C \times (A/A')$$

where 1.918 is factor to convert menadione to menadione.$NaHSO_3.3H_2O$; $C$ = μg menadione/mL std soln; and $A$ and $A'$ refer to sample and std solns, resp.

Ref.: JAOAC **54,** 593(1971).

CAS-130-37-0 (menadione sodium bisulfite)

## 986.37 Chlorpropamide in Drug Tablets

**Liquid Chromatographic Method**

**First Action 1986**

### A. Principle

Chlorpropamide is dissolved in mobile phase and detd by liq. chromatgy with UV detection at 240 nm.

### B. Apparatus

(**a**) *Liquid chromatograph*.—Equipped with sampling valve capable of introducing 20 μL injections, UV detector capable of operating at 240 nm, and recorder/integrator.
(**b**) *Column*.—Zorbax ODS, 5–6 μm diam. spherical particles, 4.6 mm × 25 cm (E.I. Dupont, or equiv.).
(**c**) *Filters*.—Millipore type HVLP, 0.45 μm porosity (Millipore Corp.), or equiv.

### C. Reagents

(**a**) *Mobile phase*.—52/48 ratio of aq./org. phases: (*1*) *Aqueous*.—Acetic acid–$H_2O$ (1 + 99). (*2*) *Organic*.—LC grade $CH_3CN$.
(**b**) *Chlorpropamide std soln*.—Transfer ca 50 mg, accurately weighed, USP Chlorpropamide Ref. Std to 100 mL vol. flask and dissolve in mobile phase. Dil. quant. to final concn of ca 0.05 mg/mL in mobile phase.
(**c**) *Resolution soln*.—Chlorpropamide + *p*-chlorobenzenesulfonamide (PCBS) (ca 0.05 mg/mL of each) in mobile phase.

### D. Preparation of Sample

Transfer accurately weighed portion of finely ground tablets equiv. to 45–55 mg chlorpropamide to 100 mL vol. flask. Add ca 70–80 mL mobile phase and shake thoroly 6–8 min (or sonicate 3–4 min) and dil. to vol. with mobile phase. Dil. quant. to final concn ca 0.05 mg/mL in mobile phase. Filter portion thru 0.45 μm filter for LC analysis.

### E. System Suitability

Set mobile phase at flow rate ca 1.5 mL/min. Retention time for chlorpropamide should not be <4.0 min. Adjust flow rate and/or solv. ratio (do not exceed 50% $CH_3CN$) for desired retention time. Column should conform to following performance parameters: theoretical plates ($n$) not <1500; tailing factor ($T$) not >1.5; resolution ($R$) between chlorpropamide and PCBS not <2.0. Relative std deviation for 4 consecutive std injections should be <2.0%.

### F. Determination

Make 20 μL injections of std and samples. Det. peak responses (area or ht) obtained and calc. amt of chlorpropamide:

$$\text{Chlorpropamide, mg/tab.} = (r/r') \times (C/W) \times \text{DF} \times \text{ATW}$$

where $r$ and $r'$ = responses for sample and std, resp.; $C$ = concn of chlorpropamide std soln, mg/mL; $W$ = sample wt, g; DF = diln factor for sample, mL; ATW = av. tablet wt, g/tab.

Ref.: JAOAC **69,** 519(1986).

CAS-94-20-2 (chlorpropamide)

# SULFONAMIDES

## 964.26★ Sulfonamide Mixtures in Drugs

**Paper Chromatographic Method**

**First Action 1964**
**Surplus 1980**

*See* **37.152–37.156**, 13th ed.

## 973.73 Trisulfapyrimidines in Drugs

**Spectrophotometric Method**

**First Action 1973**
**Final Action 1974**

***Total Trisulfapyrimidines***

### A. Principle

Total trisulfapyrimidines in sample are detd by coupling with *N*-1-naphthyl ethylenediamine.2HCl (NED), recording spectra of samples and stds between 660 and 480 nm. Individual sulfonamides are sepd by TLC and their ratios detd spectrophtric after coupling with NED.

### B. Reagents

(**a**) *Ammonia-methanol soln.*—Dil. 5 mL $NH_4OH$ to 100 mL with MeOH.

(**b**) *Sulfamerazine std soln.*—Approx. 6 μg/mL acid soln. Accurately weigh calcd amt USP Ref. Std Sulfamerazine, previously dried, and dissolve in $NH_4OH$-MeOH soln; dil. quant. and stepwise with MeOH to obtain soln contg ca 120 μg/mL. Transfer 5.0 mL to 100 mL vol. flask and dil. to vol. with 0.12*N* HCl (10 mL HCl dild to 1 L). Acidic soln is stable ≥1 month.

(**c**) *Dilute ammonia soln.*—Dil. 400 mL $NH_4OH$ to 1 L with $H_2O$.

(**d**) *N-1-Naphthyl ethylenediamine dihydrochloride (NED) soln.*—0.1%. Prep. fresh before use.

### C. Preparation of Sample

(**a**) *Tablets.*—Accurately weigh finely powd portion contg ca 180 mg total sulfonamides and transfer to 50 mL vol. flask, using 10 mL dil. $NH_4OH$, (**c**). Let stand ca 15 min, mixing occasionally, dil. to vol. with MeOH, and centrf. portion to clarify (Soln I). Dil. 5.0 mL clarified soln to 250 mL with $H_2O$; dil. 4.0 mL of this soln to 50 mL with $H_2O$ (Soln II).

(**b**) *Suspensions.*—Shake in original container to ensure homogeneity, let stand long enough for entrapped air to rise, and invert carefully just before removing portion for weighing. Det. sp gr by weighing 100 mL in tared 100 mL vol. flask. Thoroly mix and weigh portion contg 180 mg total sulfonamides and proceed as in (**a**).

### D. Determination

Pipet 5.0 mL aliquots sulfamerazine std soln and prepd Soln II into sep. 10 mL vol. flasks. Add 1.0 mL HCl (1 + 1) to each flask, mix, and cool. (Solns must be at room temp. for quant. results.) Add 1.0 mL *0.1% $NaNO_2$*, mix well, and let stand 2 min. Add 1.0 mL 0.5% *$NH_4$ sulfamate*, and mix. After 2 min, add 1.0 mL NED soln, (**d**). Mix and adjust to vol. with $H_2O$. Record spectra of samples and stds against $H_2O$ between 660 and 480 nm (peak ca 545 nm) within 15–60 min. Correct $A$ by subtracting $A$ at 660 nm from peak $A$ at ca 545 nm.

$$\text{mg Total sulfapyrimidines in sample} = (A/A') \times 31.25C$$

where $A$ and $A'$ = corrected $A$ of dild assay soln and sulfamerazine std soln, resp., and $C$ = μg sulfamerazine/mL std soln.

#### *Ratio of Sulfadiazine:Sulfamerazine:Sulfamethazine*

### E. Reagents and Apparatus

(**a**) *Chromatographic identification standards.*—Prep. sep. solns of USP Ref. Std Sulfadiazine, Sulfamerazine, and Sulfamethazine in $NH_4OH$-MeOH soln, **973.73B(a)**, to contain ca 1 mg/mL each.

(**b**) *Developing solvent.*—$CHCl_3$-MeOH-$NH_4OH$ (30 + 12 + 1).

(**c**) *Thin layer plate.*—20 × 20 cm, coated 0.25 mm thick with silica gel GF (Brinkmann Instruments, Inc.). Divide into 2 approx. equal parts by scraping thin vertical line thru coating.

### F. Thin Layer Chromatography

Line suitable chromatgc tank with blotting paper. Wet bottom of tank and paper with developing solv., seal tank, and let equilibrate 30 min. Apply ca 50 μL centrfd Soln I to starting line of thin layer plate in streak ca 8 cm long (not to extend within 1 cm of plate edge or center line), using N stream. (It is not necessary to spot accurately measured vol.) On other half of plate, spot sep. 10 μL chromatgc identification stds, evenly spaced, under N stream. Develop plate in tank equilibrated 0.5 hr, letting solv. migrate 10–15 cm above starting line. Air dry plate, locate bands under shortwave UV light, and circle with stylus. Remove silica gel from each band by scraping onto glazed weighing paper, and transfer to sep. 50 mL g-s centrf. tubes. Add 10.0 mL 0.1*N* NaOH to each tube, shake 3 min, and centrf. Transfer 5.0 mL aliquots of each supernate to 10 mL vol. flasks. Add 1.0 mL HCl (1 + 1) to each flask, mix, and cool. (Soln must be at room temp. for quant. results.) Develop color and record spectra as in **973.73D**.

### G. Calculations

Calc. fraction of each sulfapyrimidine in total sulfapyrimidines as follows:

$$\text{Sulfadiazine fraction} = 0.947A_d/T$$

$$\text{Sulfamerazine fraction} = A_r/T$$

$$\text{Sulfamethazine fraction} = 1.053A_m/T$$

where $A_d$, $A_r$, and $A_m$ = corrected $A$ of the sulfadiazine, sulfamerazine, and sulfamethazine bands, resp., and $T = 0.947A_d + A_r + 1.053A_m$.

Ref.: JAOAC **56,** 689(1973).

CAS-68-35-9 (sulfadiazine)
CAS-127-79-7 (sulfamerazine)
CAS-57-68-1 (sulfamethazine)

## 954.15 Sulfadiazine and Sulfamerazine in Drugs

**Spectrophotometric Method**

**Final Action 1962**

### A. Reagents

(**a**) *Citrate buffer soln.*—Dissolve 37 g $Na_3C_6H_5O_7 .2H_2O$ in $H_2O$, add 32 mL HCl, and dil. to 250 mL with $H_2O$.

(**b**) *2-Thiobarbituric acid (TBA) soln.*—Recrystallize acid twice from $H_2O$. Dissolve 5 g recrystd acid in 20 mL 1*N* NaOH dild with 500 mL $H_2O$. Add 250 mL citrate buffer soln and adjust to pH 2.0. Reagent is stable when stored in g-s bottle in refrigerator.

### B. Determination

(**a**) *Sulfadiazine.*—To powd sample contg ca 0.1 g mixed sulfonamides, add 50 mL 1*N* HCl. Shake intermittently 10 min, filter if necessary, and dil. filtrate and washings to 100 mL with $H_2O$. To 5 mL aliquot, add 7.5 mL 1*N* HCl and dil. to 100 mL. Designate this soln (contg ca 5 mg mixed sulfonamides/100 mL 0.1*N* HCl) as *Soln X*. To 1.0 mL aliquot *Soln X* in g-s test tube, add 10.0 mL TBA soln, stopper, and heat 1 hr at 100°. Weigh tube before and after heating, and compensate for any loss by addn of $H_2O$. Similarly treat 1.0 mL std contg 25 μg sulfadiazine in 0.1*N* HCl and blank contg 0.1*N* HCl. Det. $A$ of sample and of std, $A'$, at 532 nm against blank.

$$\text{mg Sulfadiazine in sample taken} = 50\,A/A'$$

(**b**) *Sulfamerazine.*—Det. $A_T$ of *Soln X*, and $A'_D$ and $A'_M$ of solns contg 5.0 mg pure sulfadiazine and sulfamerazine, resp., in 100 mL 0.1*N* HCl, at 305 nm against 0.1*N* HCl blank. Then $A$ of *Soln X* due to sulfadiazine ($A_D$) = $A'_D$ × (mg sulfadiazine in *Soln X*/5.0), and $A$ due to sulfamerazine ($A_M$) = $A_T - A_D$.

$$\text{mg Sulfamerazine in sample taken} = 100\,A_M/A'_M$$

Ref.: JAOAC **37,** 697(1954).

CAS-68-35-9 (sulfadiazine)
CAS-127-79-7 (sulfamerazine)

## 964.27 Sulfadiazine in Presence of Other Sulfonamide Drugs

**Final Action 1965**

Det. sulfadiazine as in **954.15B(a)** from soln prepd to contain ca 25 μg sulfadiazine/mL 0.1*N* HCl.

Refs.: JAOAC **47,** 194, 474(1964).

CAS-68-35-9 (sulfadiazine)

## 939.17★ Sulfanilamide in Drugs

**Hydrolysis Method**

**Final Action**
**Surplus 1970**

*See* **36.483**, 11th ed.

## 985.48 Sulfamethoxazole in Drug Tablets

**Liquid Chromatographic Method**

**First Action 1985**
**Final Action 1987**

### A. Principle

Sulfamethoxazole is extd with MeOH, sulfamerazine is added as internal std, and compds are detd by liq. chromatgy on normal phase silica column with isooctane–$CH_2Cl_2$–2-propanol–$CH_3CN$–HOAc (70 + 25 + 5 + 5 + 0.5) mobile phase and UV detector set at 254 nm.

### B. Apparatus and Reagents

(**a**) *Liquid chromatograph*.—Isocratic system operated at room temp., with UV detector set at 254 nm, and strip chart recorder or computing integrator.

(**b**) *Analytical column*.—Stainless steel, 300 × 3.9 (id) mm, packed with 5–10 μm particle size, uncoated silica (μPorasil, Waters Associates, or equiv.). At mobile phase flow rate of 2.0 mL/min, approx. retention times of sulfamethoxazole and sulfamerazine are 3 and 5 min, resp.

(**c**) *Mobile phase*.—Isooctane–$CH_2Cl_2$–2-propanol–$CH_3CN$–HOAc (70 + 25 + 5 + 5 + 0.5).

(**d**) *Internal std soln*.—Dissolve USP Sulfamerazine Ref. Std in MeOH in vol. flask to give soln contg ca 2.0 mg/mL.

(**e**) *Sulfamethoxazole std soln*.—Dissolve accurately weighed amt of USP Sulfamethoxazole Ref. Std in MeOH in vol. flask to obtain soln contg ca 5.0 mg/mL.

### C. Preparation of Sample Solution

Weigh and finely powder ≥20 tablets. Accurately weigh portion of powder equiv. to ca 500 mg sulfamethoxazole and transfer to 100 mL vol. flask. Dissolve in 70 mL ca 60° MeOH. Cool soln to room temp., dil. to vol. with MeOH, mix, and filter.

### D. Determination

Transfer 2.0 mL each of sample soln and std soln to individual 100 mL vol. flasks contg 5.0 mL internal std soln, add 20 mL $CH_2Cl_2$, dil. to vol. with mobile phase, and mix.

Using suitable micro syringe or sampling valve, test system suitability by making 5 replicate injections (between 10 and 20 μL) of std mixt. If necessary, adjust injection vol. and flow rate to give std peak ht of ca 60% AUFS. In properly functioning system, resolution factor between std and internal std peaks is ≥2.5, and CV for 5 replicate injections is ≤3.0% for peak ht ratios of std to internal std.

Introduce equal vols (between 10 and 20 μL) of sample soln and std soln into liq. chromatograph operated at room temp. For each injection, calc. response ratio (ht of sulfamethoxazole peak to ht of internal std peak).

$$\text{Sulfamethoxazole, mg/tab.} = 100\ C \times (R/R') \times (T/W)$$

where $C$ = concn, mg/mL, of USP Sulfamethoxazole Ref. Std in std soln; $R$ and $R'$ = ratios for sample and std solns, resp.; $T$ = av. tablet wt, g; and $W$ = sample wt, g.

Ref.: JAOAC **68,** 88(1985).

CAS-723-46-6 (sulfamethoxazole)

## 983.29 Sulfisoxazole in Drug Tablets, Solutions, and Ointments

**Liquid Chromatographic Method**

**First Action 1983**
**Final Action 1986**

### A. Principle

Sulfisoxazole content of tablets, solns, and ointments is detd by reverse phase LC using ternary aq. mobile phase, UV detection at 254 nm, and sulfadimethoxine as internal std.

### B. Apparatus

(**a**) *Liquid chromatograph*.—DuPont Model 841 solv. pump equipped with 254 nm detector (E. I. duPont de Nemours and Co.), 10 μL injection valve (Valco Instruments Co., Inc., PO Box 55603, Houston, TX 77255), and Model 3380A integrator (Hewlett-Packard). Equiv. LC system and strip chart recorder may be used.

(**b**) *Chromatographic column*.—Stainless steel, 30 cm × 3.9 mm id, packed with 10 μm μBondapak $C_{18}$ (Waters Associates, Inc.) or equiv. meeting appropriate HPLC system suitability requirements.

### C. Reagents

(**a**) *Solvents*.—UV grade MeOH and *n*-heptane (Fisher Scientific Co., or equiv.), and acetonitrile (Burdick & Jackson Laboratories, Inc., or equiv.).

(**b**) *Mobile phase*.—Acetonitrile–acetic acid–$H_2O$ (30 + 1 + 69); flow rate 2.0 mL/min. Retention times: sulfisoxazole, ca 4 min; internal std, ca 5.5 min. Vary ratio of acetonitrile to $H_2O$ to meet PLC system suitability requirements. Increased acetonitrile decreases retention time.

(**c**) *Internal std soln*.—Dissolve 80 mg USP Ref. Std Sulfadimethoxine in MeOH and dil. to 100 mL with MeOH.

(**d**) *Sulfisoxazole std soln* (*5.0 mg/100 mL*).—Transfer 100 mg accurately weighed USP Ref. Std Sulfisoxazole (previously dried 2 h at 105°) to 100 mL vol. flask and dil. to vol. with MeOH. Transfer 5.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix.

### D. Sample Preparation

(**a**) *Tablets*.—Det. av. wt and grind to pass No. 60 sieve. Transfer accurately weighed portion of powd. equiv. to 500 mg sulfisoxazole to 100 mL vol. flask, add 25 mL MeOH, stopper, mix on mech. shaker 30 min, dil. to vol. with MeOH, mix, and filter. Transfer 20.0 mL aliquot of filtrate to 100 mL vol. flask and dil. to vol. with MeOH. Transfer 5.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix.

(**b**) *Liquids* (*injections and ophthalmic solns*).—Accurately transfer vol. of dosage form contg ca 200 mg sulfisoxazole to 200 mL vol. flask and dil. to vol. with MeOH. Transfer 5.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix.

(**c**) *Ointments.*—Transfer accurately weighed amt of sample ($S$) contg ca 50 mg sulfisoxazole to 125 mL separator contg 50 mL $n$-heptane, shake to disperse ointment, and ext with three 25 mL portions of $MeOH-H_2O$ (2 + 1), passing each ext consecutively thru second 125 mL separator contg 50 mL $n$-heptane. Collect exts in 100 mL vol. flask and dil. to vol. with MeOH. Transfer 10.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln, dil. to vol. with mobile phase, and mix.

### E. Determination

Equilibrate column with mobile phase at flow rate of 2 mL/min. Make 3 replicate injections of sulfisoxazole std soln. Using either peak area or peak ht measurements, for each injection det. response ratio of sulfisoxazole to internal std. In suitable system, capacity factor, $k'$, for sulfisoxazole should be 1.0–3.0; resolution factor, $R$, for sulfisoxazole peak and internal std peak should be $\geq 1.5$; and coefficient of variation of response ratio for 3 replicate injections each of sample and sulfisoxazole std working solns should be $\leq 2.0\%$. When chromatgc requirements are met, make alternate injections of std and sample solns, and det. response ratio for each. Retention times for sulfisoxazole and internal std must be same for sample and std soln injections.

### F. Calculations

Calc. content of sulfisoxazole in dosage form as follows:

$$\text{Tablets: mg/tablet} = (RR/RR') \times C \times (T/S) \times 100$$

$$\text{Solns: mg/mL} = (RR/RR') \times (C/V) \times 40$$

$$\text{Ointments: mg/g} = (RR/RR') \times (C/S) \times 10$$

where $RR$ and $RR'$ = response ratio of sample and std solns, resp.; $C$ = amt sulfisoxazole in 100 mL final std soln, mg; $T$ = av. tablet wt, g; $S$ = sample wt, g; and $V$ = vol. soln taken, mL.

Ref.: JAOAC **66,** 1182(1983).

CAS-127-69-5 (sulfisoxazole)

## THIAZIDES

## 976.33 Bendroflumethiazide in Drugs

### Spectrophotometric Method

**First Action 1976**
**Final Action 1977**

### A. Principle

Bendroflumethiazide is eluted from 0.1$M$ $Na_2CO_3$-diat. earth column with $HOAc-CHCl_3$ and measured directly by UV spectrophotometry.

### B. Apparatus

(**a**) *Chromatographic tube and tamping rod.—See* **967.31A**.
(**b**) *Diatomaceous earth.—See* **960.53B**.

### C. Reagents

(**a**) *Sodium carbonate soln.*—0.1$M$. Dissolve 10.6 g $Na_2CO_3$ in $H_2O$, dil. to 1 L with $H_2O$, and mix.

(**b**) *Bendroflumethiazide std solns.*—(*1*) *Stock soln.*—0.4 mg/mL. Accurately weigh ca 20 mg USP Bendroflumethiazide Ref. Std into small beaker, add 5 mL DMSO, and mix with glass rod until dissolved. Transfer quant. to 50 mL vol. flask with MeOH and dil. to vol. with MeOH. (*2*) *Working soln.*—0.012 mg/mL. Transfer 3.0 mL stock soln to 100 mL vol. flask contg 2 mL MeOH and 2 drops HCl. Dil. to vol. with $CHCl_3$.

### D. Preparation of Sample

Finely powder tablets to pass No. 60 sieve. Transfer portion contg 15–20 mg bendroflumethiazide to 50 mL vol. flask. Add 5 mL DMSO, wetting entire sample. (*Caution:* DMSO can be harmful. Avoid skin contact by wearing heavy rubber gloves. Use effective fume removal device.) Let stand 10 min with frequent mixing. Dil. to vol. with 0.1$M$ $Na_2CO_3$. Filter portion thru paper, discarding first few mL.

### E. Preparation of Columns

(**a**) *Lower layer.*—Mix 2 g diat. earth with 1 mL 0.1$M$ $Na_2CO_3$ in 150 mL beaker, transfer to tube, and tamp to uniform mass.

(**b**) *Upper layer.*—Mix 4 g diat. earth with 3.0 mL sample soln, transfer to tube, and tamp. Dry-wash flask contg sample mixt. with 1 g diat. earth and 2–3 drops $H_2O$; transfer to column and tamp. Top with glass wool pad.

### F. Determination

(Use $H_2O$-washed solvs thruout. *Caution:* See safety notes on flammable solvents and isooctane.)

Let 75 mL isooctane elute thru column and discard eluate. Use 100 mL vol. flask contg 2 drops HCl in 5 mL MeOH as receiver, and elute column with 90 mL *$CHCl_3$-HOAc (98 + 2)*. Dil. to vol. with $CHCl_3$.

Det. $A$ of sample and working std solns at max., ca 271 nm ($a$ = 48.8), in 1 cm cells with spectrophtr against $CHCl_3$-HOAc (98 + 2) as ref.

### G. Identification

Macerate portion finely powd tablets contg 4–5 mg bendroflumethiazide with $H_2O$. Transfer to 125 mL separator, using small portions $H_2O$, and dil. to ca 15 mL. Ext with three 20 mL portions $CHCl_3$, collecting $CHCl_3$ in 100 mL beaker. Evap. to dryness on steam bath with aid of air current. Dissolve residue in small vol. MeOH and evap. to dryness. Compare IR spectrum in KBr matrix of residue with that of ref. std previously recrystd from alcohol.

Ref.: JAOAC **59,** 90(1976).

CAS-73-48-3 (bendroflumethiazide)

## 985.46 Methyldopa, Methyldopa-Hydrochlorothiazide, or Methyldopa-Chlorothiazide in Drug Tablets

### Liquid Chromatographic Method

**First Action 1985**
**Final Action 1987**

### A. Principle

Methyldopa, chlorothiazide, and hydrochlorothiazide are detd by comparison with stds, using liq. chromatgy with UV detection and theobromine internal std.

### B. Apparatus

(**a**) *Liquid chromatograph.*—Isothermal, isocratic pump system, photometric detector capable of monitoring $A$ at 280 nm, suitable recorder, and 20 μL injection loop.

(**b**) *Chromatographic column.*—300 × 3.9 mm id, contg μBondapak $C_{18}$, 10 μm particle size (Waters Associates).

(c) *Filter system.*—0.45 μm vac. filter app. (Millipore Corp.).

### C. Reagents

(a) *Mobile phase.*—Mix 96 mL 3% HOAc with 4 mL MeOH, both either LC or reagent grade. Filter thru filter app., (c), before use.

(b) *Stock std soln.*—Transfer ca 50 mg anhyd. USP Methyldopa Ref. Std, accurately weighed, to 100 mL vol. flask. Add accurately weighed amt of USP Hydrochlorothiazide Ref. Std or USP Chlorothiazide Ref. Std, in same ratio with USP Methyldopa Ref. Std as that in sample tablets. Add 70 mL MeOH-$H_2O$ (1 + 1) and mix by sonication with occasional swirling to dissolve. Let flask and contents cool to ambient temp. Dil. to vol. with MeOH-$H_2O$ (1 + 1). Mix thoroly and filter thru 0.45 μm membrane filter, (c), discarding first 5 mL filtrate.

(c) *Internal std soln.*—Weigh ca 22 mg theobromine into 100 mL vol. flask. Add ca 80 mL mobile phase. Warm gently on steam bath with occasional swirling to dissolve. Cool, dil. to vol. with mobile phase, and mix.

(d) *Methyldopa or methyldopa-chlorothiazide std soln.*—Pipet 4.0 mL stock std soln and 5.0 mL internal std soln into 25 mL vol. flask. Dil. to vol. with mobile phase, and mix.

(e) *Methyldopa-hydrochlorothiazide std soln.*—Pipet 10.0 mL stock std soln and 3.0 mL internal std soln into 25 mL vol. flask. Dil. to vol. with mobile phase, and mix. Do not use methyldopa-hydrochlorothiazide std soln >2 days old.

### D. Preparation of Samples

(a) *Methyldopa and methyldopa-chlorothiazide tablets.*—Weigh and finely powder ≥20 tablets. Weigh portion of powder equiv. to 125 mg methyldopa and transfer to 250 mL vol. flask. Add 170 mL MeOH-$H_2O$ (1 + 1) and mix by sonication with occasional swirling for at least the length of time needed to dissolve corresponding stds. Dil. to vol. with MeOH-$H_2O$ (1 + 1) and mix thoroly. Filter thru 0.45 μm membrane filter, (c), discarding first 5 mL filtrate. Transfer 4.0 mL filtrate to 25 mL vol. flask. Pipet 5.0 mL internal std soln into flask and mix. Dil. to vol. with mobile phase and mix.

(b) *Methyldopa-hydrochlorothiazide tablets.*—Follow procedure in (a), except transfer 10.0 mL sample filtrate and 3.0 mL internal std soln to 25 mL vol. flask.

(c) *Methyldopa drug substance.*—Accurately weigh portion of sample equiv. to ca 125 mg methyldopa, and transfer to 250 mL vol. flask. Follow procedure in (a), starting with "Add 170 mL MeOH-$H_2O$ (1 + 1). . . ."

### E. Suitability Test and Determination

Equilibrate LC column with mobile phase at 1.5 mL/min. Inject 20 μL std soln. Methyldopa, chlorothiazide, and hydrochlorothiazide peaks elute at ca 4, 6, and 7 min, resp. Retention time of theobromine internal std should be ≥9 min. Resolution factor between methyldopa and chlorothiazide and between hydrochlorothiazide and theobromine should be >3.5. Calc. resolution factor, $R$, as follows:

$$R = [2(t_2 - t_1)]/(W_2 + W_1)$$

where $t_2$ and $t_1$ = retention times of the 2 components, and $W_2$ and $W_1$ = corresponding widths of bases of peaks, obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Change flow rate or percentage of MeOH in mobile phase slightly if necessary. Adjust detector sensitivity so that peaks are ca 35–99% AUFS. Change detector sensitivity between methyldopa and hydrochlorothiazide peaks in combination samples. If necessary, adjust vol. of internal std soln added to sample soln and std soln to obtain satisfactory peak response for internal std. Make replicate injections of each std soln and compare peak responses between injections to det. reproducibility of system. In suitable system, relative std deviation, $S_r$, for 5 replicate injections is ≤2.0%. Calc. $S_r$ as follows:

$$S_r, \% = \frac{100}{\bar{x}} \left[ \frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n = 1} \right]^{1/2}$$

where $x_i$ = an individual determination of ratio of peak response of substance to peak response of internal std, and $\bar{x}$ = mean of set of $n$ measurements.

Proceed with sample analysis by injecting 20 μL each of sample soln and corresponding std soln.

### F. Calculations

Using peak response ratios ($R$ and $R'$) relative to internal std, calc. mg drug per tablet from:

$$\text{mg/tablet} = (R/R') \times C \times (D/W) \times T$$

where $R$ and $R'$ = peak response ratios for sample soln and std soln relative to internal std, resp.; $C$ = concn of std soln, mg/mL; $W$ = wt of sample taken, mg; $D$ = sample diln; and $T$ = av. tablet wt, mg.

Ref.: JAOAC **67,** 1118(1984).

CAS-58-94-6 (chlorothiazide)
CAS-58-93-5 (hydrochlorothiazide)
CAS-555-30-6 (methyldopa)

## 971.39 Methyldopa and Chlorothiazide Combination in Drugs

**Ion Exchange Method**

**First Action 1971**
**Final Action 1974**

### A. Reagents and Apparatus

(a) *Ion exchange resin.*—AG 50W-X4, 100–200 mesh, $H^+$ form (Bio-Rad Laboratories). Strongly acidic nuclear sulfonic groups on polystyrene lattice.

(b) *Acidic methanol.*—Add 1 drop HCl to 500 mL anhyd. MeOH. (Dissoln of thiazides is retarded by >2 drops.)

(c) *Methanolic hydrochloric acid.*—1*N*. Dil. 42 mL HCl to 500 mL with anhyd. MeOH.

(d) *Ion exchange tube.*—150 × 12 mm id, with replaceable coarse fritted glass disk, Teflon stopcock, and Buna-N "O" ring seal (Kontes Glass Co., No. K-422280, or equiv.).

(e) *Methyldopa std soln.*—30 μg/mL. Accurately weigh ca 3 mg std, previously dried at 105° overnight, and dissolve in 100.0 mL 1*N* methanolic HCl.

(f) *Chlorothiazide std soln.*—10 μg/mL. Accurately weigh ca 1 mg std, previously dried 1 hr at 105°, and dissolve, with heat if necessary, in 100.0 mL acidic MeOH.

### B. Preparation of Column

Prep. slurry of 2 g resin with 20–25 mL anhyd. MeOH and transfer to tube with stopcock closed and contg plug of glass wool under fritted disk. Let resin settle by gravity; then top with small pledget of glass wool. Column need not be tamped. Drain solv., wash column with several portions anhyd. MeOH, and discard all washings. Prevent column from drying before use by maintaining head of 2–3 mL MeOH or $H_2O$.

When resin is being used for first time and on completion of sepns, wash thoroly with 15–20 mL HCl-MeOH (1 + 1) to recondition resin. With stopcock closed and glass wool removed, stir resin to obtain slurry, let settle, and drain. Repeat twice. Finally, wash resin first with $H_2O$ until excess acid is removed and then with several portions anhyd. MeOH. Store under $H_2O$.

### C. Preparation of Sample

Det. av. wt/tablet and pulverize to pass No. 80 sieve. Accurately weigh amt contg ca 25 mg chlorothiazide into 50 mL beaker. Add 15 mL acidic MeOH and heat carefully on steam bath ca 30 min to dissolve active ingredients. Cool, transfer to 50 mL vol. flask, and dil. to vol. with acidic MeOH.

### D. Determination

**(a)** *Chlorothiazide.*—Collect eluate in 100 mL vol. flask at rate of 2–3 drops/sec. Transfer 10.0 mL sample soln to prepd column. After sample soln has entered column, rinse down sides with three 2 mL portions anhyd. MeOH, letting each portion sink into resin completely before next addn. Do not agitate column mech. or by addn of solv. Complete elution with 50 mL anhyd. MeOH. Rinse column tip with acidic MeOH. Add 1 drop HCl to eluate and dil. to vol. with anhyd. MeOH. Further dil. 5.0 mL to 25.0 mL with acidic MeOH. Scan sample and std solns between 235 and 360 nm against acidic MeOH. Calc. chlorothiazide from *A* at max., ca 277 nm. Proceed immediately to methyldopa detn before column dries.

**(b)** *Methyldopa.*—Place another 100 mL vol. flask under column after elution in **(a)**. Elute with 50 mL 1*N* methanolic HCl at 2–3 drops/sec. Rinse column tip with 1*N* methanolic HCl. Dil. to vol. with 1*N* methanolic HCl and further dil. to concn of ca 30 μg methyldopa/mL. Scan sample and std solns between 230 and 360 nm against 1*N* methanolic HCl. Calc. methyldopa from *A* at max., ca 280 nm.

Ref.: JAOAC **54,** 603(1971).

CAS-58-94-6 (chlorothiazide)
CAS-555-30-6 (methyldopa)

## 973.74 Thiazide Drugs

**Spectrophotometric Method**
**First Action 1973**
**Final Action 1974**

(Applicable to benzthiazide, chlorothiazide, methyclothiazide, hydrochlorothiazide, and hydroflumethiazide.)

### A. Principle

Benzthiazide, hydrochlorothiazide, or hydroflumethiazide is eluted from 0.2*N* NaOH-diat. earth column with HOAc-ethyl ether, extd into 0.2*N* NaOH, and detd by UV spectrophotometry. Chlorothiazide is eluted from 0.2*M* $K_2HPO_4$-diat. earth column with HOAc-ethyl ether, extd into 0.2*N* HCl, and detd by UV spectrophotometry. Methyclothiazide is eluted from 0.1*M* $NaHCO_3$-diat. earth column with $CHCl_3$ and measured directly by UV spectrophotometry.

### B. Apparatus and Reagents

**(a)** *Chromatographic tube and tamping rod.—See* **967.31A**.
**(b)** *Diatomaceous earth.—See* **960.53B**.
**(c)** *Dipotassium phosphate solns.*—0.2 and 0.1*M*, 34.85 and 17.43 g $K_2HPO_4$/L, resp.
**(d)** *Benzthiazide, hydrochlorothiazide, and hydroflumethiazide std solns.*—Prep. with ether-satd 0.2*N* NaOH. *(1) Benzthiazide.*—3.0 mg USP Ref. Std/200 mL. *(2) Hydrochlorothiazide.*—2.0 mg USP Ref. Std/ 200 mL. *(3) Hydroflumethiazide.*—2.0 mg USP Ref. Std/200 mL.
**(e)** *Chlorothiazide std solns.—(1) Stock soln.*—1.4 mg/mL. Accurately weigh ca 70 mg USP Chlorothiazide Ref. Std into small beaker, add 2 mL dimethylsulfoxide (DMSO), and mix with glass rod until dissolved. Transfer quant. to 50 mL vol. flask, using 0.2*M* $K_2HPO_4$, and dil. to vol. with same solv. *(2) Working soln.*—0.014 mg/mL. Dil. 2.0 mL stock soln to 200 mL with 0.2*N* HCl.
**(f)** *Methyclothiazide std solns.—(1) Stock soln.*—0.2 mg/mL. Accurately weigh 20 mg USP Methyclothiazide Ref. Std into 100 mL vol. flask and dil. to vol. with MeOH. *(2) Working soln.*—0.01 mg/mL. Dil. 10 mL stock soln to 200 mL with $CHCl_3$.

### C. Preparation of Sample

Finely powder to pass No. 60 sieve.

**(a)** *Benzthiazide, hydrochlorothiazide, or hydroflumethiazide.*—Transfer portion contg 75 mg benzthiazide or 50 mg hydrochlorothiazide or hydroflumethiazide to 50 mL vol. flask, using 0.2*N* NaOH. Shake to dissolve completely and dil. to vol.

**(b)** *Chlorothiazide.*—Transfer portion contg ca 70 mg to small beaker and add 2.0 mL DMSO. Mix thoroly 2–3 min with glass rod to dissolve completely. Transfer to 50 mL vol. flask, using 0.2*M* $K_2HPO_4$, and dil. to vol. with same solv. Mix thoroly.

**(c)** *Methyclothiazide.*—Transfer portion contg ca 2 mg to 150 mL beaker. Add 2 mL MeOH and mix thoroly. Add 2 mL 0.1*M* $NaHCO_3$ and mix.

### D. Preparation of Columns

**(a)** *Benzthiazide, chlorothiazide, hydrochlorothiazide, or hydroflumethiazide.—(1) Lower layer.*—Mix 2 g diat. earth with 1 mL 0.2*N* NaOH (1 mL 0.1*M* $K_2HPO_4$ for chlorothiazide) in 150 mL beaker, transfer to tube, and tamp to uniform mass.

*(2) Upper layer.*—Mix 3 g diat. earth with 2 mL sample soln, transfer to tube, and tamp. Dry-wash flask contg sample mixt. with 1 g diat. earth and 2–3 drops $H_2O$; transfer to column and tamp. Top with glass wool pad.

**(b)** *Methyclothiazide.—(1) Lower layer.*—Mix 3 g diat. earth with 2 mL 0.1*M* $NaHCO_3$ in 150 mL beaker, transfer to tube, and tamp to uniform mass.

*(2) Upper layer.*—Proceed as in **(a)***(2)*, except use 4 g diat. earth.

### E. Determination

(Use $H_2O$-satd solvs thruout.)

**(a)** *Benzthiazide, hydrochlorothiazide, and hydroflumethiazide.*—Pass 50 mL $CHCl_3$, followed by 50 mL ether, thru column; discard eluate. Using 250 mL separator as receiver, elute column with 0.1 mL HOAc in 100 mL ether. Wash tip of column with ether. Add 65 mL isooctane to eluate and ext org. phase with three 50 mL portions 0.2*N* NaOH; combine NaOH soln in 200 mL vol. flask and dil. to vol.

Det. *A* of sample and std solns in 1 cm cells with spectrophtr against 0.2*N* NaOH as ref.

**(b)** *Chlorothiazide.*—Proceed as in **(a)**, except use 0.25 mL HOAc in 100 mL ether, 50 mL isooctane, and 0.2*N* HCl instead of NaOH. Use 0.2*N* HCl as ref. solv.

**(c)** *Methyclothiazide.*—Pass 75 mL isooctane-ether (9 + 1) thru column; discard eluate. Use 200 mL vol. flask as receiver and elute column with 100 mL $CHCl_3$. Wash tip of column with ether. Add 10 mL MeOH and dil. to vol. with $CHCl_3$. Use $CHCl_3$ as ref. solv.

Wavelength of max. *A*, and *a* of individual compds are as follows:

| Compd | λ Max., nm | Absorptivity |
|---|---|---|
| Benzthiazide | 295 | 29.6 |
| Chlorothiazide | 278 | 32.4 |
| Methyclothiazide | 268 | 51.8 |
| Hydrochlorothiazide | 273 | 49.1 |
| Hydroflumethiazide | 273 | 45.4 |

### F. Identification

(**a**) *Benzthiazide, hydrochlorothiazide, and hydroflumethiazide.*—Acidify portion sample soln with 1*N* HCl and ext with 50 mL ether. Evap. ether to dryness, add 5 mL alcohol, and evap. again. Compare IR spectrum in KBr matrix of residue with that of ref. std previously recrystd from alcohol.

(**b**) *Chlorothiazide.*—Transfer 5 mL prepd soln, **973.74C(b)**, to 125 mL separator, add 10 mL $H_2O$, acidify with 1*N* HCl, and ext with 75 mL ether. Evap. ether to dryness. Add 5 mL alcohol to residue and evap. to dryness. Compare IR spectrum in KBr matrix of residue from 400 to 600 $cm^{-1}$ with that of ref. std previously recrystd from alcohol.

(**c**) *Methyclothiazide.*—Transfer portion sample contg ca 4 mg active ingredient to 125 mL separator, add 20 mL 0.1*M* $NaHCO_3$, and ext with ca 75 mL ether. Proceed as in (**b**).

Ref.: JAOAC **55,** 161(1972); **56,** 677(1973).

CAS-91-33-8 (benzthiazide)
CAS-58-94-6 (chlorothiazide)
CAS-58-93-5 (hydrochlorothiazide)
CAS-135-09-1 (hydroflumethiazide)
CAS-135-07-9 (methyclothiazide)

## 974.41 Polythiazide in Drugs

**Spectrophotometric Method**

**First Action 1974**
**Final Action 1976**

(Applicable to formulations contg vanillin)

### A. Principle

Vanillin, which interferes in method, is condensed thru aldehyde group with primary amine group of sulfanilic acid to form strongly polar and $H_2O$-sol. Schiff's base, which is retained in aq. immobile phase of column. Less polar polythiazide is eluted with mobile phase, ether-isooctane, and detd by UV spectrophotometry.

### B. Apparatus and Reagents

(Use $H_2O$-washed solvs thruout.)

(**a**) *Chromatographic tube and tamping rod.—See* **967.31A**.
(**b**) *Diatomaceous earth.—See* **960.53B**.
(**c**) *Dilute ammonium hydroxide.*—1*N*. Dil. 17 mL $NH_4OH$ to 250 mL with $H_2O$.
(**d**) *Ammonium sulfanilate soln.*—6%. Dissolve 6.0 g sulfanilic acid in 1*N* $NH_4OH$ and dil. to 100 mL with 1*N* $NH_4OH$.
(**e**) *Polythiazide std soln.*—10 μg/mL. Accurately weigh ca 100 mg polythiazide, transfer to 100 mL vol. flask, and dil. to vol. with MeOH. Further dil. 10 mL of this soln to 100 mL with MeOH and 10 mL dild soln to 100 mL with MeOH.

### C. Preparation of Sample

Accurately weigh sample contg ca 1 mg polythiazide and transfer to 150 mL beaker. Add 0.25 mL dimethylsulfoxide (DMSO) and mix thoroly to wet entire sample. Let stand 3–4 min.

### D. Preparation of Column

(**a**) *Lower layer.*—Mix 6 g diat. earth, (**b**), and 5 mL $NH_4$ sulfanilate soln in 150 mL beaker, transfer to tube, and tamp to uniform mass.

(**b**) *Upper layer.*—Add 4 mL $NH_4$ sulfanilate soln to sample soln, and mix. Add 4 g diat. earth, mix, transfer to tube, and tamp to uniform mass. Dry-wash beaker with 1 g diat. earth and few drops $H_2O$, transfer to tube, and tamp. Top with glass wool pad.

### E. Determination

Pass 100 mL isooctane thru column; discard eluate. Elute polythiazide with 100 mL isooctane-ether (1 + 1), receiving eluate in 250 mL beaker. Immediately evap. eluate to dryness. Dissolve residue in small amt MeOH and transfer quant. to 100 mL vol. flask. Dil. to vol. with MeOH. Filter thru glass wool, discarding first 20 mL. Det. *A* of sample and std solns against MeOH in 1 cm cell at max., ca 268 nm.

Ref.: JAOAC **57,** 716(1974).

CAS-346-18-9 (polythiazide)

# OTHER SULFUR-CONTAINING DRUGS

## 967.32 Methimazole in Drugs

**Infrared Spectrophotometric Method**

**First Action 1967**
**Final Action 1977**

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

### A. Apparatus

(**a**) *Chromatographic tube.*—200 × 22 mm.
(**b**) *Spectrophotometer.*—Recording double-beam IR spectrophtr, with 2 mm cells and NaCl windows.

### B. Reagents

(**a**) *Diatomaceous earth.—See* **960.53B**.
(**b**) *Methimazole std.*—Store in desiccator over $P_2O_5$ when not in use.

### C. Column Chromatography

Transfer amt of freshly ground tablet mixt. contg 10 mg methimazole to 100 mL beaker, add 3 mL $H_2O$, and mix thoroly to wet sample. Add 4 g diat. earth and mix thoroly. Transfer in 2 equal portions to chromatgc tube contg pledget of glass wool and pack tightly. Rinse beaker with 0.5 g diat. earth and add to column; top with glass wool pad. Rinse beaker with 150 mL $H_2O$-washed isooctane (redistd) and add rinses to column. Let last drops of isooctane drain from column before proceeding. Discard isooctane eluate.

Rinse beaker with three 5 mL portions $H_2O$-washed $CHCl_3$ and add rinses to column. Collect eluate. Elute methimazole with 200 mL $H_2O$-washed $CHCl_3$, maintaining solv. head ≤75 mm (3″) during elution. Combine $CHCl_3$ eluates, and evap. at ca 40–60° with air stream to ca 10 mL, washing down sides of beaker with small portions $CHCl_3$ during evapn. Do not heat excessively, since methimazole may oxidize. Quant. transfer conc. to 30 mL beaker with several small portions $CHCl_3$. Evap. solv. at ca 30–40° under air stream. (Make certain all traces of isooctane are removed.) Dry residue in vac. over anhyd. $P_2O_5$ 30 min. (If necessary, store residue over desiccant in dark; methimazole oxidizes on standing.)

***D. Determination***

Add 5 mL $CS_2$ to residue in beaker, cover with watch glass, and warm to dissolve. Cool and quant. transfer soln to 10 mL vol. flask with $CS_2$. Repeat with two 2 mL portions $CS_2$, cool, transfer to flask, and dil. to vol.

Prep. std soln methimazole in $CS_2$, with warming, to contain exactly 1.00 mg/mL. Record quant. IR spectra of sample and std solns between 7.6 and 8.4 μm in 2 mm NaCl cells. Measure baseline $A$ values of 7.83 μm max., using minima at 7.7 and 8.3 μm.

$$\text{mg Methimazole/tablet} = A \times (C'/A') \times 10 \times (T/W)$$

where $A$ and $A'$ = baseline values for sample and std, resp.; $C'$ = mg/mL std soln; $T$ and $W$ = av. wt/tablet and sample wt, resp., in mg.

Identify samples by comparing IR spectra of quant. solns with spectrum of std over 2–15 μm, using $CS_2$ as blank.

Ref.: JAOAC **50,** 674(1967).

CAS-60-56-0 (methimazole)

## 952.27 Propylthiouracil in Drugs
### Spectrophotometric Method
### Final Action

Start and complete detn on same day.

Transfer accurately weighed sample contg ca 150 mg propylthiouracil to 200 mL vol. flask, and transfer 150.0 mg pure propylthiouracil to another 200 mL vol. flask as std. To each flask add 150 mL $NH_4OH$ (1 + 13), washing down necks. Shake flasks moderately and continuously 1 min to dissolve propylthiouracil. Dil. each to vol. with $NH_4OH$ (1 + 13) and mix.

Filter sample soln, discarding first 25 mL filtrate. Dil. 20 mL aliquot clear filtrate to 200 mL with $H_2O$ in vol. flask (or 25 mL aliquot to 250 mL) and mix. Dil. 20 mL aliquot of this soln to 200 mL in vol. flask (or 25 mL aliquot to 250 mL) and mix. Prep. same double diln of std soln to obtain final concn of 0.0075 mg/mL.

Det. $A$ of final solns of std and sample against $H_2O$ blank in silica cells in spectrophtr at 234 nm. Apply cell corrections unless same cell is used for both std and sample. Calc. propylthiouracil content of sample.

Ref.: JAOAC **35,** 572(1952).

CAS-51-52-5 (propylthiouracil)

## 948.30★ Thiouracil in Drugs
### Bromination Method
### First Action
### Surplus 1965

*See* **32.394**, 10th ed.

## 981.25 Disulfiram in Drug Tablets
### Colorimetric Method
### First Action 1981
### Final Action 1983

(*Caution:* See safety notes on sodium hydroxide.)

***A. Principle***

Disulfiram is extd with $CH_2Cl_2$ and washed with NaOH soln to eliminate diethyldithiocarbamic acid decomposition product. Aliquot is treated with CuI to form colored cupric diethyldithiocarbamate which is measured at ca 428 nm.

***B. Reagents***

(**a**) *Sodium hydroxide soln.*—1M. Dissolve 8 g NaOH in and dil. to 200 mL with $H_2O$.

(**b**) *Cuprous iodide.*—No. C-465 (Fisher Scientific Co.), or equiv. Grind with mortar and pestle to fine powder.

(**c**) *Disulfiram ref. std soln.*—Approx. 50 μg/mL $CH_2Cl_2$. Accurately weigh NF Disulfiram Ref. Std and dissolve in $CH_2Cl_2$. Dil. quant. and stepwise with $CH_2Cl_2$ to desired concn.

(**d**) *TLC std soln.*—0.5 mg disulfiram/mL $CH_2Cl_2$.

(**e**) *TLC developing solvent.*—1% HOAc in toluene.

(**f**) *Thin layer sheets.*—*Sheet A.*—20 × 20 cm, coated 0.25 mm thick with alumina with fluorescent indicator (Analtech, Inc., Product No. 4011, or equiv.). *Sheet B.*—20 × 20 cm, coated 0.25 mm thick with silica gel with fluorescent indicator (Analtech, Inc., Product No. 2611, or equiv.).

***C. Determination***

Det. av. tablet wt. Accurately weigh portion of pulverized sample composite contg ca 100 mg disulfiram and transfer to 200 mL vol. flask. Add $CH_2Cl_2$, mix thoroly, and dil. to vol. with $CH_2Cl_2$. Transfer 10.0 mL aliquot to 125 mL separator, and add 15 mL $CH_2Cl_2$ and 10 mL 1M NaOH. Shake 1 min and transfer $CH_2Cl_2$ layer to 100 mL vol. flask thru compact pledget of absorbent cotton. Ext aq. layer with second 25 mL portion of $CH_2Cl_2$, filter $CH_2Cl_2$ layer thru same cotton plug, and combine with first ext. Dil. ext to vol. with $CH_2Cl_2$ and mix.

Transfer 10.0 mL $CH_2Cl_2$ ext and 10.0 mL ref. std soln to sep. 25 mL g-s flasks. To each flask add ca 50 mg powdered CuI. Shake flasks and let stand 1 h. Quant. filter each soln into sep. 50 mL vol. flasks thru tight pledget of absorbent cotton previously wet with $CH_2Cl_2$. Dil. to vol. with $CH_2Cl_2$. Scan sample and std solns from 600 to 325 nm, against $CH_2Cl_2$ blank. Det. max. $A$ at ca 428 nm, and calc. amt disulfiram as follows:

$$\text{mg Disulfiram/tablet} = (A/A') \times C \times 200 \times (D/W)$$

where $A$ and $A'$ = values for sample and std, resp., $C$ = mg disulfiram in 10 mL ref. std soln; $D$ = av. tablet wt (g); $W$ = wt sample (g); 200 = diln factor.

***D. TLC Confirmatory Test***

Line suitable chromatgc tank with filter paper and add developing solv. Apply sep. spots of 5 μL of original 200 mL sample soln and 5 μL TLC std soln to either TLC sheet. Develop 10–15 cm above starting line, and air dry. Locate and mark spots under shortwave UV light. $R_f$ of sample and std spots should be ca 0.60 and 0.25 for alumina and silica gel, resp.

Ref.: JAOAC **64,** 554(1981).

CAS-97-77-8 (disulfiram)

## 968.47 Phenothiazine in Drugs
### Gas Chromatographic Method
### First Action 1968
### Final Action 1982

***A. Reagents and Apparatus***

(All $CHCl_3$ solns must be protected from light and assay must be completed within 8 hr.)

(**a**) *Phenothiazine std soln.*—Dissolve phenothiazine in 10 parts toluene with heat. Add 0.1 g activated charcoal for each

4 g phenothiazine. Boil 10 min under reflux and filter while hot thru heated filter. Cool soln, and collect phenothiazine crystals on buchner or fritted glass filter. Dry crystals at 100° and then in vac. desiccator contg paraffin chips. Repeat re-crystn, if necessary, until mp is 184–185°. Dissolve 100.0 mg purified phenothiazine in $CHCl_3$ in 50 mL vol. flask and dil. to vol. with $CHCl_3$.

(**b**) *Internal std soln.*—Dissolve 125 mg promethazine.HCl in $CHCl_3$ in 25 mL vol. flask and dil. to vol. with $CHCl_3$.

(**c**) *Chromatographic column.*—Slurry 20 g Gas-Chrom Q, 100–120 mesh, with 100 mL $CHCl_3$ in 500 mL r-b flask. Add, with stirring, 1.0 g Apiezon L dissolved in 50 mL $CHCl_3$. Evap. to dryness in 70° $H_2O$ bath, using rotary vac. evaporator. Apply vac. (ca 50 cm Hg) to one end of 1.2 m (4′) glass column (4 mm id) and, with gentle tapping only, fill tube with coated support. Condition column by heating 48 hr at 240° with N flow of ca 10 mL/min.

(**d**) *Gas chromatograph.*—Any gas chromatograph with H flame ionization detector capable of using specified column.

### *B. Determination*

Grind representative sample portion to pass No. 60 sieve. Accurately weigh sample contg ca 200 mg phenothiazine and transfer to 100 mL vol. flask. Add 80 mL $CHCl_3$ and shake vigorously until phenothiazine is completely dissolved (ca 20 min). Dil. to vol. with $CHCl_3$, mix thoroly, and let stand 15 min. Pipet 5 mL aliquots of clear supernate and phenothiazine std soln into sep. 25 mL g-s erlenmeyers. Pipet 4 mL aliquots of promethazine.HCl internal std soln into each flask.

About 1 hr before initial injection, adjust app. to following temps: column 215°, detector 230°, injector 230°. Set N carrier gas flow rate to give phenothiazine retention time of ca 8 min (ca 20 psig regulator outlet pressure). Retention time of internal std will be ca 17 min. Inject similar vol. of sample and std soln contg ca 10 μg phenothiazine, using sensitivity setting that gives 70–90% of full-scale deflection.

% Phenothiazine in original sample

$$= (M/W)(P_u/P_a) \times (P_b/P_p) \times 200$$

where $M$ = mg phenothiazine used to prep. std soln, $W$ = mg sample, $P_u$ = phenothiazine sample soln peak area, $P_a$ = promethazine.HCl sample soln peak area, $P_b$ = promethazine.HCl std soln peak area, and $P_p$ = phenothiazine std soln peak area.

Refs.: JAOAC **49,** 857(1966); **50,** 682(1967); **51,** 273(1968).

CAS-92-84-2 (phenothiazine)

## 985.47 Allopurinol in Drug Tablets

**Liquid Chromatographic Method**

**First Action 1985**
**Final Action 1987**

### *A. Principle*

Allopurinol content is detd by liq. chromatgy on reverse phase column with aq. ammonium phosphate mobile phase, UV detection, and hypoxanthine internal std.

### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Model 950 solv. pump with Model 970A variable wavelength detector capable of monitoring $A$ at 254 nm (Tracor Instruments, Inc., replacement models 951 and 971, resp.), 20 μL injection valve (Valco Instruments, Inc., PO Box 55603, Houston, TX 77255) and suitable strip chart recorder, or equiv. LC system.

(**b**) *Chromatographic column.*—Stainless steel, 300 × 3.9 mm id, packed with μBondapak 10 μm (Waters Associates, Inc.) or equiv. column meeting LC system suitability requirements.

### *C. Reagents*

(**a**) *Ammonium phosphate.*—Monobasic, FW 115.031 (Fisher Scientific Co.), or equiv.

(**b**) *Hypoxanthine.*—Reagent grade (Fisher Scientific Co.), or equiv.

(**c**) *Mobile phase.*—Prep. 0.05M ammonium phosphate (monobasic) soln, 5.75 g $(NH_4)H_2PO_4$/L $H_2O$. Degas under vac. or by ultrasonic treatment 5–10 min. (Do not leave mobile phase in column overnight; after draining mobile phase, flush entire system ≥20 min with $H_2O$, followed by MeOH for 20 min.)

(**d**) *Internal std preparation.*—Accurately weigh ca 50 mg hypoxanthine and transfer to 50 mL vol. flask. Add 10 mL 0.1N NaOH and shake mech. 10 min, or until completely dissolved. Dil. with $H_2O$ to vol., and mix. Prep. this soln fresh daily.

(**e**) *Std preparation.*—Accurately weigh ca 50 mg USP Allopurinol Ref. Std and transfer to 50 mL vol. flask. Add 10 mL 0.1N NaOH and shake mech. 10 min. Dil. with $H_2O$ to vol., and mix. Prep. this soln fresh daily. Transfer 4.0 mL of this soln to 200 mL vol. flask, add 2.0 mL internal std prepn, dil. with mobile phase to vol., and mix.

### *D. Sample Preparation*

Weigh and finely powder ≥20 tablets. Accurately weigh portion of powder equiv. to ca 50 mg allopurinol, and transfer to 50 mL vol. flask. Add 10 mL 0.1N NaOH, shake mech. 10 min, dil. with $H_2O$ to vol., and mix. From this point, proceed with detn without delay. Filter portion of soln thru suitable paper or 0.45 μm membrane filter, discarding first 10 mL filtrate. Transfer 4.0 mL filtrate to 200 mL vol. flask, add 2.0 mL internal std prepn, dil. with mobile phase to vol., and mix.

### *E. Suitability Test and Determination*

Inject equal vols (10–20 μL) of sample prepn and std prepn into liq. chromatograph by means of sampling valve or high pressure microsyringe. Operate chromatograph at ambient temp. while pumping mobile phase at 1.5 mL/min. Adjust detector sensitivity so that peak response for allopurinol and hypoxanthine is 40–60% AUFS. In suitable chromatogram, lowest and highest peak response ratios of 3 successive injections of std prepn do not differ >3.0%, and resolution factor, $R$, for sample peak and internal std peak is ≥5. Retention times are hypoxanthine 7 min, allopurinol 12 min.

Calc. resolution factor, $R$, as follows:

$$R = [2(t_2 - t_1)]/(W_2 + W_1)$$

where $t_2$ and $t_1$ = retention times of the 2 components, and $W_2$ and $W_1$ = corresponding widths of bases of peaks, obtained by extrapolating the relatively straight sides of the peaks to the baseline.

Standard peak response ratio, $R'$, is ratio of std allopurinol peak to internal std peak.

### *F. Calculation*

Calc. amt of allopurinol in dosage form, using response ratios based on either peak hts or peak areas, according to following equation:

$$\text{Allopurinol, mg/tablet} = (R/R') \times (W'/W) \times T$$

where $R$ and $R'$ = response ratios of allopurinol peak to internal std peak for sample and std, resp.; $W$ and $W'$ = mg taken for sample and std prepns, resp.; and $T$ = av. tablet wt, mg.

Ref.: JAOAC **67,** 1121(1984).

CAS-315-30-0 (allopurinol)

## 986.36 Primidone in Drug Tablets

**Liquid Chromatographic Method**
**First Action 1986**
**Final Action 1988**

### A. Principle

Sample is dissolved in mobile phase, filtered, injected into liq. chromatgc system, and quantitated by comparison with external std.

### B. Reagents

(**a**) *Solvents*.—LC grade $H_2O$ and MeOH (J.T. Baker Inc., Nos. 4218 and 9093, resp.).

(**b**) *LC mobile phase*.—In suitable flask, combine 500 mL $H_2O$ and 500 mL MeOH; stir mag. Filter thru 0.4 μm membrane filter wetted with MeOH. Place in ultrasonic bath 10 min to deaerate.

(**c**) *Std soln*.—Accurately weigh 50 mg USP Ref. Std Primidone, previously dried 2 h at 105°, and transfer to 50 mL vol. flask. Add 35 mL mobile phase, place in ultrasonic bath 15 min, cool, and dil. to vol. with mobile phase. Place in ultrasonic bath for addnl 15 min and cool. Soln is stable 1 week.

### C. Apparatus

(**a**) *Liquid chromatograph*.—System equipped with injector, solv. delivery system, and UV detector. Operating conditions: flow rate 1.0 mL/min; 254 nm detector, 0.2 AUFS; temp. ambient; 20 μL injection.

(**b**) *LC column*.—Macherey-Nagel Nucleosil C-8, 10 μm particle size, 25 cm × 3.2 mm, or equiv.

(**c**) *Recorder*.—10 mV with 0.5 cm/min chart speed.

(**d**) *Membrane filters*.—Nylon-66 pore size 0.45 μm (Rainin Instrument Co., Mack Rd, Woburn, MA 01801), or equiv.

### D. Preparation of Samples

Det. av. wt of 20 tablets and grind to pass No. 60 sieve. Transfer accurately weighed portion of powder equiv. to 50 mg primidone to 50 mL vol. flask. Add 35 mL mobile phase, place in ultrasonic bath 15 min, cool, and dil. to vol. with mobile phase. Place in ultrasonic bath addnl 15 min and cool. Filter soln thru 0.45 μm membrane filter and use as sample prepn.

### E. Determination

Equilibrate system with column in instrument and mobile phase set at 1.0 mL/min. Inject 20 μL std soln and adjust flow rate and sensitivity so that peak response is ca 45% full scale, with retention time ca 3 min. In suitable system, coefficient of variation (CV) of peak responses of 5 replicate injections is ≤2.0%. Proceed with sample analysis, using 20 μL injections for each std and sample soln.

### F. Calculation

Det. peak responses of std and sample peaks and calc. amt of primidone in tablets.

$$\text{Primidone, mg/tablet} = (R/R') \times 50 \times C \times (T/W)$$

where $R$ and $R'$ = peak response of sample and std solns, resp.; $C$ = concn of primidone std soln in mg/mL; $T$ = av. tablet wt in mg; and $W$ = sample wt in mg.

Ref.: JAOAC **68,** 85(1985).

CAS-125-33-7 (primidone)

## 988.23 Diethylpropion Hydrochloride in Drug Substance and Tablets

**Liquid Chromatographic Method**
**First Action 1988**

### A. Principle

DEPH content of drug substance and tablets is detd by reverse phase liq. chromatgy using anthracene as internal std, UV detection at 254 nm, and MeOH–$H_2O$–phosphate mobile phase. Purity is confirmed by UV, LC, and spot tests.

### B. Apparatus

(**a**) *Liquid chromatograph*.—Equipped with 30 cm × 4 mm id stainless steel column packed with ODS bonded to microparticulate silica; UV detector; and suitable integrator. Operating conditions: column temp. ambient; mobile phase flow rate ca 1.5 mL/min; vol. injected 20 μL; detector wavelength 254 nm.

(**b**) *UV-visible scanning spectrophotometer*.

### C. Reagents

Use deionized, purified $H_2O$ and anal. reagent grade chemicals unless otherwise specified.

(**a**) *Mobile phase*.—Dissolve 0.40 g $KH_2PO_4$ and 2.26 g anhyd. $Na_2HPO_4$ in $H_2O$ to make 1000 mL. Mix 1 part of this soln with 4 parts of MeOH, and filter thru suitable membrane of ≤1 μm porosity. pH of resulting soln should be ca 7.7. Degas soln by sparging with He. Make adjustments, if necessary, to obtained desired retention times and resolution.

(**b**) *$H_3PO_4$ soln*.—(1 in 2000). Dil. 1 mL $H_3PO_4$ (85%) to 2 L with $H_2O$.

(**c**) *Internal std soln*.—Dissolve 20 mg anthracene in 500 mL MeOH. Store in air-tight container.

(**d**) *DEPH std soln*.—Dry USP Diethylpropion Hydrochloride Ref. Std over silica gel ≥4 h. Accurately weigh ca 25 mg dried std and transfer to 200 mL vol. flask contg 40 mL $H_3PO_4$ soln. Pipet 40.0 mL internal std soln into flask, dil. to vol. with MeOH, and mix.

### D. Chromatographic System Suitability Test

Let chromatgc system equilibrate and inject replicate 20 μL portions of DEPH std soln. Typical retention times for diethylpropion and anthracene are ca 6 and 9 min, resp., at flow rate between 1.0 and 2.0 mL/min. In suitable system, resolution, $R$, between these peaks is >3.0 and relative std dev. (RSD) for ratios of peak responses, $R_s$, for 6 replicate injections of DEPH std soln is ≤1.0%.

### E. Sample Preparation

(**a**) *Bulk drug assay soln*.—Dry DEPH bulk drug over silica gel ≥4 h. Accurately weigh ca 25 mg dried sample and transfer to 200 mL vol. flask contg 40 mL $H_3PO_4$ soln. Pipet 40.0 mL internal std soln into flask, dil. to vol. with MeOH, and mix.

(**b**) *Tablet assay soln*.—Weigh and finely powder ≥20 DEPH tablets. Into 200 mL vol. flask, transfer accurately weighed amt of powder equiv. to ca 25 mg DEPH. Add 40 mL $H_3PO_4$ soln and shake mech. ≥15 min. Transfer 40.0 mL internal std soln to flask, dil. to vol. with MeOH, and mix. Centrifuge portion of soln, and filter supernate thru suitable membrane filter ≤1 μm porosity.

### F. Determination

(**a**) *Bulk drug*.—Sep. inject equal vols (ca 20 μL) of DEPH std soln and bulk drug assay soln in duplicate into LC system, record chromatograms, and measure response for major peaks. Rel. retention times are ca 0.7 for diethylpropion and 1.0 for anthracene. Calc. amt DEPH, mg, in portion of DEPH drug substance taken as follows:

$$\text{mg DEPH} = F \times C \times (R_u/R_s)$$

where $F$ = vol. sample soln/1000 μg/mg = 200 mL/1000 μg/mg = 0.2; and where $C$ = concn, μg/mL, of USP DEPH Ref. Std in DEPH std soln, and $R_u$ and $R_s$ are ratios of peak responses of diethylpropion to anthracene obtained from bulk drug assay soln and DEPH std soln, resp.

(**b**) *Tablets.*—Use tablet assay soln and follow procedure for bulk drug detn. Calc. amt DEPH, mg/tablet, as follows:

$$\text{DEPH, mg/tablet} = \{[0.2C \times (R_u/R_s)]/\text{mg sample}\} \times W$$

where $W$ = av. tablet wt, mg.

### G. Identification Tests

(**a**) *Bulk drug.*—UV spectrum of 1 in 100 000 soln in 0.1N HCl exhibits max. and min. at same wavelengths as that of similar prepn of USP DEPH Ref. Std concomitantly measured, and molar $a$ values (anhyd. basis) at wavelength max. at ca 253 nm do not differ by >3.0%.

(**b**) *Tablets.*—Chromatogram of tablet assay soln obtained as directed for tablet assay exhibits major peak for diethylpropion, with retention time that corresponds with that exhibited in chromatogram of DEPH std soln, both relative to internal std.

### H. Chromatographic Purity

(**a**) *Test preparations.*—*Test prepn A.*—Dissolve 250 mg bulk drug in 20 mL $H_3PO_4$ soln in 100 mL vol. flask, dil. to vol. with MeOH, and mix. *Test prepn B.*—Transfer 1.0 mL test prepn A to 100 mL vol. flask, dil. to vol. with 4:1 mixt. of MeOH and $H_3PO_4$ soln, and mix.

(**b**) *LC system.*—Use system described in *Apparatus*.

(**c**) *Procedure.*—Inject 20 μL test prepn B into LC system, adjust detector output to obtain peak ht for diethylpropion ≥ 40% but <100% full scale deflection on chart, adjust integrator sensitivity accordingly, and det. area of diethylpropion peak. Inject 20 μL test prepn A, chromatograph for total time equal to 3 times retention time of diethylpropion, and det. sum of areas of impurity peaks between solv. front peak and diethylpropion peak. Perform solv. blank detn by injecting 20 μL of 4:1 mixt. of MeOH and $H_3PO_4$ soln. Calc. % chromatgc impurities by formula:

$$\%\ \text{impurities} = 100\ (r_A - r_s)/[100 r_B + (r_A - r_s)]$$

where $r_A$ = sum of areas of impurity peaks in chromatogram obtained from test prepn A, $r_s$ = sum of areas of peaks in chromatogram obtained from solv. blank, and $r_B$ = area of diethylpropion peak in chromatogram obtained from test prepn B. Impurities found should be ≤0.5%.

### I. Secondary Amines

(**a**) *Acetaldehyde test soln.*—Mix 4 mL acetaldehyde, 3 mL alcohol, and 1 mL $H_2O$. Prep. soln fresh.

(**b**) *Procedure.*—Dissolve 100 mg bulk drug in 2 mL $CH_2Cl_2$ in centrf. tube. Transfer to second tube 2 mL std soln of diethylamine·HCl (DEAH) (dried 2 h at 105° before using) in $CH_2Cl_2$, which has known concn of 250 μg/mL. Treat each soln as follows: Ext with 2 mL buffer soln contg 5.7 g $Na_2CO_3$ and 3.0 g $NaHCO_3$/100 mL $H_2O$. Centrifuge, if necessary, to clarify upper phase, and immediately transfer 0.5 mL of soln to spot plate. Immediately add 2 drops of acetaldehyde test soln, and then, in rapid succession, add 1 drop of Na nitroferricyanide soln (1 in 100) to each spot. Immediately and simultaneously, briefly stir both spots to mix reagents. Any blue color formed within 3 min by bulk drug soln is not visibly more intense than that of DEAH std soln (<0.5% of secondary amines as DEAH). *Note:* Failure of DEPH std to form blue color has been shown to be due to decomposed acetaldehyde.

Ref.: Pharm. Forum Sept.–Oct. 1985, p. 791.

CAS-90-84-6 (diethylpropion)
CAS-134-80-5 (diethylpropion HCl)

**Common and Chemical Names of Drugs in this Chapter**

| Common Name | Chemical Name |
|---|---|
| Acenocoumarol | 4-Hydroxy-3-[1-(4-nitrophenyl)-3-oxybutyl]-2*H*-1-benzopyran-2-one |
| Acetaminophen | *N*-(4-Hydroxyphenyl)acetamide |
| Acetanilide | *N*-Phenylacetamide |
| Acetylcarbromal | 1-Acetyl-3-(α-bromo-α-ethylbutyryl)urea |
| Allopurinol | 1,5-Dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidin-4-one |
| Aminophylline | 3,7-Dihydro-1,3-dimethyl-1*H*-purine-2,6-dione with 1,2-ethanediamine |
| Aminopyrine | 4-Dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one |
| *p*-Aminosalicylic Acid | 4-Amino-2-hydroxybenzoic acid |
| Amobarbital | 5-Ethyl-5-(3-methylbutyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Aspirin | 2-(Acetyloxy)benzoic acid |
| Bendroflumethiazide | 3,4-Dihydro-3-(phenylmethyl)-6-(trifluoromethyl)-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide |
| Benzthiazide | 6-Chloro-3-[[(phenylmethyl)thio]-methyl]-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide |
| Bromisovalum | 2-Bromo-3-methylbutyrylurea |
| Butabarbital sodium | 5-Ethyl-5-(1-methylpropyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Caffeine | 3,7-Dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione |
| Carbromal | 2-Bromo-2-ethylbutyrylurea |
| Chloral hydrate | 2,2,2-Trichloro-1,1-ethanediol |
| Chlorothiazide | 6-Chloro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide |
| Chlorpropamide | 1-[(*p*-Chlorophenyl)sulfonyl]-3-propylurea |
| Codeine | (5α, 6α)-7,8-Didehydro-4,5-epoxy-3-methoxy-17-methylmorphinan-6-ol |
| Dicumarol | 3,3′-Methylenebis[4-hydroxy-2*H*-1-benzopyran-2-one] |
| Diethylpropion Hydrochloride | 2-(Diethylamino)propiophenone hydrochloride |
| Diphenylhydantoin | *See* phenytoin |
| Disulfiram | Tetraethylthioperoxydicarbonic diamide |
| Ethchlorvynol | 1-Chloro-3-ethyl-1-penten-4-yn-3-ol |
| Guaiacol | *o*-Methoxyphenol |
| Guaiafenesin | 3-(2-Methoxyphenoxy)-1,2-propanediol |
| Hexylresorcinol | 4-Hexyl-1,3-benzenediol |
| Hydrochlorothiazide | 6-Chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide |
| Hydroflumethiazide | 3,4-Dihydro-6-(trifluoromethyl)-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide |
| Isoniazid | 4-Pyridinecarboxylic acid, hydrazide |
| Mandelic Acid | α-Hydroxybenzeneacetic acid |

(*Continued*)

**Common and Chemical Names of Drugs in this Chapter (*Continued*)**

| Common Name | Chemical Name |
|---|---|
| Menadione Sodium Bisulfite | 1,2,3,4-Tetrahydro-2-methyl-1,4-dioxo-2-naphthalenesulfonic acid sodium salt |
| Meprobamate | 2-Methyl-2-propyl-1,3-propanediol dicarbamate |
| Methimazole | 1,3-Dihydro-1-methyl-2*H*-imidazole-2-thione |
| Methyclothiazide | 6-Chloro-3-(chloromethyl)-3,4-dihydro-2-methyl-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide |
| Methyldopa | 3-Hydroxy-α-methyl-L-tyrosine |
| Methyl salicylate | 2-Hydroxybenzoic acid methyl ester |
| Morphine | 7,8-Didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol |
| Oxyquinoline sulfate | 8-Quinolinol sulfate (2:1) (salt) |
| Paraldehyde | 2,4,6-Trimethyl-1,3,5-trioxane |
| Pentobarbital | 5-Ethyl-5-(1-methylbutyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Phenacetin | *N*-(4-Ethoxyphenyl)acetamide |
| Phenaglycodol | 2-(*p*-Chlorophenyl)-3-methyl-2,3-butanediol |
| Phenobarbital | 5-Ethyl-5-phenyl-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione |
| Phenolphthalein | 3,3-Bis(4-hydroxyphenyl)-1(3*H*)-isobenzofuranone |
| Phenothiazine | Thiodiphenylamine |
| Phenprocoumon | 4-Hydroxy-3-(1-phenylpropyl)-2*H*-1-benzopyran-2-one |
| Phenyl salicylate | 2-Hydroxybenzoic acid phenyl ester |
| Phenytoin | 5,5-Diphenyl-2,4-imidazolidinedione |
| Polythiazide | 6-Chloro-3,4-dihydro-2-methyl-3-[[(2,2,2-trifluoroethyl)thio]methyl]-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide |
| Primidone | 5-Ethyldihydro-5-phenyl-4,6(1*H*,5*H*)pyrimidinedione |
| Propylthiouracil | 2,3-Dihydro-6-propyl-2-thioxo-4(1*H*)-pyrimidinone |
| Quinine | 6′-Methoxycinchonan-9-ol |
| Salicylamide | *o*-Hydroxybenzamide |
| Salicylic Acid | 2-Hydroxybenzoic acid |
| Secobarbital sodium | 5-(1-Methylbutyl)-5-(2-propenyl)-2,4,6(1*H*,3*H*,5*H*)-pyrimidinetrione monosodium salt |
| Sodium salicylate | 2-Hydroxybenzoic acid monosodium salt |
| Sulfadiazine | 4-Amino-*N*-2-pyrimidinyl-benzenesulfonamide |
| Sulfamerazine | 4-Amino-*N*-(4-methyl-2-pyrimidinyl)-benzenesulfonamide |
| Sulfamethazine | 4-Amino-*N*-(4,6-dimethyl-2-pyrimidinyl)-benzenesulfonamide |
| Sulfamethoxazole | $N^1$-(5-Methyl-3-isoxazolyl)sulfanilamide |
| Sulfanilamide | *p*-Aminobenzenesulfonamide |
| Sulfisoxazole | 4-Amino-*N*-(3,4-dimethyl-5-isoxazolyl)-benzenesulfonamide |
| Sulfonethylmethane | 2,2-Bis(ethylsulfonyl)butane |
| Sulfonmethane | 2,2-Bis(ethylsulfonyl)propane |
| Theobromine | 3,7-Dihydro-3,7-dimethyl-1*H*-purine-2,6-dione |
| 2-Thiouracil | 2,3-Dihydro-2-thioxo-4(1*H*)-pyrimidinone |
| Thymol | 5-Methyl-2-(1-methylethyl)phenol |
| Warfarin potassium | 4-Hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-1-benzopyran-2-one potassium salt |
| Warfarin sodium | 4-Hydroxy-3-(3-oxo-1-phenylbutyl)-2*H*-1-benzopyran-2-one sodium salt |

Source: *USAN and the USP Dictionary of Drug Names* (1983; 1989) *U.S. Pharmacopeial Convention, Rockville, MD.*

# 20. Drugs: Part III


## Edward Smith, *Associate Chapter Editor*

*Food and Drug Administration*


## OPIUM ALKALOIDS

### 961.18 Opium Alkaloid Drugs

**First Action 1961**
**Final Action 1965**

***A. Microchemical Tests***

*See* **930.40** and Table **980.34**, and **960.57**.

***B. General Titration Method***

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Det. av. wt/tablet or other unit and grind to fine powder. Accurately weigh sample equiv. to 100–200 mg alkaloid and transfer to separator with ca 20 mL $H_2O$. Add 1 mL $H_2SO_4$ (1 + 9) and ext with three 25 mL portions $CHCl_3$. (Extn from acid soln is not necessary in absence of $CHCl_3$-sol. acidic or neut. components.) Add ca 1 mL $NH_4OH$ (use excess solid $NaHCO_3$ for apomorphine or physostigmine) and ext with four 25 mL portions $CHCl_3$ (use $CH_2Cl_2$ for ephedrine and $CHCl_3$-isopropanol (4 + 1) for morphine). Use correspondingly larger vols of solv. if larger vols aq. soln are required, as in case of sirups or with excessive amts of excipients. Check alky of soln after first extn by touching indicator paper to stopper. If not distinctly alk., add addnl $NH_4OH$. Check for complete extn by evapg 1 mL final ext to dryness; if more than trace of residue remains, ext with addnl portions solv. Filter ext thru plug of cotton or fine glass wool, previously wet with $CHCl_3$, into 200 mL erlenmeyer. Complete detn by either of following methods (***C*** must be used for arecoline and cocaine).

***C. Determination***

Evap. combined exts on steam bath with air current to ca 10 mL. Add measured excess 0.02*N* $H_2SO_4$ and continue evapn to remove solv. Cool, add Me red, and titr. excess acid with 0.02*N* NaOH.

***D. Alternative Determination***

Evap. combined exts on steam bath with air current to dryness. Dissolve residue in ca 2 mL MeOH, heating if necessary. Add Me red, and titr. with 0.02*N* $H_2SO_4$ to faint pink. If alkaloid is not completely dissolved, heat gently to complete soln. Add ca 40 mL freshly boiled, cooled $H_2O$, and complete titrn.

***E. Titration Factors***

*See* Table **961.18** for titrn factors.

Ref.: JAOAC **44,** 293(1961).

### Apomorphine in Drug Tablets

*See* **961.18B**.

### Codeine in Drug Tablets

*See* **961.18B**.

### 961.19 Codeine in Presence of Antihistamines in Drugs

**Chromatographic Method**

**First Action 1961**
**Final Action 1965**

(Applicable to sirups contg codeine with pyrilamine, methapyrilene, prophenpyridamine, and similar antihistamines)

***A. Apparatus***

*See* **967.31A**.

***B. Reagents***

(**a**) *Triethylamine*.—If blank *A*, **961.19D**, is >0.010, purify as follows: Reflux 100 mL $Et_3N$ with 20 mL $H_2O$ and 2 g Na hydrosulfite ≥8 hr. Wash with four or five 20 mL portions $H_2O$, dry by either distg into Dean-Stark trap or by salting-out with anhyd. $K_2CO_3$, and then distil, collecting first 75 mL. Store over anhyd. $Na_2CO_3$ or $K_2CO_3$. (*Caution:* See safety notes on distillation, toxic solvents, and triethylamine.)

(**b**) *Codeine std soln*.—Accurately weigh ca 100 mg codeine sulfate.$5H_2O$ (or other salt), dissolve in MeOH, transfer to 100 mL vol. flask, and dil. to vol. with MeOH. 1 mL = 0.8067 mg codeine.$H_2O$.

(**c**) *Chloroform*.—Use $CHCl_3$ satd with $H_2O$ thruout.

(**d**) *Diatomaceous earth*.—*See* **960.53B**.

***C. Preparation of Sample***

Prep. following 3 columns (columns II and III need not be quant.):

(**a**) *Column I*.—Pipet 2.0 mL sample, draining thoroly, into small beaker. Add 0.5 mL 1*N* NaOH and 3 g diat. earth. Mix thoroly and transfer quant. to tube, **967.31A(a)**. Dry-wash beaker with small portion diat. earth and few drops $H_2O$, and add to tube. Tamp column firmly with tamping rod, and press pad of glass wool on top.

(**b**) *Column II*.—Mix 3 g diat. earth and 2 mL 1*N* $HNO_3$, and prep. column as in (**a**).

(**c**) *Column III*.—Mix 3 g diat. earth and 2 mL 1*N* $H_2SO_4$, and prep. column as in (**a**).

***D. Determination***

Arrange columns so that effluent from *I* flows into *II* and then into *III*. Pass 100 mL $CHCl_3$ over columns. Discard Column *I* which retains excipients.

Pass 50 mL $CHCl_3$ thru Column *II* (which retains antihistamine) onto *III*; then pass 25 mL $CHCl_3$ over *III*. Remove Column *II*.

To recover codeine, place 50 mL vol. flask contg 10 mL MeOH and 1 mL HCl under Column *III*. Pass 5 mL $CHCl_3$ contg 1 mL $Et_3N$ over column followed by 32 mL 1% $Et_3N$ in $CHCl_3$. Dil. to vol. with $CHCl_3$ and det. *A* at 287 nm against $CHCl_3$. (Film of $Et_3N$.HCl may adhere to walls of cells. Rinse cells carefully with $H_2O$ and alcohol; then wipe clean before use.) Correct for blank *A* of mixt. of 10 mL MeOH, 1 mL $Et_3N$, and 1 mL HCl dild to 50 mL with $CHCl_3$. Also det. *A′* of std prepd by dilg 5 mL std soln contg 1 mg codeine salt/mL MeOH, to 50 mL with $CHCl_3$ and 5 drops HCl.

**Table 961.18 Titration Factors for Alkaloids**

| Alkaloid | Formula | mg/mL 0.02*N* $H_2SO_4$ |
|---|---|---|
| Apomorphine hydrochloride | $C_{17}H_{17}O_2N.HCl.{}^1/_2H_2O$ | 6.25 |
| Arecoline hydrobromide | $C_8H_{13}O_2N.HBr$ | 4.72 |
| Atropine | $C_{17}H_{23}NO_3$ | 5.79 |
| Atropine sulfate | $(C_{17}H_{23}NO_3)_2.H_2SO_4.H_2O$ | 6.95 |
| Cocaine hydrochloride | $C_{17}H_{21}O_4N.HCl$ | 6.80 |
| Codeine sulfate | $(C_{18}H_{21}O_3N)_2.H_2SO_4.5H_2O$ | 7.87 |
| Codeine phosphate | $C_{18}H_{21}O_3N.H_3PO_4.1{}^1/_2H_2O$ | 8.49 |
| Emetine hydrochloride | $C_{29}H_{40}O_4N_2.2HCl$ | 5.54 |
| Ephedrine | $C_{10}H_{15}ON$ | 3.30 |
| Ephedrine hydrochloride | $C_{10}H_{15}ON.HCl$ | 4.03 |
| Ephedrine sulfate | $(C_{10}H_{15}ON)_2.H_2SO_4$ | 4.29 |
| Ethylmorphine hydrochloride | $C_{19}H_{23}O_3N.HCl.2H_2O$ | 7.72 |
| Homatropine hydrobromide | $C_{16}H_{21}O_3N.HBr$ | 7.13 |
| Homatropine hydrochloride | $C_{16}H_{21}O_3N.HCl$ | 6.24 |
| Hydrocodone hydrochloride | $C_{18}H_{21}O_3N.HCl.H_2O$ | 7.08 |
| Hydrocodone bitartrate | $C_{18}H_{21}O_3N.C_4H_6O_6.2{}^1/_2H_2O$ | 9.89 |
| Morphine hydrochloride | $C_{17}H_{19}O_3N.HCl.3H_2O$ | 7.52 |
| Morphine sulfate | $(C_{17}H_{19}O_3N)_2.H_2SO_4.5H_2O$ | 7.59 |
| Physostigmine salicylate | $C_{15}H_{21}O_2N_3.C_7H_6O_3$ | 8.27 |
| Physostigmine sulfate | $(C_{15}H_{21}O_2N_3)_2.H_2SO_4$ | 6.49 |
| Pilocarpine hydrochloride | $C_{11}H_{16}O_2N_2.HCl$ | 4.89 |
| Pilocarpine nitrate | $C_{11}H_{16}O_2N_2.HNO_3$ | 5.43 |
| Procaine hydrochloride | $C_{13}H_{20}O_2N_2.HCl$ | 5.46 |
| Strychnine | $C_{21}H_{22}O_2N_2$ | 6.69 |
| Strychnine sulfate | $(C_{21}H_{22}O_2N_2)_2.H_2SO_4.5H_2O$ | 8.57 |
| Strychnine nitrate | $C_{21}H_{22}O_2N_2.HNO_3$ | 7.95 |

$$\text{mg Codeine salt in sample} = 5AC/A'$$

where $A$ and $A'$ refer to corrected $A$ of sample and std, resp., and $C$ = mg codeine salt/mL std soln.

Ref.: JAOAC **44,** 285(1961).

CAS-76-57-3 (codeine)
CAS-52-28-8 (codeine phosphate)
CAS-1420-53-7 (codeine sulfate)

## 965.44 Codeine and Terpin Hydrate in Drug Elixirs

### Spectrophotometric Method

**First Action 1965**
**Final Action 1966**

### *A. Reagents*

(**a**) *Color reagent.*—Either Folin-Denis reagent, **952.03A(a)**, or phosphotungstic-phosphomolybdic acid reagent prepd as follows: To 100 g pure Na tungstate and 20 g phosphomolybdic acid (free from nitrates and $NH_4$ salts), add 100 g $H_3PO_4$ and 700 mL $H_2O$. Boil over free flame 1.5–2 hr, cool, filter if necessary, and dil. to 1 L with $H_2O$. Equiv. amt of pure molybdic acid may be substituted for phosphomolybdic acid.

(**b**) *Terpin hydrate std soln.*—Accurately weigh ca 80 mg terpin hydrate, add 2 mL HOAc, and stir until terpin hydrate is almost dissolved. Add 10 mL alcohol, stir, and transfer to 100 mL vol. flask. Rinse dish with three 10 mL portions alcohol. Finally rinse few times with $H_2O$ and dil. to vol. with $H_2O$. Soln keeps indefinitely.

(**c**) *Codeine std soln.*—*See* **961.19B(b)**.

(**d**) *Water-saturated ether.*—Add 100 mL $H_2O$ to 200 mL ether in separator, shake, let stand 30 min, and discard $H_2O$.

(**e**) *Acidified water-saturated chloroform.*—Sat. 300 mL $CHCl_3$ with $H_2O$. After 30 min standing, transfer $CHCl_3$ to flask contg 3 mL HOAc.

(**f**) *Diatomaceous earth.*—*See* **960.53B**.

### *B. Determination of Terpin Hydrate*

Pipet 5 mL sample into distg flask and add 100 mL satd NaCl soln, 35 mL alcohol, 2 mL HOAc, and 10 mL $H_2O$. Distil, collecting 100 mL distillate.

Pipet 5 mL color reagent into 50 mL vol. flask. Cool under running $H_2O$ while slowly adding 5 mL $H_2SO_4$. Let mixt. come to room temp. and then add exactly 2 mL sample distillate. Place flask in boiling $H_2O$ 20 min. Cool under $H_2O$ to room temp. and dil. to vol. with dil. alcohol (1 + 3). Shake every few min until soln is clear (10–15 min). (If soln fails to clear, phosphomolybdic acid used to prep. color reagent is unsatisfactory.)

Let stand 0.5 hr and det. $A$ at 725 nm against reagent blank prepd without sample. Det. $A'$ of std soln prepd simultaneously with sample, beginning "Pipet 5 mL color reagent . . ."

Terpin hydrate (g/100 mL elixir) = $A \times C \times 20/A'$; where $C$ = g terpin hydrate/100 mL std soln.

### *C. Determination of Codeine*

Pipet 5 mL sample into 100 mL beaker, add 0.5 g *p-toluenesulfonic acid,* and stir with glass rod. Add 6 g diat. earth, mix to fluffy mass, and transfer to tube, **967.31A(a)**, contg plug of glass wool at base. Tamp flrmly, and cover with glass wool. Pass $H_2O$-satd ether over column and discard ether (Column *I*).

Mix 2 g diat. earth and 1 mL 1*N* $NaHCO_3$ (8.4 g/100 mL). Add to second tube (*II*), tamp, and cover with glass wool. Mount Column *I* over Column *II* and place 100 mL vol. flask contg 10 mL MeOH and 4 drops HCl under *II*. Add in 4 equal portions enough acidified $H_2O$-satd $CHCl_3$ to Column *I* to fill vol. flask to mark. Completely drain each portion before adding next.

Prep. std codeine soln contg 10 mL std soln, (**c**), and 2 drops HCl dild to 50 mL with $H_2O$-satd $CHCl_3$.

Det. $A$ and $A'$ at 287 nm of sample and std solns, resp., against mixt. of 10 mL MeOH and 2 drops HCl dild to 50 mL with $H_2O$-satd $CHCl_3$.

Codeine.$H_2O$, mg/100 mL elixir = $A \times C \times 20/A'$, where $C$ = mg codeine.$H_2O$ in 100 mL std soln.

Refs.: JAOAC **42,** 459(1959); **48,** 607(1965).

CAS-76-57-3 (codeine)
CAS-6059-47-8 (codeine monohydrate)
CAS-2451-01-6 (terpin hydrate)

## 972.53 Codeine in APC Drug Tablets

**Chromatographic Method**

**First Action 1972**
**Final Action 1973**

(Wash all ether with $H_2O$ and wash 0.1*N* $H_2SO_4$ with ether before use in prepn of reagents and in detn.)

### A. Reagents

(**a**) *Phosphate-citrate buffer.*—pH 5.1. *See* **970.78A** (**d**).
(**b**) *Di-(2-ethylhexyl) phosphoric acid (DEHP) soln.*—Prep. daily 1% soln in ether. (*Caution:* Avoid contact with skin.)
(**c**) *Codeine std soln.*—120 μg codeine salt/mL 0.1*N* $H_2SO_4$. Prep. fresh daily from phosphate or sulfate salt. Dissolve 60 mg codeine salt in 0.1*N* $H_2SO_4$ and dil. to 50 mL. Dil. 10 mL aliquot of this soln to 100 mL with 0.1*N* $H_2SO_4$.

### B. Determination

Grind sample to pass No. 60 sieve.

Pack small glass wool plug in base of 200 × 22 mm id chromatgc tube. Mix 2 g diat. earth, **960.53B**, and 1 mL pH 5.1 buffer, transfer to tube, and tamp. Accurately weigh ground sample contg 12 mg codeine for 0.5 and 1 grain tablets, 6 mg for 0.25 and 0.125 grain tablets, and 2.5 mg for 1 mg tablets into 100 mL beaker. Add 2.0 mL pH 5.1 buffer and mix to smooth suspension. Add 3 g diat. earth and mix well. Quant. transfer mixt. to tube, above pH 5.1 layer, with aid of scoop-type spatula. Wipe beaker and spatula with glass wool, add to tube, and tamp.

Add 50 mL ether to sample beaker, swirl, and transfer to column. Elute with 4 addnl 50 mL portions ether, letting each portion pass into column before adding next. Rinse tip of column with 2–3 mL $CHCl_3$. Discard rinse and eluate which contain aspirin, acetophenetidin, and caffeine (APC).

Wash column with 50 mL ether and discard eluate. Rinse tip of column with 2–3 mL $CHCl_3$ and dry tip with tissue paper. Place 125 mL separator under column and elute codeine with 50 mL 1% DEHP soln. After soln passes into column, elute with 50 mL ether. Rinse column tip with 5–10 mL ether into separator. Ext with three 10 mL portions 0.1*N* $H_2SO_4$, collecting exts in vol. flask (100 mL flask for 1 and 0.5 grain codeine, 50 mL flask for 0.25 and 0.125 grain codeine), and dil. to vol. with 0.1*N* $H_2SO_4$. (For tablets contg 1 mg codeine, use 10, 10, and 4 mL 0.1*N* $H_2SO_4$ and dil. to 25 mL.) Det. *A* of sample and std solns at max., ca 284 nm, against 0.1*N* $H_2SO_4$. Calc. codeine salt content of tablets.

Ref.: JAOAC **55,** 142(1972).

CAS-76-57-3 (codeine)
CAS-52-28-8 (codeine phosphate)
CAS-1420-53-7 (codeine sulfate)

## Codeine, Acetanilid, and Caffeine in Drugs

*See* **916.04★**.

## Ethylmorphine (Dionine) in Drug Sirups

*See* **961.18B**.

## 967.33 Hydrocodone (Dihydrocodeinone) in Drugs

**First Action 1967**
**Final Action 1968**

### A. General Method

*See* **961.18B**.

**In Presence of Antihistamines**

(*Caution:* See safety notes on distillation, toxic solvents and chloroform.)

### B. Apparatus and Reagents

(**a**) *Chromatographic tubes and tamping rod.*—*See* **967.31A**.
(**b**) *Column I.*—Thoroly mix 4 g acid-washed diat. earth, **960.53B**, and 3 mL ca 2*N* HCl. Transfer to tube and tamp to uniform mass, using gentle pressure.
(**c**) *Column II.*—Mix and tamp layers as in (**b**). (*1*) *Lower layer.*—2 g diat. earth and 1 mL 1*N* $NaHCO_3$. (*2*) *Upper layer.*—4 g diat. earth and 3 mL 6% succinic acid.
(**d**) *Equilibrated sulfuric acid.*—Thoroly shake 1*N* $H_2SO_4$ with small vol. $H_2O$-satd $CHCl_3$.
(**e**) *Hydrocodone std soln.*—175 μg hydrocodone bitartrate/mL. Dissolve 17.5 mg hydrocodone bitartrate in equilibrated $H_2SO_4$ in 100 mL vol. flask and dil. to vol. with equilibrated $H_2SO_4$. Shake ca 20 mL std soln with ca 75 mL $H_2O$-satd $CHCl_3$. Det *A′* of aq. phase from 360 to 250 nm.

### C. Determination

Mount Column *I* directly over Column *II*. Transfer 10.0 mL sample (or vol. contg ca 3–4 mg hydrocodone bitartrate) to separator. Add 5 mL $H_2O$ and 1 mL ca 1*N* NaOH, and ext with four 30 mL portions of $CHCl_3$. Pass each ext thru columns; let individual exts drain completely into both columns. Wash with 50 mL $H_2O$-satd $CHCl_3$. Discard Column *I*. Wash Column *II* with addnl 100 mL $H_2O$-satd $CHCl_3$. Discard eluate.

Add mixt. of 3.5 g diat. earth and 3 mL $NH_4OH$ to Column *II*, directly onto packing. Tamp. Pass 150 mL $H_2O$-satd $CHCl_3$ thru column. Evap. eluate to ca 75 mL or until $NH_3$ is completely removed (test vapors with moistened indicator paper). Quant. transfer to separator contg 20.0 mL equilibrated $H_2SO_4$ and shake thoroly. Det. *A* of aq. phase from 360 to 250 nm, using max., ca 282 nm, for calcn.

$$\text{mg Hydrocodone bitartrate in mL sample taken} = (A/A') \times C \times V$$

where *A* and *A′* refer to sample and std, resp., $C$ = mg hydrocodone bitartrate/mL in std, and $V$ = vol. $H_2SO_4$ (20 mL).

Refs.: JAOAC **50,** 655(1967); **51,** 494(1968).

CAS-143-71-5 (hydrocodone bitartrate)

## Morphine in Drug Sirups and Tablets

**Final Action**

See **961.18B**.

## 970.82 Morphine in Opium and Paregoric

**Chromatographic Method**

**First Action 1970**
**Final Action 1972**

(*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, triethylamine, diethyl ether, and chloroform.)

### A. Apparatus

(**a**) *Chromatographic tubes.*—*See* **967.31A**.
(**b**) *Diatomaceous earth.*—*See* **960.53B**.

### B. Reagents

(**a**) *Triethylamine.*—Purified as in **961.19B(a)**.
(**b**) *Morphine std soln.*—0.08 mg anhyd. morphine/mL. Accurately weigh morphine base or salt equiv. to 4 mg anhyd. morphine into 50 mL vol. flask. Add 10 mL MeOH, 1 mL HCl, and 1 mL $Et_3N$, and dil. to vol. with $CHCl_3$. Alternatively, prep. stock soln by dissolving accurately weighed std equiv. to ca 40 mg anhyd. morphine in 0.5 mL $Et_3N$ in 100 mL vol. flask, and dil. to vol. with MeOH. Pipet 10 mL of this stock soln into 50 mL vol. flask, add 1 mL $Et_3N$ and 1 mL HCl, and dil. to vol. with $H_2O$-satd $CHCl_3$.
(**c**) *Citrate buffer.*—0.1*M*, pH 4.4. Mix equal vols 0.1*M* Na citrate (2.94 g $Na_3C_6H_5O_7.H_2O$/100 mL) with 0.1*M* citric acid (2.10 g $H_3C_6H_5O_7.H_2O$/100 mL).

### C. Preparation of Sample

(**a**) *Opium.*—Accurately weigh ca 2 g opium into 100 mL vol. flask. Add 20 mL dimethyl sulfoxide (DMSO) and heat in beaker of boiling $H_2O$ or in steam bath ca 15 min. Swirl gently to dissolve, keeping opium particles in contact with DMSO and not letting particles remain on walls. Inspect soln carefully. If undissolved material remains, continue heating. Small amt insol. material, such as fine leaf fragments, sandlike particles, and gelatinous particles, may remain undissolved; add more DMSO, if necessary. Cool, add $H_2O$ to ca 90 mL, and mix. Let soln reach room temp., dil. to vol. with $H_2O$, and mix. (If foaming occurs on mixing, use 1 drop ether or alcohol to dispel foam.)

If sample is in pieces too large to fit in neck of vol. flask, accurately weigh into 250 mL beaker, add 20 mL DMSO, and heat in boiling $H_2O$ or steam bath. Use stirring rod to disperse sample while heating. Decant into 100 mL vol. flask. If undissolved opium remains in beaker, heat with addnl 3 mL portions DMSO as needed until soln is complete as possible (DMSO concn in final soln can vary over wide range without adverse effect.) Dil. to vol. with $H_2O$ as above.

Filter prepd soln thru paper, rejecting first 20 mL filtrate. Use 2 mL aliquot for prepn of Column *I*.

(**b**) *Paregoric.*—Evap. 10.0 mL paregoric, contg ca 4 mg morphine, to ca 2 mL on steam bath under stream of air. If evapn continues beyond 2 mL, dil. to 2 mL with $H_2O$. Cool soln to room temp. and then use for prepn of Column *I*.

### D. Preparation of Columns

(**a**) *Column I.*—(*1*) *Lower layer.*—Mix 3 g diat. earth and 2 mL citrate buffer; transfer to tube and tamp as in **961.19C**. (*2*) *Upper layer.*—Add 0.5 mL citrate buffer to 2.0 mL aliquot of sample ext, **970.82C(a)** or (**b**). Add 3 g diat. earth, mix, and transfer to tube. Dry-wash beaker with 1 g diat. earth and add to column; tamp and add glass wool pad.

(**b**) *Column II.*—Mix 3 g diat. earth and 2 mL 1.0*M* $K_2HPO_4$ (17.42 g/100 mL); transfer to tube, tamp, and add glass wool pad.

(**c**) *Column III.*—Mix 3 g diat. earth and 2 mL 0.5*M* NaOH; transfer to tube, tamp, and add glass wool pad.

### E. Determination

(Use $H_2O$-satd solvs thruout. Rinse each column tip with $CHCl_3$ before discarding columns or changing receivers.)

Pass 100 mL ether, followed by 100 mL $CHCl_3$, thru Column *I*. Discard eluates. Mount Columns *II* and *III* in series below Column *I*. Pass thru columns 5 mL 20% (v/v) $Et_3N$ in $CHCl_3$, followed by four 10 mL portions 1% $Et_3N$ in $CHCl_3$. Let each portion pass thru completely before next addn. Continue elution without delay. Discard Column *I*, and pass three 5 mL portions 1% $Et_3N$ in $CHCl_3$ thru remaining columns. Discard Column *II*. Wash Column *III* successively with 10 mL 1% $Et_3N$ in $CHCl_3$, 50 mL $CHCl_3$, 2 mL 10% HOAc in $CHCl_3$, and 50 mL 1% HOAc in $CHCl_3$. Discard all eluates.

Place as receiver under Column *III* 50 mL vol. flask contg 10 mL MeOH and 1 mL HCl. (Remove metal leashes from vol. flasks to prevent contamination during transfer to cells.) Elute column with 5 mL 20% $Et_3N$ in $CHCl_3$, followed by 33 mL 1% $Et_3N$ in $CHCl_3$. Dil. eluate to vol. with $CHCl_3$.

Scan spectrum of eluate and morphine std from 360 to 255 nm, using $CHCl_3$ as ref. (Film of $Et_3N.HCl$ may adhere to walls of cells. Rinse cells carefully with $H_2O$ and alcohol; then wipe clear before scanning.) Correct *A* at max., ca 285 nm, by extrapolating baseline from 340 to 310 nm to this wavelength.

$$\text{mg Anhyd. morphine in aliquot taken} = (W' \times A/A') \times f$$

where $W'$ = mg morphine in std soln, $A$ and $A'$ = corrected *A* of sample and std, resp., and $f$ = factor to convert wt std to its equiv. in anhyd. morphine (if hydrated morphine or morphine salt is used as std).

Refs.: JAOAC **51,** 1315(1968); **53,** 603(1970).

CAS-57-27-2 (morphine)

## 989.08 Morphine Sulfate in Bulk Drug and Injections
### Liquid Chromatographic Method
### First Action 1989

Method Performance:
$s_r = 0.91$; $s_R = 1.41$; $RSD_r = 0.9\%$; $RSD_R = 1.4\%$
Injection, 2 mg/mL:
$s_r = 0.027$; $s_R = 0.035$; $RSD_r = 1.3\%$; $RSD_R = 1.7\%$
Injection, 8 mg/mL:
$s_r = 0.050$; $s_R = 0.173$; $RSD_r = 0.6\%$; $RSD_R = 2.1\%$
Injection, 10 mg/mL:
$s_r = 0.061$; $s_R = 0.185$; $RSD_r = 0.6\%$; $RSD_R = 1.8\%$

### A. Principle

Bulk drug and injection samples are prepd by direct diln in modified LC mobile solv. Morphine sulfate is quantitated and preservative phenol is identified by UV detection at 284 nm. Degradation product pseudomorphine and contaminant 2-mercaptobenzothiazole (2-MCBT) are detected and identified at 230 nm.

### B. Apparatus

(Equiv. app. may by substitued.)

(**a**) *Liquid chromatograph.*—Equipped with injection valve with 20 µL sample loop, solv. delivery system, recording integrator, and variable wavelength UV detector capable of monitoring at 230 and 284 nm. Operating conditions: flow rate 1.5–2.0 mL/min (morphine sulfate should be eluted between 5 and 8 min).; temp. ambient; injection vol. 20 µL. (System must meet system suitability requirements.)

(**b**) *LC column.*—Stainless steel, 30 cm × 3.9 mm id, packed with µBondapak $C_{18}$, 10 µm (Waters Associates, Inc.). Use of different $C_{18}$ column may require addn of amine modifier to meet system suitability requirements.

(c) *Membrane filters*.—Nylon 66, pore size 0.45 μm, 25 and 47 mm diam.

### C. Reagents

(Protect all morphine sulfate solns from direct light.)

(a) *Methanol*.—LC grade.
(b) *Acetic acid*.—Anal. grade.
(c) *1-Heptanesulfonic acid Na salt*.—Monohydrate. ≥98% (Eastman Kodak Co., or equiv.).
(d) *Morphine sulfate reference std*.—USP Morphine Sulfate (Pentahydrate) Ref. Std. Do *not* dry before use.
(e) *Phenol*.—Crystals, anal. grade.
(f) *Pseudomorphine*—Prep. as follows: Add 10 g morphine to hot soln of 2.0 g KOH in 1 L $H_2O$, and let mixt. cool to room temp. Then add soln of 11.58 g $K_3Fe(CN)_6$ in 400 mL $H_2O$ during 50 min, with stirring, and continue stirring addnl 30 min. Collect solid matter and stir with hot MeOH to remove morphine (1 g). Dissolve MeOH-insol. material (8.4 g) in concd $NH_3$ soln, dil. with $H_2O$ to 700 mL, and boil mixt. 7.7 g pseudomorphine is deposited as almost colorless rods that decompose at ca 330°. *A* maxima are at 231 and 261 nm in 0.1N $H_2SO_4$. (From Bentley, K.W., & Dyke, S.F. J. Chem. Soc. 2574(1959).)
(g) *2-Mercaptobenzothiazole (2-MCBT)*.—98% (Aldrich Chemical Co.).
(h) *Mobile solvent*.—Mix 240 mL MeOH with 720 mL 0.005M 1-heptanesulfonic acid Na salt monohydrate in $H_2O$ and add 10 mL HOAc. Pass soln thru 0.45 μm filter and degas before use. Adjust MeOH or 1-heptanesulfonic acid Na salt soln content so that system meets suitability test requirements.
(i) *Dilution solvent*.—Mobile solv. (h) prepd without 1-heptanesulfonic acid Na salt.

### D. Preparation of Standard Solutions

(a) *Morphine sulfate std soln*.—Dissolve accurately weighed amt USP Morphine Sulfate Ref. Std in diln solv. to prep. soln contg ca 0.24 mg morphine sulfate/mL.
(b) *Morphine sulfate–phenol std soln*.—Dissolve accurately weighed amts USP Morphine Sulfate Ref. Std and phenol in diln solv. to prep. soln contg ca 0.24 mg morphine sulfate and ca 0.15 mg phenol/mL.
(c) *Phenol std solns*.—*Stock soln*.—About 2.0 mg phenol/mL $H_2O$. *Working soln*.—Add diln solv. to stock soln to prep. soln contg ca 0.15 mg phenol/mL.
(d) *Pseudomorphine std solns*.—*Stock soln*.—0.2–0.3 mg pseudomorphine/mL diln solv. *Working soln*.—Add diln solv. to stock soln to prep. soln contg ca 20 μg pseudomorphine/mL.
(e) *2-MCBT std solns*.—About 80 μg 2-MCBT/mL MeOH. *Working soln*.—Add diln solv. to stock soln to prep. soln contg ca 5–6 μg 2-MCBT/mL.
(f) *Mixed std soln*.—Prep. soln in diln solv. to contain morphine sulfate, phenol, pseudomorphine, and 2-MCBT at concns approx. equal to individual working std solns.

### E. Preparation of Samples

(a) *Bulk drug*.—Accurately weigh ca 100 mg bulk drug into 25 ml vol. flask and dil. to vol. with diln solv. Dil. soln with diln solv. to final concn of ca 0.24 mg morphine sulfate/mL.
(b) *Injections*.—Dil. accurately measured vol. morphine sulfate injection with diln solv. to final concn of ca 0.24 mg morphine sulfate/mL.

### F. Determination

Filter all solns thru 0.45 μm filter before injection.

Let LC system equilibrate 1 h with mobile solv. flow rate ca 1.5 mL/min. Set wavelength at 284 nm. Inject 20 μL morphine sulfate–phenol std soln (b). Coeff. of var. (CV) of morphine sulfate peak area for 5 replicate injections should be <2%. Resolution factor for phenol–morphine sulfate pair should be >2. Tailing factor at 5% peak ht for morphine sulfate peak should be <2. Proceed with analysis by alternating duplicate 20 μL injections of samples and morphine sulfate std soln (a).

If contaminants such as 2-MCBT and pseudomorphine are suspected in sample, det. identity by using stds (c)–(f). Detect phenol and morphine sulfate and quantitate latter at 284 nm. Detect 2-MCBT and pseudomorphine at 230 nm. Approx. retention items are 10–20 min for 2-MCBT and 20–45 min for pseudomorphine.

### G. Calculations

Calc. results as follows:

Morphine sulfate bulk drug, % purity

$$= (R/R') \times (W'/W) \times (D'/D) \times 100$$

Morphine sulfate, mg/mL injection

$$= (R/R') \times W' \times (D'/D)$$

where $R$ and $R'$ = av. peak areas for duplicate injections of sample and std, resp.; $W'$ and $W$ = wt of std and sample, resp.; and $D'$ and $D$ = diln of std and sample, resp.

Ref.: JAOAC **71,** 1046(1988).

CAS-6211-15-0 (morphine sulfate, pentahydrate)

## TROPANE ALKALOIDS

### 958.16★ Atropine in Drug Tablets

**Infrared Spectroscopic Method**

**Final Action 1965**
**Surplus 1983**

*See* **38.028**, 14th ed.

### 932.23★ Belladonna and Stramonium Alkaloids in Drug Ointments

**Final Action**
**Surplus 1965**

*See* **32.037** and **32.038**, 10th ed.

### Homotropine in Drug Tablets

**Final Action**

*See* **961.18B**.

### 973.75 Benztropine Mesylate in Drugs

**Spectrophotometric Method**

**First Action 1973**
**Final Action 1975**

(Not applicable in presence of compds reacting with bromophenol blue, e.g., quaternary ammonium compds)

### A. Principle

Benztropine is extd from acid soln by bromophenol blue-$CHCl_3$ soln, forming dye complex with max. *A* at ca 410 nm.

### B. Reagents and Apparatus

(a) *Dye soln*.—Weigh 100 mg reagent bromophenol blue into 1 L vol. flask, add ca 750 mL $CHCl_3$, stir mech. 10 min to dissolve, and dil. to vol. with $CHCl_3$. Filter thru small pad of glass wool. Dil. 50 mL to 500 mL with $CHCl_3$. Prep. fresh daily.

(**b**) *Benztropine mesylate std soln*.—1 mg/100 mL. Weigh 100 mg USP Ref. Std into 100 mL vol. flask and dissolve and dil. to 100 mL with 0.2$N$ $H_2SO_4$. Dil. 10 mL aliquot to 100 mL with 0.2$N$ $H_2SO_4$ and further dil. 10 mL dild soln to 100 mL with 0.2$N$ $H_2SO_4$. Prep. fresh daily.

(**c**) *Spectrophotometer*.—Recording, with 5 cm matched cells.

### C. Preparation of Sample

(**a**) *Tablets*.—Transfer accurately weighed ground portion contg ca 1 mg benztropine mesylate to 100 mL vol. flask, using ca 70 mL 0.2$N$ $H_2SO_4$. Shake mech. 15 min and filter thru Whatman No. 541 paper wetted with 0.2$N$ $H_2SO_4$ into 100 mL vol. flask. Rinse flask and filter with three 5 mL portions 0.2$N$ $H_2SO_4$, rinse filter with several small portions 0.2$N$ $H_2SO_4$, adding rinses to soln, and dil. to vol. with 0.2$N$ $H_2SO_4$.

(**b**) *Injections*.—Transfer aliquot contg ca 1 mg benztropine mesylate to 100 mL vol. flask and dil. to vol. with 0.2$N$ $H_2SO_4$.

### D. Determination

Perform detn on same day sample and std solns are prepd. Place 25 mL each sample soln and std soln and 0.2$N$ $H_2SO_4$ for blank into sep. 250 mL separators and treat similarly. Add 50 mL dye soln and shake vigorously 1 min. Let sep. and drain lower layer into 125 mL separator contg 25 mL 0.2$N$ $H_2SO_4$. Wash by inverting 5 times and let stand ca 20 min. Filter lower $CHCl_3$ layer thru glass wool wetted with $CHCl_3$ into 100 mL vol. flask, covering funnel with watch glass. Re-ext aq. soln in 250 mL separator with 50 mL dye soln, shake vigorously 1 min, drain into same 125 mL separator, and wash and filter as before, rewetting glass wool with $CHCl_3$ if necessary. Dil. to vol. with $CHCl_3$, mix, and place in dark 40 min.

Record spectra of std and sample solns against blank in matched 5 cm cells, and det. $A$ at max., ca 410 nm.

$$\text{mg Benztropine mesylate/100 mL} = (A/A') \times C$$

where $A$ and $A'$ refer to sample and std solns, resp; and $C$ = concn std soln in mg/100 mL.

Ref.: JAOAC **56,** 681(1973).

CAS-132-17-2 (benztropine mesylate)

## 927.12★ Mydriatic and Myotic Drugs

**Cat-Eye Bioassay Method**

**Final Action**
**Surplus 1972**

*See* **36.084–36.088**, 12th ed.

## XANTHINE ALKALOIDS

### Caffeine in Drugs

(**a**) *Microchemical tests*.—*See* Table **960.56**.
(**b**) *With acetanilid*.—*See* **916.03★**.
(**c**) *With acetanilid and codeine*.—*See* **916.04★**.
(**d**) *With acetanilid and quinine*.—*See* **916.05★**.
(**e**) *With acetanilid, morphine, and quinine*.—*See* **916.06★**.
(**f**) *With phenacetin*.—*See* **916.08★**.
(**g**) *With phenacetin and aminopyrine*.—*See* **941.22★**.
(**h**) *With phenacetin, aminopyrine, and phenobarbital*.—*See* **942.30★**.
(**i**) *With phenacetin and aspirin*.—*See* **960.59★**.
(**j**) *With phenacetin, aspirin, and codeine*.—*See* **972.53**.
(**k**) *With effervescent potassium bromide*.—*See* **980.35E★**.

## 936.18 Theobromine in Theobromine-Calcium Salicylate Drugs

**Final Action**

### A. Method I

Dry ca 0.5 g sample at 110° to const wt. Weigh 0.2 g dried substance into g-s 100 mL vol. flask, add 2 mL HOAc, and warm on steam bath. Add 10 mL boiling $H_2O$ and shake until dissolved, adding more boiling $H_2O$ if necessary. Cool soln to room temp. (Soln should be clear or nearly so.) Add 50 mL 0.1$N$ I, 20 mL satd NaCl soln, and 2 mL HCl. Shake well and dil. to vol. with $H_2O$. Shake again and let stand overnight. Filter, discarding first 10 mL filtrate. Titr. 50 mL filtrate with 0.1$N$ $Na_2S_2O_3$, using starch soln, as indicator (mix ca 2 g finely powd. potato starch with cold $H_2O$ to thin paste; add ca 200 mL boiling $H_2O$, stirring const., and immediately discontinue heating; add ca 1 mL Hg, shake, and let soln stand over the Hg). 1 mL 0.1$N$ I = 0.00450 g theobromine, $C_7H_8O_2N_4$.

Ref.: JAOAC **19,** 534(1936).

***Method II***

### B. Indicator

*Phenol red indicator*.—Triturate 0.1 g phenol red in agate mortar with 15 mL 0.02$N$ NaOH until dissolved and dil. soln to 200 mL with recently boiled $H_2O$.

### C. Determination

Weigh 0.5 g powd tablets, 0.4 g powder, or 0.2 g theobromine alkaloid into 300 mL beaker and add 100 mL $H_2O$. Warm moderately over flame and add 15 mL ca 0.1$N$ $H_2SO_4$. Heat to boiling to ensure complete soln and to remove $CO_2$. Cool to room temp. Add 1.5 mL phenol red indicator and make slightly alk. with ca 0.1$N$ NaOH (violet-red); then titr. carefully to acid reaction with 0.1$N$ $H_2SO_4$ (yellow). To this soln add 25 mL (an excess) neut. 0.1$N$ $AgNO_3$, **941.18**, and immediately titr. liberated $HNO_3$ with 0.1$N$ NaOH to distinct violet-red. Cautiously titr. dropwise with const stirring near end point. 1 mL 0.1$N$ NaOH = 0.01802 g $C_7H_8O_2N_4$.

Refs.: JAOAC **21,** 555(1938); **22,** 729(1939).

CAS-83-67-0 (theobromine)

## 937.14★ Theophylline in Drugs

**Gravimetric Method**

**Final Action**
**Surplus 1974**

(Applicable to solns and tablets)

*See* **38.101**, 12th ed.

## IPECAC ALKALOIDS

### Emetine Hydrochloride in Drug Tablets

**Final Action**

*See* **961.18B**.

## 971.40 Ipecac Alkaloid in Drugs
### Chromatographic Method
**First Action 1971**
**Final Action 1973**

(Applicable to sirup, fluidextract, and powd prepns)

(*Caution:* See safety notes on distillation, triethylamine, diethyl ether, chloroform, and isooctane.)

### *A. Principle*

Principal ipecac alkaloids, phenolic cephaeline and its Me ether emetine, constitute over 90% of total alkaloids of ipecac. They occur in ratios varying from 3:1 to 1:3 in the several species and constitute total of ca 2–3% of wt of root. Minor alkaloids, mainly psychotrine, *o*-methyl psychotrine, and emetamine, are closely related structurally to emetine, differing principally by presence of addnl double bonds, which affect their UV spectra.

Four-column system isolates emetine and cephaeline from ipecac sirup, fluidext, and powd root. Prepn of sample itself, made alk. with $NaHCO_3$, constitutes immobile phase in first column. Ether eluate of this column, contg total alkaloids together with other ether extractives from sample, is passed thru phosphate buffer column 1*N* with respect to chloride ion. Alkaloids are retained in column while nonalkaloidal extractives are partly washed thru column. Major purification is achieved in next step in which mixt. of $CHCl_3$ and ether selectively removes emetine and cephaeline (with perhaps trace amts of other alkaloids) from phosphate column, and carries them onto pH 4.0 column, on which they are retained. Purification achieved in this step is 2-fold: (*1*) Phosphate buffer column retains alkaloids (presumably emetamine and psychotrine) which absorb in UV region between 380 and 300 nm, in which region emetine and cephaeline do not absorb. (Retained alkaloids can be recovered in part by elution with $CHCl_3$, and remainder with ether soln of di(ethylhexyl)phosphoric acid, which is very effective counter-ion for extn of alkaloids.) (*2*) Eluate from phosphate column carries thru pH 4.0 column material which absorbs in UV. This material, if not removed, would accompany emetine and thus give spuriously high assay values. This is especially significant in the case of ipecac sirup, which contains large amt of substance, provisionally identified as 5-hydroxymethyl-2-furaldehyde, spectrum of which closely resembles that of emetine.

Emetine and cephaeline are sepd in final partition step. Combined alkaloids are eluted from pH 4.0 column after raising pH to >8 *in situ* with soln of $Et_3N$ in mixt. of ether and isooctane. This eluate continues thru 0.5*N* NaOH column which retains phenolic alkaloid cephaeline. Finally, cephaeline is eluted directly with $CHCl_3$ from NaOH column, with no adjustment of pH.

Respective eluates are extd with 0.5*N* $H_2SO_4$ and *A* of acid solns are measured at 283 nm. Since only emetine std is available, and inasmuch as cephaeline and its Me ether emetine have essentially same molar *A*, this std is used for both emetine and cephaeline.

### *B. Reagents*

(**a**) *Triethylamine.*—Must pass following test: Transfer 3.0 mL to 50 mL graduate or vol. flask contg 15 mL 4*N* $H_2SO_4$, dil. to vol. with 0.5*N* $H_2SO_4$, and mix. Scan spectrum from 350 to 240 nm against 0.5*N* $H_2SO_4$ as blank. If *A* at ca 250 nm is >0.040, purify as in **961.19B(a)**.

(**b**) *Dimethyl sulfoxide* (*DMSO*).—Spectral grade (Fisher Scientific Co., or equiv.).

(**c**) *Ethyl ether.*—Peroxide-free. *See* Definitions of Terms and Explanatory Notes, ether.

(**d**) *Phosphate buffer.*—Mix 3 vols 0.5*M* $KH_2PO_4$ with 1 vol. 0.5*M* $K_2HPO_4$, and adjust to pH 6.0 ± 0.05. Dissolve 7.46 g KCl/100 mL mixt.

(**e**) *Citrate buffer.*—Mix equal vols 0.5*M* citric acid and 0.5*M* Na citrate, and adjust to pH 4.0 ± 0.05.

(**f**) *Emetine std soln.*—Accurately weigh ca 3 mg emetine.2HCl.$3H_2O$ and dissolve in 50 mL 0.5*N* $H_2SO_4$ (1 mg emetine.2HCl.$3H_2O$ is equiv. to 0.79 mg emetine base). If alkaloid content of std is not known, det. as in **961.18B**.

### *C. Preparation of Sample*

(**a**) *Sirup.*—Pipet 10 mL $H_2O$ into 25 mL vol. flask. Using 20 mL pipet, add sirup to vol., avoiding wetting neck of flask above graduation mark, and mix. Use 4.0 mL for prepn of Column *I*.

(**b**) *Fluidextract.*—Pipet 5 mL fluidext into 50 mL vol. flask, dil. to vol. with $H_2O$, and mix. Pipet 2 mL into 150 mL beaker. Evap. almost to dryness on steam bath, using gentle stream of air to remove alcohol. Add 3 mL $H_2O$ and ca 1 g $NaHCO_3$, and mix. Proceed as in **971.40D(a)**, beginning ". . . add 6 g diat. earth, and mix."

(**c**) *Powdered ipecac.*—Accurately weigh ca 200 mg powd ipecac (60 mesh) in 150 mL beaker, add 2 mL DMSO, and mix thoroly with flattened stirring rod to assure complete wetting of powder. Let stand ca 30 min. Add 2 mL $H_2O$ and ca 1 g $NaHCO_3$, and mix. Proceed as in **971.40D(a)**, beginning ". . . add 6 g diat. earth, and mix."

### *D. Preparation of Columns*

Transfer specified soln to 150 mL beaker, add specified wt acid-washed diat. earth, and mix until uniform fluffy mixt. is obtained.

(**a**) *Column I.*—To 4.0 mL sample soln, add 1 g $NaHCO_3$, mix, add 6 g diat. earth, and mix. Dry-wash beaker with 1 g diat. earth and add to column. (Since emetine is unstable in alk. soln and in $CHCl_3$, proceed with detn without delay.)

(**b**) *Column II.*—Add 3 g diat. earth to 2 mL phosphate buffer and mix.

(**c**) *Column III.*—Add 3 g diat. earth to 2 mL citrate buffer and mix.

(**d**) *Column IV.*—Add 3 g diat. earth to 2 mL 0.5*N* NaOH and mix.

Quant. transfer mixts to sep. chromatgc tubes, **967.31A**. Tamp each to uniform mass and top with glass wool pad.

### *E. Determination*

(Use $H_2O$-satd solvs thruout. Rinse tips of columns with ether before discarding columns and when changing solvs. Remove metal leashes from separators and vol. flasks.)

Mount Column *II* under Column *I*. Pass three 50 mL portions ether thru columns. Discard eluate and Column *I*. Mount Column *III* below Column *II*. Pass three 50 mL portions $CHCl_3$-ether (3 + 1) thru columns. Let each portion pass thru completely before next addn. Continue elution without delay. Discard eluate and Column *II*. Pass 25 mL $CHCl_3$-ether (3 + 1) thru Column *III*. Discard eluate. Pass 25 mL ether-*isooctane* (1 + 1) thru Column *III*. Discard eluate. Prewash Column *IV* with 20 mL 2% $Et_3N$ in ether-isooctane (1 + 1) and discard eluate. Mount Column *IV* below Column *III*. Collect emetine eluate in 125 mL separator contg 15 mL 4*N* $H_2SO_4$ by passing 10 mL 20% $Et_3N$ in ether-isooctane (1 + 1), followed by three 10 mL portions 2% $Et_3N$ in ether-isooctane (1 + 1) thru Columns *III* and *IV*. Discard Column *III*. Pass two 10 mL portions of 2% $Et_3N$ in ether-isooctane (1 + 1) thru Column *IV*, collecting eluate in same 125 mL separator. Shake separator, and transfer acid layer to 50 mL vol. flask. Ext solv. with two 10 mL portions 0.5*N* $H_2SO_4$ and combine in vol. flask. Dil. to vol. with 0.5*N* $H_2SO_4$.

Elute cephaeline from Column *IV* with 75 mL $CHCl_3$. Collect eluate in 250 mL separator contg 20 mL 0.5*N* $H_2SO_4$ and 150 mL ether. Shake and transfer acid layer to 50 mL vol. flask. Ext solv. with 2 addnl 10 mL portions 0.5*N* $H_2SO_4$ and combine in vol. flask. Dil. to vol. with 0.5*N* $H_2SO_4$.

Scan spectra of emetine std and sample fractions from 350 to 240 nm against 0.5*N* $H_2SO_4$. Correct *A* at 283 nm by subtracting *A* at 350 nm for samples ($\Delta A$) and for stds ($\Delta A'$).

#### F. Calculations

(**a**) *Sirup*.—Calc. mg drug/100 mL = $0.1 \times (\Delta A/\Delta A') \times C \times D \times F$, where *C* = concn std in µg/mL, *D* = diln factor = $(25/15) \times (50/4) = 20.8$, and *F* for emetine = 0.79 if emetine.2HCl.$3H_2O$ is used as std; if $H_2O$ content differs from $3H_2O$, recalc. factor from MW of anhyd. salt = 554. *F* for cephaeline = $0.79 \times 0.971 = 0.767$, where 0.971 is ratio of MW of emetine and cephaeline.

(**b**) *Fluidextract*.—Calc. mg drug/100 mL as in (**a**), using $D = (50/2) \times (50/5) = 250$.

(**c**) *Powdered ipecac*.—Calc. % drug = $(\Delta A/\Delta A') \times (F \times 5C/W)$, where *C* and *F* are defined in (**a**), and *W* = mg sample.

Refs.: JAOAC **54,** 609, 614(1971).

CAS-483-17-0 (cephaeline)
CAS-483-18-1 (emetine)

## EPHEDRA ALKALOIDS

### 929.13★ Alkaloids in Ephedra

**Final Action**
**Surplus 1965**

*See* **32.047**, 10th ed.

### 931.13 Ephedrine in Drug Inhalants

**Final Action**

#### A. Method I★
#### —Surplus 1970

*See* **36.067**, 11th ed.

#### B. Method II★
#### —Surplus 1983

*See* **38.050**, 14th ed.

### 950.93★ Ephedrine in Water-Soluble Jellies, Sirups, and Solutions of Ephedrine Salts

**Gravimetric Method**

**Final Action**
**Surplus 1972**

*See* **38.039**, 12th ed.

### 947.14 Ephedrine in Drug Tablets and Capsules

**Final Action**

#### A. Method I

See **961.18B**.

#### B. Method II★
#### —Final Action 1972
#### —Surplus 1975

*See* **38.041**, 12th ed.

### 980.35 Ephedrine in Solid Dosage Drugs

**Spectrophotometric Method**

**First Action 1980**
**Final Action 1982**

#### A. Principle

Ephedrine is eluted with $CH_2Cl_2$ from weakly basic diat. earth column, retained on weakly acidic column, and converted to benzaldehyde by on-column periodate reaction. Benzaldehyde is detd by UV spectrophotometry.

#### B. Reagents

(**a**) *Phosphate-chloride soln*.—Dissolve 5 g $KH_2PO_4$ and 7.5 g KCl in 100 mL $H_2O$. (Omit KCl for pseudoephedrine assay.)

(**b**) *Sodium metaperiodate soln*.—Dissolve 2 g $NaIO_4$ in 20 mL $H_2O$. Store in dark.

(**c**) *Water-saturated methylene chloride*.—Sat. ca 400 mL $CH_2Cl_2$ by shaking with equal vol. $H_2O$ 1 min. Use thruout method.

(**d**) *Ephedrine std soln*.—0.4 mg/mL. Accurately weigh ca 100 mg std ephedrine salt and transfer to 250 mL vol. flask. Dissolve and dil. to vol. with $H_2O$.

#### C. Apparatus

(**a**) *Recording spectrophotometer*.—For UV, with matched 1 cm cells.

(**b**) *Chromatographic tubes and tamping rod*.—*See* **967.31A**(**a**) and (**b**).

#### D. Preparation of Sample and of Chromatographic Columns

Weigh amt well mixed and ground sample contg ca 100 mg ephedrine salt into 250 mL vol. flask. Add ca 100 mL $H_2O$, let stand ≥10 min with occasional shaking, dil. to vol. with $H_2O$, mix well, and let solids settle. Prep. columns as follows:

(**a**) *Column I*.—Add 2.0 mL sample soln to 300 mg $K_2HPO_4$ in 150 mL beaker. Swirl to dissolve. Add 3 g diat. earth, **960.53B**, mix, transfer quant. to column, and tamp. Dry wash beaker with 1 g diat. earth, add to column, and tamp. Cover with small pad of glass wool. (If acidic compds such as acetaminophen or theophylline are present, underlay column mixt. with 3 g diat. earth mixed with 2.0 mL 10% NaOH soln.)

(**b**) *Column II*.—Mix 3 g diat. earth and 2 mL phosphate-chloride soln and transfer to column. Tamp and cover with small pad of glass wool.

(**c**) *Column III*.—Mix 0.5 mL $H_2O$ and 1 g diat. earth, transfer to column, and tamp. Mix 3 g diat. earth and 2.0 mL $NaIO_4$ soln, transfer to column, tamp, and cover with pad of glass wool.

#### E. Determination

Arrange columns so that effluent from I flows into II. Elute combined columns with four 25 mL portions of $CH_2Cl_2$, letting each portion sink entirely into surface of both columns. Rinse tip of column I into column II with $CH_2Cl_2$, and discard column I. Elute column II with addnl 25 mL $CH_2Cl_2$. Discard all eluates.

Mount column II over column III and place 100 mL vol. flask under column III. With pipet, evenly distribute 1.0 mL conc. $NH_4OH$ directly onto surface of column II. Elute combined columns with four 25 mL portions $CH_2Cl_2$, letting each portion sink entirely into surface of each column. Rinse tip of column II into column III with ca 1 mL $CH_2Cl_2$. Discard column II and continue elution of column III to vol.

Prep. sep. column III, omitting glass wool at top. Mix 2.0 mL std soln and 3 g diat. earth, transfer quant. to same column, and tamp. Dry wash beaker with 1 g diat. earth, transfer to column, tamp, and cover with pad of glass wool. Place 100 mL vol. flask under column. Wet column with 10 mL $CH_2Cl_2$. With pipet, evenly distribute 1.0 mL concd $NH_4OH$ directly onto surface of column. Elute column with four 25 mL portions $CH_2Cl_2$, letting each portion sink entirely into surface of column. Rinse tip of column into flask, and dil. to vol. with $CH_2Cl_2$. Elute std column with addnl 25 mL $CH_2Cl_2$ and collect eluate for use as blank.

Scan spectra of sample and std eluates from 350 to 230 nm against column blank eluate. If cloudy, let soln clear (ca 1 min) before detg $A$. Det. $A$ and $A'$ of sample and std solns, resp., at min., ca 267 nm, and at max., ca 246 nm and det. difference, $\Delta A$ and $\Delta A'$.

$$\text{mg Ephedrine sulfate/dosage unit} = (\Delta A/\Delta A') \times C \times 250 \times D/(2 \times W)$$

where $C$ = concn std, mg/mL; $D$ = av. wt/dosage unit; and $W$ = mg sample.

Ref.: JAOAC **63**, 692(1980).

CAS-134-72-5 (ephedrine sulfate)

## 981.26 Pseudoephedrine HCl and Triprolidine HCl or Chlorpheniramine Maleate in Drug Combinations

**Liquid Chromatographic Method**

**First Action 1981**
**Final Action 1983**

(*Caution:* See safety notes on acetonitrile.)

### A. Principle

Pseudoephedrine HCl and triprolidine HCl or chlorpheniramine maleate in syrups or tablets are detd by reverse phase LC using ion-pairing. Column chromatgc cleanup of syrup removes preservatives.

### B. Apparatus

(Equiv. app. may be substituted.)

(**a**) *Liquid chromatograph*.—Equipped with Model U6K injector, Model 6000A solv. delivery system, and Model 440 UV $A$ detector (Waters Associates, Inc.). Operating conditions: flow rate 1.5 mL/min; 254 nm detector, 0.05 AUFS; temp. ambient; 10 μL injection.

(**b**) *LC column*.—μBondapak Phenyl, 10 μm particle size, 3.9 mm (id) × 30 cm long (Waters Associates, Inc.). Wash column daily after use with $H_2O$ followed by MeOH.

(**c**) *Recorder*.—10 mV with 0.5 cm/min chart speed (Omniscribe B-5000, Houston Instrument, 8500 Cameron Rd, Austin, TX 78753).

(**d**) *Integrator*.—Chromatopac Data Processor Model E1A (Shimadzu Scientific Instruments, Inc., 7102 Riverwood Rd, Columbia, MD 21046).

(**e**) *Filters*.—Millipore types HA and FH (pore size 0.45 μm) and type AP prefilter (Millipore Corp.).

(**f**) *Chromatographic tubes*.—25 × 2.5 cm od, glass column with one restricted end.

### C. Reagents

(**a**) *Solvents*.—Distd-in-glass (Burdick & Jackson Laboratories, Inc.), or equiv.

(**b**) *Sodium hydroxide–sodium chloride soln*.—1.0N NaOH-0.5N NaCl. Dil. 2.0 g NaOH and 1.46 g NaCl to 50 mL with $H_2O$.

(**c**) *Diatomaceous earth*.—*See* **960.53B**.

(**d**) *Internal std soln*.—0.4 mg pheniramine maleate/mL $H_2O$.

(**e**) *Antihistamine soln*.—Transfer 31.25 mg accurately weighed NF Ref. Std Triprolidine HCl or 50 mg USP Ref. Std Chlorpheniramine Maleate to 100 mL vol. flask and dil. to vol. with $H_2O$.

(**f**) *LC std solns*.—(*1*) *Stock soln*.—Transfer 30 mg accurately weighed NF Ref. Std Pseudoephedrine HCl to 10 mL vol. flask. Pipet 4.0 mL antihistamine soln and 5.0 mL internal std soln into flask and dilute to volume with $H_2O$. (*2*) *Working soln*.—Transfer 2.0 mL aliquot stock soln and 2.0 mL $H_2O$ to small g-s flask and mix.

(**g**) *LC mobile phase*.—Transfer 1 bottle PIC Reagent B-5 (Waters Associates, Inc.) to 1 L vol. flask. Add 350 mL $CH_3CN$, and dil. to vol. with $H_2O$. Mag. stir 5 min. Filter through type FH filter prewetted with $CH_3CN$. Place in ultrasonic bath 10 min to deaerate. (PIC Reagent B-5 contains enough pentanesulfonic acid and HOAc so that when 1 bottle is dild to 1 L, concns are 0.005M and 1%, resp.)

### D. Preparation of Samples

(**a**) *Tablets*.—Det. av. wt of tablets and grind to pass No. 60 sieve. Transfer accurately weighed portion of powder contg 60 mg pseudoephedrine HCl to 50 mL g-s flask. Pipet 10 mL internal std soln and 30 mL $H_2O$ into flask and place in ultrasonic bath 1 min. Stopper and shake flask vigorously. Filter portion of soln thru type HA filter into small g-s flask. Use type AP prefilter if necessary.

(**b**) *Syrups*.—Pipet 5.0 mL internal std soln into 10 mL vol. flask. Carefully add sample syrup to vol. (do not wet flask above mark) and mix. Place small glass wool plug in base of chromatgc tube as support. Mix 0.7 mL NaOH-NaCl soln and 1.0 g diat. earth. Transfer mixt. to tube and tamp to uniform firm mass. Piept 2.0 mL aliquot dild sample soln into 100 mL beaker. Add 0.3 mL NaOH-NaCl soln and swirl to mix. Add 3.5 g diat. earth and mix thoroly. Transfer mixt. to tube in 3 portions, tamping after addn of each portion. Dry-wash beaker with 1.0 g diat. earth, add wash to column, and tamp. Cover with small glass wool plug. Elute column with four 50 mL portions of $H_2O$-satd $CHCl_3$ into 250 mL g-s flask contg 5 mL alcohol and 5 drops of HCl. Evap. to dryness on steam bath with stream of air. Add 5 mL alcohol and evap. to dryness again. Pipet 4.0 mL $H_2O$ into flask and swirl to dissolve residue. Filter soln through type HA filter into small g-s flask.

### E. Determination

Let LC system equilibrate with column in instrument and set mobile phase at flow rate of 1.5 mL/min. Inject 10 μL LC working std soln. The 4 peaks (maleic acid, pseudoephedrine, pheniramine, and triprolidine or chlorpheniramine) should be completely resolved and symmetrical. Make replicate injections of LC working std soln and compare area response ratios relative to internal std area response to ascertain reproducibility of system. In a suitable system, the coefficient of variation for 3 replicate injections is not >2.0%. Proceed with sample analysis, using three 10 μL injections each of sample and LC std working solns. If interfering peak (methylparaben) is observed in chromatogram of syrup sample in region of pheniramine internal std peak, repeat column chromatgc cleanup, using another 2.0 mL aliquot of dild sample soln. Pack column firmly. If baseline resolution is obtained between any residual methylparaben and pheniramine peaks, repeated cleanup is unnecessary.

***F. Calculations***

Calc. results, using area response ratios ($R$ and $R'$) relative to internal std:

$$\text{mg Amine salt/tablet} = (R/R') \times C \times (T/S) \times 2$$
$$\text{mg Amine salt/5 mL syrup} = (R/R') \times C$$

where $R$ and $R'$ = area response ratio for sample and std solns, respectively; $C$ = mg amine salt in initial LC std stock soln; $T$ = av. tablet wt; $S$ = sample wt.

Ref.: JAOAC **64,** 564(1981).

CAS-113-92-8 (chlorpheniramine maleate)
CAS-345-78-8 (pseudoephedrine hydrochloride)
CAS-550-70-9 (triprolidine hydrochloride)

## ERGOT ALKALOIDS

### 985.49 Colchicine in Drugs
**Liquid Chromatographic Method**
**First Action 1985**
**Final Action 1987**

(*Caution:* Colchicine is extremely poisonous.)

***A. Principle***

Colchicine is dild or extd with MeOH–$H_2O$ (1 + 1) and detd directly by LC with UV detection at 254 nm.

***B. Apparatus***

(**a**) *Liquid chromatograph.*—Model 100A pump, Model 153 UV detector (replacement Model 116 Programmable Solvent Module and Model 160, resp.) (Beckman Instruments, Inc., Altex Div., 2300 Camino Ramon, PO Box 5101, San Ramon, CA 94583-0701) equipped with injection valve with 20.0 µL sample loop, reporting integrator (Hewlett-Packard Co., Avondale Div) or equiv. system equipped with strip chart recorder, Operating conditions: mobile phase flow rate 1.0 mL/min, detector set at 254 nm, sensitivity 0.005 AUFS, temp. ambient, chart speed 0.5 cm/min.

(**b**) *LC column.*—Ultrasphere Octyl ($C_8$), 5 µm particle size, 4.6 mm id × 25 cm stainless steel (Beckman Instruments, Inc., Altex Div.) or equiv. column packed with 5–6 µm octylsilane bonded spherical particles which meets chromatgc system suitability requirements. Wash column after use with MeOH–$H_2O$ (1 + 1), and then with MeOH.

(**c**) *Filters.*—Cellulose triacetate membrane filter, pore size 0.45 µm.

(**d**) *Glassware.*—Use low-actinic vol. flasks for all sample and std solns.

***C. Reagents***

(**a**) *Methanol.*—UV or LC grade (Burdick & Jackson Laboratories, Inc.) or equiv.

(**b**) *Extraction and dilution solvent.*—MeOH–$H_2O$ (1 + 1). Mix equal vols of MeOH and $H_2O$, cool to room temp., and pass soln thru 0.45 µm membrane filter.

(**c**) *Potassium phosphate (monobasic) buffer.*—0.5M. Dissolve 68.0 g $KH_2PO_4$ (LC grade, Fisher Scientific Co., or equiv.) in $H_2O$ and dil. to 1 L.

(**d**) *Phosphoric acid.*—0.5M. Dil. 3.4 mL $H_3PO_4$ (AR grade, 85%) to 100 mL with $H_2O$.

(**e**) *LC mobile phase.*—MeOH–0.05M $KH_2PO_4$ (55 + 45), pH 5.5. Dil. 45 mL 0.5M $KH_2PO_4$ buffer (**c**) to 450 mL with $H_2O$, then further dil. to ca 1 L (e.g., 980 mL) with MeOH. Cool to room temp. and adjust vol. to 1 L with MeOH. Adjust pH to 5.5 (±0.05) with 4–10 drops 0.5M $H_3PO_4$ (**d**). Filter thru 0.45 µm membrane filter.

(**f**) *Colchicine std soln.*—0.006 mg/mL. Prep. all dilns with MeOH–$H_2O$ (1 + 1). Transfer 30 mg accurately weighed (3 mg if using microbalance) USP Colchicine Ref. Std, previously dried 3 h at 105°, to 50 mL vol. flask; dissolve and dil. to vol. Dil. this soln quant. to 0.006 mg/mL. Soln is stable ≥4 months when stored tightly stoppered and in dark.

***D. Chromatographic System Suitability Test***

(**a**) *Repeatability.*—Let chromatgc system equilibrate with mobile phase ≥45 min. Inject 20 µL colchicine std soln and adjust sensitivity to provide 50–70% FDS. Inject 5 replicate 20 µL vols of same soln and compare peak response values (peak ht or area). Coeff. of var. should be <2%.

(**b**) *Retention.*—Retention time for colchicine should be between 5.5 and 9.5 min (depending on brand and age of column).

(**c**) *Column efficiency.*—No. of theoretical plates ($N$) detd from colchicine response is ≥4500 when calcd by following expression:

$$N = 5.54(t_R/W_{0.5})^2$$

where $t_R$ and $W_{0.5}$ are retention time and peak width at 50% ht measured in mm, resp. Use chart speed of 2–4 cm/min to facilitate accurate peak width measurement. *Note:* If small impurity peak elutes just before colchicine response, it should be completely resolved from major peak.

***E. Preparation of Sample***

Carry out all extns and dilns with MeOH–$H_2O$ (1 + 1) in low-actinic glassware.

(**a**) *Tablets.*—Weigh and finely powder, to pass No. 60 sieve, ≥20 tablets. Transfer accurately weighed portion of composite equiv. to 0.6 mg colchicine into 100 mL vol. flask, add ca 50 mL MeOH–$H_2O$ (1 + 1) and mech. shake for total of 15 min, rinsing down walls of flask at ca 8 min. Dil. to vol. and filter portion thru 0.45 µm membrane filter.

(**b**) *Injections.*—Transfer accurately measured vol. ($V$ of liq. equiv. to 1 mg colchicine into 50 mL vol. flask and dil. to vol. Transfer 30.0 mL of this soln into 100 mL vol. flask and dil. to vol.

(**c**) *Bulk drug.*—Accurately weigh ca 60 mg well mixed sample into 500 mL vol. flask, dissolve, and dil. to vol. Transfer 5.0 mL of this soln to 100 mL vol. flask and dil. to vol.

***F. Determination***

Immediately after extn and diln of sample, inject duplicate 20 µL vols of colchicine std soln and sample soln in alternating sequence. Calc. results by using av. peak response values as follows:

$$\text{Tablets: mg colchicine/tablet} = (R/R') \times C \times 100 \times (T/W)$$

$$\text{Injections: mg colchicine/mL} = (R/R') \times C \times (166.7/V)$$

Bulk drug: % colchicine (as-is basis)

$$= (R/R') \times C \times 10\,000 \times (100/W)$$

where $R$ and $R'$ = peak response values of sample and std solns, resp.; $C$ = concn of colchicine std soln, mg/mL; $T$ = av. tablet wt, mg; $W$ = sample wt, mg; $V$ = vol. injection taken for analysis, mL.

Ref.: JAOAC **68,** 1051(1985).

CAS-64-86-8 (colchicine)

## 960.60 Ergotamine in Drugs

### Chromatographic Method

**First Action 1960**
**Final Action 1962**

(Applicable in presence of caffeine, phenacetin, phenobarbital, and belladonna alkaloids)

### A. Reagents

(**a**) *Tartaric acid soln.*—1%. Dissolve 10 g tartaric acid in $H_2O$ and dil. to 1 L.

(**b**) *Alcoholic tartaric acid soln.*—Mix equal vols tartaric acid soln, (**a**), with alcohol. Prep. fresh daily.

(**c**) *Sodium bicarbonate soln.*—10%. Dissolve 100 g $NaHCO_3$ in $H_2O$ and dil. to 1 L.

(**d**) *Citric acid soln.*—(1 + 1). Mix equal wts of citric acid and $H_2O$.

(**e**) *Alum soln.*—0.25*M*. Dissolve 12 g $KAl(SO_4)_2.12H_2O$ in $H_2O$ and dil. to 1 L. pH should be 3.5 ± 0.2.

(**f**) *Color reagent.*—Dissolve 1.25 g *p*-dimethylaminobenzaldehyde in cooled mixt. of 650 mL $H_2SO_4$ and 350 mL $H_2O$. Add 0.5 mL 9% $FeCl_3$ soln.

(**g**) *Diatomaceous earth.*—*See* **960.53B**.

(**h**) *Ergotamine tartrate std soln.*—50 μg/mL. Dissolve 25 mg ergotamine tartrate, USP, in enough tartaric acid soln, (**a**), to make 500 mL.

### B. Preparation of Chromatographic Column

*Chromatographic tube.*—Prep. chromatgc tube as in **967.31A(a)**. Fit with packing rod, **967.31A(b)**. Place small wad of glass wool in bottom of tube.

*Ergotamine-retaining layer.*—Add ca 4 g diat. earth to 3 mL citric acid soln in beaker. Mix thoroly with scoop-shaped spatula until mixt. appears fluffy and uniform, and transfer to chromatgc tube. Tap side of tube gently to settle mixt. Press down firmly with packing rod.

*Ergonovine-retaining layer.*—Add ca 2 g diat. earth to 2 mL alum soln, mix, and transfer to tube on top of citric acid layer. Press down firmly and evenly.

*Water layer.*—Add ca 2 g diat. earth to 2 mL $H_2O$, mix, and transfer to tube on top of alum layer. Press down firmly and evenly and top with pad of glass wool.

### C. Preparation of Sample

(**a**) *Tablets.*—Det. av. wt and reduce to fine powder. Accurately weigh portion contg ca 2.5 mg ergotamine tartrate into beaker. Mix thoroly with 5 mL 1% tartaric acid soln and let stand 30 min. Add 5 mL $CHCl_3$ and 1 mL 10% $NaHCO_3$ soln, and mix. (Aq. phase must be alk.) Add ca 7 g diat. earth and stir thoroly until mass appears uniform and does not stick to beaker. (It may be necessary to wash down sides of beaker with small amts of $CHCl_3$.) Add and mix more diat. earth as may be necessary to make mixt. workable. Quant. transfer mixt. to another chromatgc tube fitted with glass wool plug, in several portions, pressing down firmly with packing rod. Wash packing rod, spatula, and sides of beaker with small amt (ca 5 mL) of $CHCl_3$. Add enough diat. earth to make mixt. workable. Scrub sides of beaker and add mixt. to tube. Again rinse rod, spatula, and beaker with $CHCl_3$ and pour wash onto column.

(**b**) *Suppositories.*—Place suppositories contg 3–5 mg ergotamine tartrate in 125 mL separator. Add 10 mL 0.2*N* $H_2SO_4$ and 75 mL ether. Shake until sample dissolves and then 1 min more. Drain acid layer into second separator. Complete extn with three 10 mL portions acid. Discard ether layer. Combine exts and make alk. with $NH_4OH$. Promptly ext alkaloids with four 10 mL portions $CHCl_3$. Pass each $CHCl_3$ ext directly onto prepd column, **960.60B**. Let column drain completely between addn of successive exts. Proceed as in **960.60D**, second par.

### D. Separation of Ergotamine

Place tube contg sample so that effluent will flow directly onto water layer of second column. Add 50 mL $H_2O$-satd ether to top column and receive eluate from bottom column in 250 mL erlenmeyer. Follow with 50 mL $H_2O$-satd $CHCl_3$. (Since effluent may flow faster thru sample column than thru second column, do not add too much $CHCl_3$ at a time.) Rinse tip of sample column with $CHCl_3$ from wash bottle and discard sample column.

Let column drain completely and then rinse down sides with small amt of $CHCl_3$. Pass thru addnl 25 mL $H_2O$-satd $CHCl_3$ into same flask and rinse tip of column with alcohol. Discard effluent if ergotamine was properly retained. (*See Note.*)

Inspect column for proper retention of ergotamine and for presence of $H_2O$-sol. alkaloids by holding column under UV light *very briefly*. (*Caution:* See safety notes on hazardous radiations.) Blue fluorescent band must not be at bottom of column. (*See Note.*) Extrude column into 400 mL beaker. Rinse tube with $H_2O$. Add 8 g $NaHCO_3$ and ca 25 mL $H_2O$ to form aq. liq. layer. Break up column with spatula and mix. Wash mixt. with $H_2O$ from wash bottle into 250 mL separator. Add 10 mL $CHCl_3$ and shake. Check aq. layer to assure that it is alk. Be sure that layers are well sepd. It may be necessary to break $CHCl_3$ bubbles with wire. Drain $CHCl_3$ layer thru glass wool filter into 100 mL vol. flask. Ext aq. layer with four 10 mL portions $CHCl_3$ and filter solv. layers into the 100 mL vol. flask. Dil. to vol. with $CHCl_3$, and mix.

### E. Determination

Evap. 10 mL aliquot $CHCl_3$ soln in 50 mL erlenmeyer to dryness with air current. Do not heat. (Ergotamine is easily decomposed. If assay cannot be completed in 1 day, dried residue after evapn of $CHCl_3$ may be stored in refrigerator overnight.) Dissolve residue, equiv. to 0.25 mg ergotamine tartrate, in 5.0 mL alc. tartrate soln. Pipet 5.0 mL std soln into 50 mL erlenmeyer. Add, to each, 10 mL color reagent dropwise while swirling continuously in ice-$H_2O$ bath. After 30, but <60 min, det. *A* of sample and of std, *A'*, at 550 nm against blank prepd by mixing 5 mL $H_2O$ and 10 mL reagent.

$$\text{mg Ergotamine tartrate in sample weighed} = (A/A') \times 2.5$$

*Note:* Ergot alkaloids fluoresce bright blue when exposed to UV light at ca 360 nm. If fluorescent band has reached bottom of trap layer, sample must be discarded. If desired, sample can be salvaged by combining column and effluent, shaking with $CHCl_3$, and passing thru another acid trap. Use of eluant which is not $H_2O$-satd will cause loss of ergotamine from column. Blue fluorescent ring at top of alum layer indicates presence of $H_2O$-sol. ergot alkaloids. If detn of $H_2O$-sol. alkaloid content is desired, repeat detn on new portion of sample, changing citric acid trap to one prepd by mixing 3 g diat. earth with 3 mL alum soln. Cover alum layer with mixt. of 2 g diat. earth and 2 mL $H_2O$.

Refs.: JAOAC **43**, 224(1960); **46**, 634(1963).

CAS-379-79-3 (ergotamine tartrate)

## 961.20 Ergotamine in Drugs

### Paper Chromatographic Identification

**First Action 1961**
**Final Action 1962**

### A. Reagents

(**a**) *Mobile solvent.*—Dissolve 7.1 g Na citrate in $H_2O$ and dil. to 100 mL. Adjust pH to ca 4.7 with 2*N* HCl and transfer to separator. Add 70 mL formamide and 9 mL dimethylphthalate. Shake vigorously, let sep., and drain and discard lower layer. Adjust to pH 5.2 with 2*N* HCl or NaOH.

(**b**) *Immobile solvent*.—Dimethylphthalate-$CHCl_3$ (1 + 9). Prep. immediately before use.

(**c**) *Ergotamine std solns*.—Accurately weigh 10 mg ergotamine tartrate, USP, into small separator contg 5 mL 1% tartaric acid soln and mix gently. Make alk. with few drops 10% $NaHCO_3$ soln, add 2.0 mL $CHCl_3$, and shake vigorously. Draw off $CHCl_3$ layer (*std soln 1*). Dil. 1.0 mL *std soln 1* to 25 mL with $CHCl_3$ (*std soln 2*).

### B. Identification

For details of app. and technic *see* **970.52G** and **970.52BB★**. Blotter paper liners must be used in tank and tank must be sealed.

Equilibrate mobile solv. in sealed tank ≥3 hr with liners dipping into solv. Just before use, quickly dip marked 8 × 8″ paper once in freshly prepd immobile solv. and let dry 15 min.

Prep. soln of ergotamine in $CHCl_3$, as in **960.60D**, contg 2.5 mg/0.5 mL (remainder of $CHCl_3$ soln from assay may be evapd to this concn). Spot 10 μL each of sample and std solns on paper and let solv. evap. Place paper in tank, seal tank, and let chromatogram develop until solv. front is ca 2.5 cm from top (ca 3 hr). Let paper dry overnight in hood and examine under UV light. There should be one yellow primary spot (and there may be a "tail," probably as result of ergotamine changing to ergotaminine during developing) corresponding to 50 μg ergotamine spot (*std 1*) in position and intensity. If any other spot is more intense than that of *std 2*, >2 μg other ergot alkaloids, expressed as ergotamine tartrate, are present.

*Note:* Ergot alkaloids produce blue fluorescence which on overnight contact with formamide and air changes to yellow fluorescence. Paper must be completely dry and *std 2* spot clearly visible. In humid weather it may be necessary to dry developed paper 2–3 days in well ventilated hood.

Ref.: JAOAC **44**, 288(1961).

CAS-379-79-3 (ergotamine tartrate)

## PHYSOSTIGMINE ALKALOIDS

## 982.37 Physostigmine Salicylate and Physostigmine Sulfate in Drug Solutions and Ointments

### Chromatographic Method

**First Action 1982**
**Final Action 1987**

### A. Principle

Physostigmine salicylate or sulfate is dild with $CH_3CN$ and detd by LC with UV (254 nm) detector and with flurazepam as internal std.

### B. Apparatus

(**a**) *Liquid chromatograph*.—Model 204 equipped with 2 Model 6000 pumps, Model 660 solv. programmer, 254 nm UV detector, Model U6K injector (Waters Associates, Inc.) and Model 3380A integrator (Hewlett-Packard). Equiv. LC system with strip chart recorder may be used.

(**b**) *LC column*.—μBondapak $C_{18}$, 3.9 mm id × 30 cm (Waters Associates, Inc.) or equiv. reverse phase column providing appropriate retention times and sepn for physostigmine and internal std.

### C. Reagents

(**a**) *Ammonium acetate*.—0.05*M*. Dissolve 3.85 g $NH_4OAc$ in $H_2O$ and dil. to 1 L. Filter thru 4.7 cm Whatman GF/F glass microfiber filter, or equiv., in Millipore-type filter holder. Adjust filtrate to pH 6.0 ± 0.1 with HOAc or $NH_4OH$.

(**b**) *Solvents*.—UV grade hexane and $CH_3CN$ (Burdick & Jackson Laboratories, Inc., or equiv.); filtered thru same filter as in (**a**).

(**c**) *Mobile phase*.—$CH_3CN$–0.05*M* $NH_4OAc$ (1 + 1) at flow rate of ca 2.0 mL/min. Mobile phase ratio and flow rate may be varied to give retention time of ca 3–4 min for physostigmine peak (first) and sepn of flurazepam internal std peak (second).

(**d**) *Internal std soln*.—Dissolve 50 mg flurazepam HCl in MeOH and dil. to 100 mL with MeOH.

(**e**) *Physostigmine std solns*.—3.0 mg/100 mL. Transfer 60 mg accurately weighed USP Physostigmine, Physostigmine Salicylate, or Physostigmine Sulfate to 100 mL vol. flask and dil. to vol. with $CH_3CN$. Transfer 5.0 mL aliquot to 100 mL vol. flask contg 5.0 mL internal std soln and dil. to vol. with $CH_3CN$. Use physostigmine and salicylate stds without drying. Dry sulfate std 2 h at 105°.

### D. Sample Preparation

(**a**) *Solutions*.—Transfer aliquot of sample ($V$) contg ca 3 mg physostigmine or its salts to 100 mL vol. flask contg 5.0 mL internal std soln and dil. to vol. with $CH_3CN$.

(**b**) *Ointments*.—Transfer accurately weighed sample ($W$) contg ca 3 mg physostigmine or its salts to 60 mL separator. Add 20 mL *n*-hexane and ext with four 20 mL portions of $CH_3CN$. Collect exts in 100 mL vol. flask contg 5.0 mL internal std soln and dil. to vol. with $CH_3CN$.

### E. System Suitability (Chromatographic System) Check

(**a**) *Repeatability*.—Let system equilibrate with flow rate of ca 2 mL/min. Then make four 10.0 μL injections of any std. soln. Measure coeff. of variation of peak response for 4 injections by following formula:

$$CV, \% = 100\sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}} \div \bar{x}$$

where $x$ = ratio of area of physostigmine peak divided by area of internal std peak, $\bar{x}$ = mean of these ratios, and $n$ = number of injections.

Coeff. of variation should be ≤2%. If reproducibility is unsatisfactory, let system equilibrate longer and repeat test.

(**b**) *Resolution*.—Retention time for physostigmine peak should be 2.5–4.5 min. Resolution factor, $R$, for physostigmine peak and internal std peak should be ≥3.0, using following formula:

$$R = 2(t' - t)/(PW + PW')$$

where $t$ and $t'$ = mm retention of physostigmine and internal std peaks, respectively; and $PW$ and $PW'$ = mm peak widths measured at baseline of physostigmine and internal std, respectively.

### F. Determination

Make duplicate 10 μL injections each of sample soln and appropriate std soln, alternating sample and std solns. Calc. results by using response ratios ($RR$) rel. to internal std, based on peak areas:

For solns:

Physostigmine (or salt), mg/mL

$$= 100 \times (RR/RR') \times (C/V)$$

For ointments:

$$\text{Physostigmine (or salt), mg/g} = 100 \times (RR/RR') \times (C/W)$$

where $RR$ and $RR'$ = response ratio of sample and std, resp.; $C$ = concn of std (mg/100 mL); $V$ = mL soln; $W$ = g ointment. Identification is based on same retention times for samples and stds.

Refs.: JAOAC **65**, 132(1982); **66**, 339(1983).

## 948.31★ Physostigmine in Drug Ointments

**Final Action**
**Surplus 1965**

*See* **32.089**, 10th ed.

## Physostigmine Salicylate in Drug Tablets

**Final Action**

*See* **961.18B**.

## 942.31★ Neostigmine in Drugs

**Distillation Method**
**Final Action**
**Surplus 1974**

*See* **38.159**, 12th ed.

## 974.42 Neostigmine in Drugs

**Chromatographic Method**
**First Action 1974**
**Final Action 1976**

### A. Apparatus

(**a**) *Spectrophotometer*.—Suitable for measurement in range 265–400 nm.

(**b**) *Chromatographic tube and tamping rod*.—*See* **967.31A**.

### B. Reagents

(**a**) *Phosphate buffer*.—pH 5.8. Mix 1 vol. 1$M$ $K_2HPO_4$ (17.4 g/100 mL) with 4 vols. 1$M$ $KH_2PO_4$ (13.6 g/100 mL). Adjust pH, using pH meter, to 5.80 ± 0.05 with either component.

(**b**) *Washed chloroform*.—Shake equal vols $CHCl_3$ and $H_2O$ in separator. Let layers sep. 5 min and discard upper layer.

(**c**) *Washed ether*.—Shake equal vols ether and $H_2O$ in separator. Let layers sep. 5 min and discard lower layer.

(**d**) *Bis(2-ethylhexyl) hydrogen phosphate* (*DEHP*) *soln*.—2.5% Mix 2.5 mL DEHP with 97.5 mL $H_2O$-washed $CHCl_3$.

(**e**) *Diatomaceous earth*.—*See* **960.53B**.

(**f**) *Neostigmine std soln*.—Dry neostigmine bromide 3 hr in 105° oven. Accurately weigh ca 5 mg dry std, using microbalance, and transfer to 150 mL beaker. Add 2.0 mL pH 5.8 phosphate buffer, mix by swirling gently, and proceed as in **974.42D**.

### C. Preparation of Sample

(**a**) *Tablets*.—Accurately weigh portion powd tablets contg 5 mg neostigmine bromide into 150 mL beaker, add 2.0 mL pH 5.8 phosphate buffer, mix by swirling gently, and proceed as in **974.42D**.

(**b**) *Individual tablets*.—Transfer tablet to 50 mL centrf. tube, powder if coated, and add 6.0 mL pH 5.8 phosphate buffer by pipet. Stopper, shake mech. 30 min, and centrf. 5 min at high speed. Pipet 2.0 mL clear supernate into 150 mL beaker and proceed as in **974.42D**.

(**c**) *Ophthalmic soln*.—Dil. accurately measured vol. sample soln to 2.5 mg neostigmine bromide/mL with pH 5.8 phosphate buffer soln. Pipet 2.0 mL dild sample soln into 150 mL beaker and proceed as in **974.42D**.

### D. Determination

(*Caution:* See safety notes on distillations, diethyl ether, chloroform, and isooctane. Use $H_2O$-washed solvs thruout.)

Treat std and sample solns similarly. Add 3.0 g diat. earth, mix with metal spatula until fluffy, and transfer quant. in 3 portions to chromatgc tube contg 1 g diat. earth mixed with 0.5 mL pH 5.8 phosphate buffer. Pack uniformly. Dry-wash beaker with 0.2 g diat. earth, transfer wash to tube, and pack uniformly. Wipe beaker and all app. used in column prepn with glass wool and add to column. Proceed without delay.

Wash column with 75 mL ether and then with 75 mL $CHCl_3$. Discard washings. Elute neostigmine bromide with 75 mL 2.5% DEHP soln into 500 mL separator contg 20 mL 0.1$N$ $H_2SO_4$. Complete elution with 25 mL $CHCl_3$. Add 175 mL isooctane to eluate and shake vigorously 2 min. Let stand ≥5 min to completely sep. layers. Transfer lower aq. layer to 250 mL beaker. Repeat extn with two 20 mL portions 0.1$N$ $H_2SO_4$, and combine aq. layers in the 250 mL beaker.

Add 10 mL *10% NaOH soln* to beaker, mix by swirling gently, cover with watch glass, and heat 45 min on vigorous steam bath. Cool, transfer quant. to 100 mL vol. flask, dil. to vol. with $H_2O$, and mix. Centrf. portions of std and sample solns. Record spectra of clear sample and std solns between 400 and 255 nm against 1% NaOH soln in 1 cm cells. Det. $\Delta A$ of each soln by subtracting $A$ at 340 nm from $A$ at max., ca 293.5 nm.

$$\text{mg Neostigmine bromide in final soln} = (\Delta A/\Delta A') \times C \times 100$$

where $C$ = mg neostigmine bromide std/mL, and $\Delta A$ and $\Delta A'$ refer to sample and std, resp.

Ref.: JAOAC **57**, 725(1974).

CAS-114-80-7 (neostigmine bromide)

## 971.41 Neostigmine Methylsulfate in Drugs

**Spectrophotometric Method**
**First Action 1971**
**Final Action 1975**

(Applicable only to injections)

### A. Apparatus and Reagents

(**a**) *Recording spectrophotometer*.—Suitable for measurement in range 230–350 nm.

(**b**) *Neostigmine methylsulfate std soln*.—0.5 mg/mL. Accurately weigh 50 mg neostigmine methylsulfate of known purity, transfer to 100 mL vol. flask, add 1 mL 1$N$ $H_2SO_4$, and dil. to vol. with $H_2O$.

### B. Preparation of Sample

(**a**) *Interfering UV-absorbing preservatives absent*.—Transfer aliquot contg 5.0 mg neostigmine methylsulfate to 150 mL beaker and hydrolyze as in **971.41D**.

(**b**) *Injection solns containing phenol or parabens.*—Proceed as in **971.41C**.

### C. Extraction of Interferences

Transfer sample aliquot (or sample diln if necessary) contg 5.0 mg neostigmine methylsulfate to 125 mL separator, add 1 mL 1*N* $H_2SO_4$, and add $H_2O$ to total vol. of 21 mL. Add 35 mL $CHCl_3$ and shake vigorously 2 min. Transfer $CHCl_3$ layer into second 125 mL separator contg wash soln of 10 mL $H_2O$ and 1 mL 1*N* $H_2SO_4$; shake, let layers sep. completely, and discard $CHCl_3$ phase. Repeat extn with 5 addnl 35 mL portions $CHCl_3$. Rinse stem of each separator with $CHCl_3$ after last extn and discard $CHCl_3$. Combine aq. layers in 150 mL beaker. Rinse each separator in succession with two 5 mL portions $H_2O$, rinse stem of each separator with $H_2O$, transfer rinsings to beaker, and proceed as in **971.41D**.

### D. Hydrolysis

Add 25 mL 10% NaOH soln and $H_2O$ to ca 80 mL. Cover with watch glass and heat 30 min on vigorous steam bath. Cool, quant. transfer soln thru loose glass wool plug, prewashed with 1% NaOH soln, to 250 mL vol. flask, and dil. to vol. with $H_2O$. In sep. beaker, similarly treat 10 mL aliquot std neostigmine methylsulfate soln. Perform blank detn, omitting neostigmine methylsulfate. Proceed as in **971.41E**.

### E. Determination

Record spectra of sample and std solns, relative to blank, in 1 cm cells, from 350 to 230 nm. Det. $\Delta A$ of each soln by subtracting $A$ at 350 nm from $A$ at max., ca 239 nm.

$$\text{mg/mL Neostigmine methylsulfate in sample} = (\Delta A/\Delta A') \times 0.5 \times (10/\text{mL sample aliquot})$$

where $\Delta A$ and $\Delta A'$ refer to sample and std, resp.

Ref.: JAOAC **54**, 21(1971).

CAS-51-60-5 (neostigmine methylsulfate)

## CHINCHONA ALKALOIDS

## Quinine in Drugs

**Final Action**

(**a**) *Microchemical tests.—See* Table **930.40**.

(**b**) *Optical crystallographic properties.—See* **955.52** and **955.58**.

(**c**) *With acetanilid and caffeine.—See* **916.05★**.

(**d**) *With acetanilid, caffeine, and morphine.—See* **916.06★**.

(**e**) *With diacetylmorphine.—See* **955.56★**.

## 942.32★ Quinine in Drugs

**Spectrophotometric Method**

**Final Action**
**Surplus 1974**

*See* **38.066–38.067**, 12th ed.

## 947.15★ Quinine Ethylcarbonate in Drugs

**Titrimetric Method**

**Final Action**
**Surplus 1974**

*See* **38.068**, 12th ed.

## 962.22 Elixirs of Iron, Quinine, and Strychnine in Drugs

**First Action 1962**
**Final Action 1965**

### A. Method I—Surplus 1974★

*See* **38.069–38.074**, 12th ed.

### Method II

### B. Apparatus

*See* **967.31A**.

### C. Reagents

(**a**) *Strychnine sulfate std solns.*—(*1*) *Stock soln.*—250 µg/mL. Dissolve 25.0 mg strychnine sulfate in MeOH and dil. to 100 mL with MeOH. (*2*) *Working soln.*—50 µg/mL. To 10 mL stock soln add 5 drops HCl and dil. to 50 mL with $CHCl_3$.

(**b**) *Triethylamine.—See* **961.19B(a)**.

### D. Preparation of Sample and Columns

*Sample.*—Pipet 10 mL sample into 100 mL beaker, add 0.2 g *p*-toluenesulfonic acid, and heat on steam bath under gentle air current to remove alcohol.

*Column I.*—Add 2 mL 2*N* NaOH to 3 g diat. earth, **960.53B**. Mix thoroly by kneading with flexible spatula, transfer to column, and tamp, using gentle pressure, to uniform mass. Add 8 g diat. earth to alcohol-free sample. (If sample is too sirupy from excess evapn of $H_2O$, add small amt of $H_2O$.) Mix thoroly, transfer to column above NaOH layer, and tamp. Dry-wash with ca 1 g diat. earth for quant. transfer.

*Column II.*—Mix 3 g diat. earth and 2 mL 2*N* NaOH, and tamp as above. Mix 8 g diat. earth and 7 mL 1*N* HCl, transfer to column above NaOH layer, and tamp.

*Column III.*—Mix 3 g diat. earth and 2 mL 1*N* tartaric acid, and tamp as above.

Place small pad of glass wool above each column.

### E. Determination

(Use $H_2O$-satd solvs thruout.)

Pass 100 mL ether thru Column *I*, discarding eluate contg aromatic flavoring components and bulk of transformation product of quinine which forms in aged prepns.

Mount columns so that eluate from *I* passes thru *II* onto *III*. Pass 100 mL $CHCl_3$ thru columns and discard Column *I*. Pass 50 mL $CHCl_3$ thru Column *II* onto *III* and finally pass 50 mL $CHCl_3$ thru Column *III*. Discard eluate. Column *II* may be used for quinine detn as in **38.074★**, 12th ed.

Place 50 mL vol. flask contg 10 mL MeOH and 1 mL HCl under Column *III* which contains strychnine. Pass thru column 5 mL $CHCl_3$ contg 1 mL triethylamine, followed by 32 mL 1% triethylamine in $CHCl_3$. Dil. to vol. with $CHCl_3$. Det. $A$ at 350, 320, and 288 nm against $CHCl_3$ or, preferably, record spectrum over this region. (Film of $Et_3N.HCl$ may adhere to walls of cells. Rinse cells carefully with $H_2O$ and alcohol; then wipe clean before use.) Background $A$ at 310–360 nm should be <0.02. Deduct av. reading at 320 and 350 nm from reading at inflection at 288 nm. (Max. $A$ of strychnine is at ca 255 nm but nature of solvs makes it undesirable to use this wavelength.)

Deduct $A$ of blank of 10 mL MeOH, 1 mL triethylamine, and 1 mL HCl dild to 50 mL with $CHCl_3$. Compare net $A$ with that of dild strychnine sulfate std soln, $A'$, and calc. strychnine content.

Ref.: JAOAC **45,** 595(1962).

CAS-130-95-0 (quinine)
CAS-57-24-9 (strychnine)

## 947.16★ Pamaquine in Drugs

**Titrimetric Method**

**Final Action**
**Surplus 1974**

*See* **38.169–38.170**, 12th ed.

## 944.15★ Quinacrine Hydrochloride in Drugs

**Volumetric Method**

**Final Action**
**Surplus 1972**

*See* **38.216–38.217**, 12th ed.

## 970.83 Quinacrine Hydrochloride in Drugs

**Fluorometric Method**

**First Action 1970**
**Final Action 1974**

(*Caution:* See safety notes on photofluorometers.)

### A. Apparatus

*Spectrophotofluorometer.*—Scanning, with 1 cm cell path, Xe lamp, excitation wavelength 420 nm, and sensitivity to produce 80% fluorescence intensity ($F$) for std soln.

### B. Reagent

*Quinacrine hydrochloride std soln.*—0.00050 mg/mL. Weigh 5.0 mg USP Quinacrine.HCl Ref. Std in 1 L vol. flask and dil. to vol. with $H_2O$. Mix well and dil. 10.0 mL to 100 mL with 0.1$N$ HCl. Alternatively, weigh 50.0 mg quinacrine.HCl into 1 L vol. flask and dil. to vol. with $H_2O$. Mix well and dil. 10.0 mL to 1 L with 0.1$N$ HCl. Prep. fresh daily.

### C. Preparation of Sample

(**a**) *Tablets and powders.*—Weigh amt of well mixed or well ground sample contg 100 mg quinacrine.HCl into 200 mL vol. flask. Dil. to vol. with 0.1$N$ HCl, mix 2 min, and filter if necessary. Dil. 10.0 mL clear sample soln to 1 L with $H_2O$ and mix. Finally dil. 10 mL to 100 mL with 0.1$N$ HCl to obtain sample soln.

(**b**) *Liquids.*—Pipet accurate sample contg ca 100 mg quinacrine.HCl into 200 mL vol. flask. Proceed as in (**a**), beginning "Dil. to vol. with 0.1$N$ HCl, . . ."

### D. Determination

Adjust spectrophotofluorometer to ca 80% fluorescence intensity ($F$) at 500 nm with std soln. Transfer ca 3 mL 0.1$N$ HCl to clean 10 × 10 mm cell and record the blank scan between 350 and 650 nm. Repeat with std and sample solns. In each case, draw baseline from 350 to 650 nm. Det. $\%F$ at peak max. (ca 500 nm) of sample and std solns relative to 0.1$N$ HCl blank.

Calc. as follows:

Liqs: mg quinacrine.HCl/mL
$$= 200{,}000 \times C \times (F/F') \times (1/V)$$

Solids: % by wt quinacrine.HCl
$$= 200{,}000 \times C \times (F/F') \times (100/W)$$

where $C$ = mg/mL std soln; $F$ and $F'$, resp., = fluorescence of sample and std solns at 500 nm, each corrected for blank; $W$ = mg sample; and $V$ = mL sample.

### E. Identification

Set emission wavelength monochromator at wavelength of max. fluorescence, i.e., 500 nm. Scan std and sample solns used for quantitation with excitation wavelength monochromator from 200 to 750 nm.

Use same instrument parameters as for quantitation except set sensitivity at ca 40. Sample and std spectra exhibit identical max. and min.

Refs.: JAOAC **53,** 117(1970); **65,** 484(1982).

CAS-69-05-6 (quinacrine hydrochloride)

# RAUWOLFIA ALKALOIDS

## 958.17 Reserpine in Drugs

**Spectrophotometric Method I**

**Final Action 1965**

### A. Reagents

(**a**) *Sulfamic acid soln.*—2.5%. Prep. fresh every 2–3 days.

(**b**) *Alcoholic sodium nitrite soln.*—Dissolve 10 g $NaNO_2$ in 100 mL $H_2O$. Store in refrigerator. Mix 1 mL of this aq. soln with 50 mL alcohol.

(**c**) *Reserpine std soln.*—50 μg/mL. Dissolve 25 mg USP Reserpine Ref. Std, previously dried 3 hr at 60°, in ca 40 mL boiling alcohol, cool, and dil. to 100 mL with alcohol. Dil. 10 mL of this stock soln to 50 mL with alcohol. When stored in tightly stoppered brown bottle in dark, solns are stable for weeks.

### B. Determination

(**a**) *Crystalline reserpine.*—Accurately weigh ca 25 mg reserpine, dissolve in ca 40 mL boiling alcohol, cool, and dil. to 100 mL with alcohol. Transfer 10.0 mL to separator contg 50 mL *1% $NaHCO_3$ soln*. Ext with 20, 10, and 10 mL $CHCl_3$, washing each $CHCl_3$ ext in second separator with 50 mL *2% citric acid soln*. Filter $CHCl_3$ exts thru cotton into 50 mL vol. flask contg 5 mL alcohol, dil. to 50 mL with $CHCl_3$, and mix.

Transfer duplicate 5.0 mL aliquots to 25 mL vol. flasks contg 15 mL alcohol. Transfer duplicate 5.0 mL aliquots dil. reserpine std soln to 25 mL vol. flasks contg 10 mL alcohol and 4.5 mL $CHCl_3$. Add 1.0 mL alc. $NaNO_2$ soln to 1 std and 1 sample soln. Add 10 drops HCl to all flasks, swirl, and let stand 30 min. Add 1.0 mL sulfamic acid soln, dil. with alcohol to 25 mL, and mix. Let stand 15 min and det. $A$ in matched 1 cm cells at 390 nm against alcohol.

mg Reserpine in sample weighed
$$= 25 \times (A - A_0)/(A' - A_0')$$

where $A$ and $A_0$ refer to nitrite-treated and untreated sample, resp., and $A'$ and $A_0'$ refer to corresponding std aliquots.

(**b**) *Tablets.*—Transfer accurately weighed portion powd tablets contg ca 5 mg reserpine to 100 mL beaker. Add 20 mL alcohol, cover with watch glass, and heat to simmering. Boil gently 20 min, stirring occasionally, adding small portions alcohol to maintain vol. Cool to <50°, add 10 mL $CHCl_3$, and mix. Filter thru pledget of cotton, and collect filtrate in 50 mL vol. flask. Wash filter and solids with several portions $CHCl_3$. Cool, dil. to 50 mL, and mix. Transfer 25 mL aliquot to separator contg 50 mL 1% $NaHCO_3$. Add 5 mL $CHCl_3$ and shake vigorously. Transfer $CHCl_3$ layer to separator contg 50 mL *2% citric acid soln,* and shake. Repeat extns with two 10 mL portions $CHCl_3$. Filter exts thru cotton and collect in 50 mL vol. flask contg 5 mL alcohol. Proceed as in (**a**), second par., after dilg to vol. with $CHCl_3$.

mg Reserpine in portion powd tablets weighed
$$= 5 \times (A - A_0)/(A' - A'_0)$$

Ref.: JAOAC **41,** 488(1958).

CAS-50-55-5 (reserpine)

## 969.51 Reserpine in Drugs

### Spectrophotometric Method II

**First Action 1969**
**Final Action 1976**

### A. Reagents

(Prep. std, sample, and blank solns from same lots of $CHCl_3$ and MeOH.)

(**a**) *Treated fiberglass.*—Soak Pyrex fiberglass, Corning Glass Works No. 3950, in $CHCl_3$, rinse several times with $CHCl_3$, and air dry on filter paper or dry in forced-draft oven.

(**b**) *Dimethylsulfoxide (DMSO).—See* **972.50A(f)**.

(**c**) *Sodium nitrite in dilute methanol soln.*—0.3% in MeOH (1 + 1). Stable ≥1 month when stored in refrigerator. Bring to room temp. before use.

(**d**) *Methanolic hydrochloric acid soln.*—Dil. 6.0 mL HCl to 100 mL with MeOH.

(**e**) *Reserpine std soln.*—20 µg/mL. Dissolve 25.0 mg accurately weighed USP Reserpine Ref. Std, previously dried 3 hr at 60°, in 0.25 mL $CHCl_3$. Mix with ca 30 mL MeOH, previously warmed to 50°; transfer mixt. to 250 mL vol. flask with warm MeOH. Cool soln to room temp., dil. to vol. with MeOH, and mix. Protect soln from light. Just prior to use, pipet 10 mL into 50 mL vol. flask, add 36 mL $CHCl_3$, and dil. to vol. with MeOH.

(**f**) *Diatomaceous earth.—See* **960.53B**.

### B. Preparation of Column

Place small pledget of treated fiberglass in base of 200 × 22 mm id tube. *Lower layer:* Mix 1 g diat. earth, **960.53B**, with 0.5 mL freshly prepd 2% $NaHCO_3$, transfer to column, and tamp to uniform mass. *Acid layer:* Mix 1 g diat. earth with 0.5 mL freshly prepd 0.5% citric acid soln, transfer to column, and tamp. *Water layer:* Mix 1 g diat. earth with 0.5 mL $H_2O$; transfer to column, and tamp.

Proceed with entire assay *quickly,* without interruption, avoiding exposure of sample to direct light. Read UV spectrum immediately after column elution is completed.

### C. Chromatography

Powder tablets and pass thru No. 60 sieve. Transfer accurately weighed amt, contg ca 1 mg reserpine, but ≤1 g of the powder, to 150 mL beaker. Dry-mix powder with ca 500 mg diat. earth. Add 1 mL DMSO and wet sample thoroly by mixing with spatula. Let mixt. stand, with spatula remaining in beaker, 5 min. Add addnl 500 mg diat. earth and mix thoroly. Add addnl diat. earth to total wt of 2 g and mix thoroly. Quant. transfer to column thru wide-mouth funnel. Dry-wash beaker with ca 1 g diat. earth and transfer to column. Wipe beaker, spatula, and funnel with small pledget of treated glass wool. Tamp sample, drywash, and glass wool firmly. Pass ca 45 mL $CHCl_3$ thru sample column. Collect eluate in 50 mL vol. flask contg 14 mL MeOH, rinsing tip of column with $CHCl_3$, and dil. sample to vol. with $CHCl_3$.

Prep. and elute blank column exactly as above, replacing sample layer with 1 mL DMSO + 2 g diat. earth.

### D. UV Assay

Scan UV absorption spectrum of sample eluate from 250 to 350 nm, against column blank eluate. Likewise, scan spectrum of std soln in same range against ref. blank of 3.6 parts $CHCl_3$ and 1.4 parts MeOH.

$$\text{mg Reserpine in sample portion} = (A/A') \times (C \times 50)$$

where $A$ and $A'$ refer to sample and std, resp., at 268 nm, and $C$ = mg reserpine/mL in std soln (0.02).

### E. Colorimetric Assay

Pipet duplicate 5.0 mL aliquots of sample eluate and std soln into sep. 10 mL vol. flasks. Add 2.0 mL methanolic HCl soln to each flask and swirl. To one std and one sample flask, add 1.0 mL MeOH (1 + 1) (blanks). To remaining std and sample flasks, add 1.0 mL 0.3% $NaNO_2$ soln and mix. Let stand exactly 30 min. Add 0.5 mL freshly prepd 5% *$NH_4$ sulfamate* soln to each flask, dil. with MeOH, and let stand ≥10 min. Read $A$ from 450 to 350 nm for each soln against blank of 3.6 parts $CHCl_3$, 5.4 parts MeOH, and 1 part $H_2O$.

mg Reserpine in sample portion
$$= [(A - A_0) \times C \times 50]/(A' - A'_0)$$

where A, $A_0$, $A'$, and $A'_0$ refer to sample, sample blank, std, and std blank, resp., at 390 nm and $C$ = mg reserpine/mL in std soln (0.02).

Refs.: JAOAC **52,** 113(1969); **53,** 1106(1970).

CAS-50-55-5 (reserpine)

## 972.54 Reserpine in Drugs

### Single Tablet Assay

**First Action 1972**
**Final Action 1973**

### A. Reagents

(**a**) *Vanadium pentoxide-phosphoric acid (VP-PA) solns.*—(*1*) *Stock soln.*—Sat. 85% $H_3PO_4$ with $V_2O_5$ by shaking mech. 2 hr. Filter thru medium porosity fritted glass funnel. (Satd soln contains ca 0.8 mg $V_2O_5$/mL.) Soln is stable ca 1 month. (*2*) *Working soln.*—Dil. 10 mL stock soln to 100 mL with $H_2O$. Prep. fresh daily.

(**b**) *Reserpine std solns.*—(*1*) *Stock soln.*—0.1 mg/ mL. Accurately weigh ca 10 mg USP Reserpine Ref. Std, previously dried 3 hr at 60°, into 100 mL vol. flask. Dissolve in 0.1 mL $CHCl_3$; then add 30 mL alcohol previously warmed to 50°. Cool to room temp. and dil. to vol. with alcohol. (*2*) *Working soln I.*—0.002 mg/mL. Transfer 2.0 mL stock soln to 100 mL vol. flask contg ca 50 mL alcohol. Add following vols $CHCl_3$: for 1 mg tablets, 1.0 mL; 0.5 mg, 2 mL; 0.25 mg, 2.4 mL. Dil. to vol. with alcohol. (*3*) *Working soln II.*—0.001 mg/mL. Transfer 1.0 mL stock soln to 100 mL vol. flask contg ca 50 mL alcohol, add 2.0 mL $CHCl_3$, and dil. to vol. with alcohol. Protect all std solns from light as in **961.20B(c)**.

### B. Apparatus

*Spectrophotofluorometer.*—Adjusted so that reserpine working std solns *II* and *I* give ca 40 and 80% *F*, resp. Wavelengths of max. excitation and fluorescence of reserpine treated with VP-PA are 400 and 500 nm, resp. (*Caution:* See safety notes on photofluorometers.)

### C. Preparation of Sample

Drop single tablet into 100 mL vol. flask. Add 2 mL $H_2O$, crush tablet with fire-polished glass rod, and, leaving rod in flask, heat on steam bath ca 15 min or until tablet is dispersed. Frequently crush particles with rod to aid soln. Cool. Rinse rod into flask with following vols $CHCl_3$: 2 mL for 0.1 mg tablets; 3 mL for 0.25; and 5 mL for 0.5 and 1. Remove rod. Protect $CHCl_3$ solns of reserpine from light. Vigorously shake flask ca 2 min. Dil. to vol. with alcohol, shake vigorously, and filter thru rapid paper, discarding first 25 mL filtrate. Collect remaining filtrate in g-s erlenmeyer. Further dil. filtrate as follows: For 0.1 mg tablets, use directly; 0.25 mg, dil. 20 mL to 25 mL; 0.5, 20 to 50; and 1, 10 to 50.

### D. Determination

Transfer 5.0 mL std soln *II* (for 0.1 mg tablets) or std soln *I* (for all others) and 5.0 mL final sample diln to sep. 50 mL g-s erlenmeyers. Add 5.0 mL VP-PA working soln to each flask, shake vigorously, and let stand 15–60 min. Det. fluorescence of sample and std solns. (Blank is unnecessary, since its reading is negligible compared to sample and std solns.)

$$\text{Relative fluorescence} = \%F \times \text{meter multiplier reading}$$

$$\text{mg Reserpine/tablet} = (R/R') \times C \times f$$

where $R$ and $R'$ = relative fluorescence of sample and std solns, resp., $C$ = concn reserpine *stock* soln, and factor $f$ = 1, 2.5, 5, and 10 for 0.1, 0.25, 0.5, and 1 mg tablets, resp.

Ref.: JAOAC **55,** 149(1972).

CAS-50-55-5 (reserpine)

## 976.34 Reserpine in Drugs

**Semiautomated Fluorometric Method**

**First Action 1976**
**Final Action 1978**

### A. Principle

Reserpine is dissolved in 0.25*M* $H_3PO_4$ soln contg 20% MeOH and mixed with $V_2O_5$, and fluorescence of oxidized reserpine is detd.

### B. Apparatus

(Other equiv. instruments may be used.)

(**a**) *Automatic analyzer*.—AutoAnalyzer with following modules (Technicon Instruments Corp.): Sampler II with 30/hr (2:1) cam; proportioning pump I; manifold (*see* Fig. **976.34**).

(**b**) *Ratio fluorometer*.—Equipped with flowcell and Kopp Glass No. C5113 filter for 395 nm excitation and Wratten No. 8 filter for 495 nm emission.

(**c**) *Recorder*.—Texas Instruments Servo/Riter II (Texas Instruments, Inc., 24500 Hwy 290, PO Box 1444, Sypress, TX 77429), or equiv.

### C. Reagents

(**a**) *Phosphoric acid-methanol soln*.—Add 20 mL $H_3PO_4$ to 200 mL MeOH and dil. to 1 L with $H_2O$.

(**b**) *Vanadium pentoxide-phosphoric acid* (*VP-PA*) *solns*.—(*1*) *Stock soln*.—Sat. 85% $H_3PO_4$ with $V_2O_5$ by stirring mag. 3 hr. Let settle overnight. (Satd soln contains ca 0.8 mg $V_2O_5$/mL.) Soln is stable ca 1 month. (*2*) *Working soln*.—Dil. 200 mL stock soln to 1 L with $H_2O$. Prep. fresh daily.

(**c**) *Reserpine std solns*.—(*1*) *Stock soln*.—0.125 mg/mL. Accurately weigh ca 25 mg USP Reserpine Ref. Std, previously dried 3 hr at 60°, into 200 mL vol. flask, dissolve in $H_3PO_4$-MeOH soln, and dil. to vol. (*2*) *Working soln I*.—0.0025 mg/mL. Dil. 5.0 mL stock soln to 250 mL with $H_3PO_4$-MeOH soln. (*3*) *Working soln II*.—0.002 mg/mL. Dil. 4.0 mL stock soln to 250 mL with $H_3PO_4$-MeOH soln.

### D. Preparation of Sample

Place tablet in suitable vol. flask to give reserpine concn of 0.002–0.0025 mg/mL. Add $H_3PO_4$-MeOH soln to ca $^1/_2$ vol. of flask and place in ultrasonic generator to disintegrate tablet. After complete disintegration, agitate 15 min on mech. shaker. Dil. to vol. and mix. Let soln settle 2 hr.

### E. Analytical System

Sample is withdrawn, segmented with air, and dild with $H_3PO_4$-MeOH soln. Soln is resampled into stream of solv. that has been segmented with air and then mixed with VA-PA working soln. After flowing thru full delay coil (ca 10 min delay), soln is debubbled and passed thru flowcell, and fluorescence is measured at excitation and emission wavelengths of 395 and 495 nm, resp.

### F. Start-Up and Shut-Down Operations

Place all tubes in resp. solns and pump until steady baseline is obtained. To shut down system, place all lines in $H_2O$ and pump 15 min. Remove lines from $H_2O$ reservoir and pump system dry.

### G. Determination

Fill 3 mL sample cups in following order: 5 cups std soln, 5 cups sample solns, 1 cup std soln, 5 cups sample solns, etc., ending with 2 cups std soln. Start Sampler II. After last cup has been sampled, let system operate until steady baseline is obtained. Draw tangent to initial and final baselines. Subtract baseline to det. net fluorescence for each sample, $F$, and std, $F'$, peak, resp. Using av. of 2 stds which bracket sample peak, calc. reserpine as follows:

$$\text{mg Reserpine in sample taken} = (F/F') \times C \times D$$

where $C$ = concn of std in mg/mL and $D$ = diln factor.

Ref.: JAOAC **59,** 289(1976).

CAS-50-55-5 (reserpine)

## 977.31 Reserpine-Rescinnamine Group Alkaloids in *Rauwolfia serpentina* Drugs

**Spectrophotofluorometric Method**

**First Action 1977**
**Final Action 1979**

### A. Principle

Reserpine-rescinnamine is extd with DMSO-MeOH. After addn of $H_2SO_4$, drug is extd into $CHCl_3$, then sepd from interfering materials by chromatgy on 0.1*N* NaOH-diat. earth and silica gel columns. Reserpine-rescinnamine is eluted from latter column with $CHCl_3$-MeOH and detd by spectrophotofluorometry against std treated similarly.

### B. Apparatus

(**a**) *Chromatographic tube and tamping rod*.—200 × 22 (id) mm. *See* **967.31A**.

(**b**) *Shaker*.—Wrist action (Model BT, Burrell Corp., or equiv.).

(**c**) *Spectrophotofluorometer*.—Excitation and emission wavelengths 400 and 502 nm (uncorrected), resp. Use excitation and emission slit widths consistent with good quantitation according to manufacturer's recommendations. Sensitivity setting depends upon slit widths. (*Caution:* See safety notes on photofluorometers.)

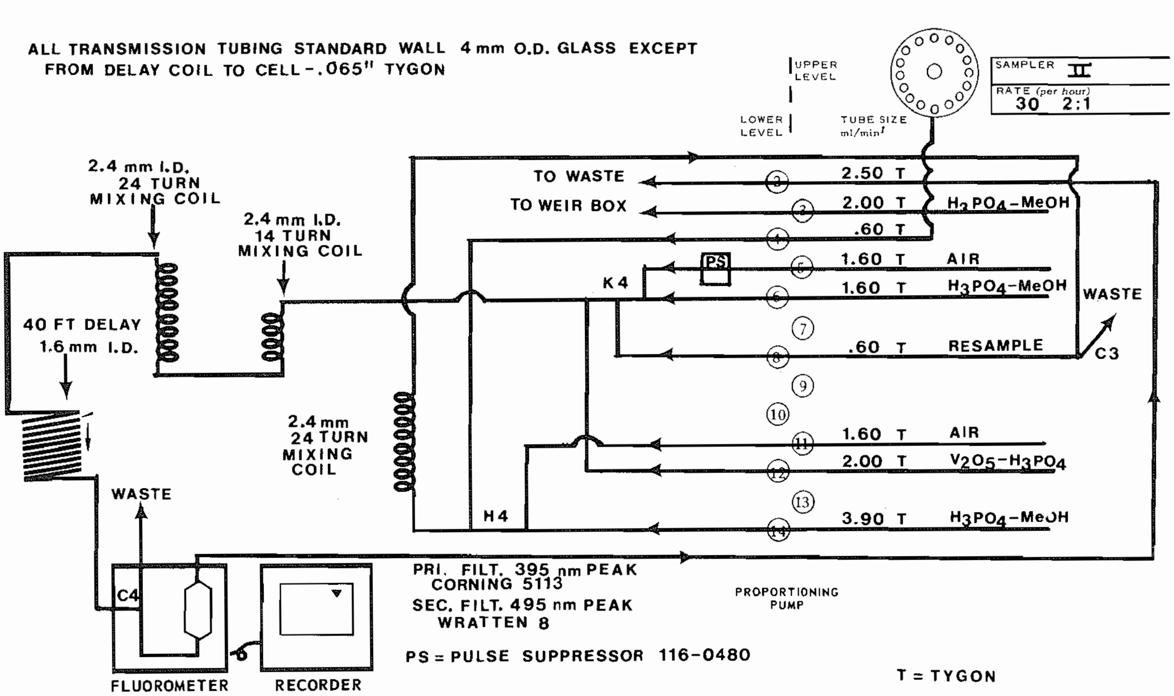



**FIG. 976.34—Flow diagram for semiautomated fluorometric analysis for reserpine**

### C. Reagents

(**a**) *Acidic alcohol soln.*—Add 10 mL 85% $H_3PO_4$ to 40 mL $H_2O$ and 50 mL alcohol. Mix well and cool before use.

(**b**) *Chloroform-methanol mixture.*—(1 + 1). Mix equal vols $CHCl_3$ and MeOH; 105 mL/detn is required.

(**c**) *Diatomaceous earth.—See* **960.53B**.

(**d**) *Dimethyl sulfoxide-methanol mixture.*—(1 + 1). Mix equal vols DMSO and MeOH. 10 mL/detn is required. (*Caution:* DMSO can be harmful. Avoid skin contact by wearing heavy rubber gloves. Use effective fume removal device.)

(**e**) *Silica gel for column chromatography.*—0.063–0.2 mm. *See* **968.22B**(**a**).

(**f**) *Sulfuric acid.*—0.5*N*. Add 7 mL $H_2SO_4$ to 500 mL $H_2O$, and mix well.

(**g**) *Vanadium pentoxide-phosphoric acid* (*VP-PA*) *solns.*—(*1*) *Stock soln.*—0.8 mg $V_2O_5$/mL. Sat. 85% $H_3PO_4$ with $V_2O_5$ by shaking mech. 2 hr. Filter thru medium porosity fritted glass funnel. Soln is stable ca 1 month. (*2*) *Working soln.*—0.08 mg $V_2O_5$/mL. Dil. 10 mL stock soln and 40 mL $H_2O$ to 100 mL with alcohol. Prep. fresh daily.

(**h**) *Reserpine std solns.*—(*1*) *Stock soln.*—40 μg/mL. Accurately weigh 20 mg USP Reserpine Ref. Std, previously dried 3 hr at 60°, into 500 mL vol. flask. Dissolve with 50 mL hot alcohol, cool, and dil. to vol. with alcohol. Protect soln from direct sunlight. Soln, when stored in g-s brown glass bottle, is stable for weeks. (*2*) *Working soln.*—2 μg/mL. Pipet 5 mL stock soln into 100 mL vol. flask, and dil. to vol. with alcohol. Prep. fresh daily.

### D. Preparation of Sample

Grind tablets to pass No. 60 sieve. Mix well. Use powd root samples as received.

### E. Preparation of Columns

(**a**) *Column I.*—Mix 2 mL 0.1*N* NaOH with 3 g diat. earth. Transfer to chromatgc tube plugged with glass wool, and tamp. Cover with pad of glass wool.

(**b**) *Column II.*—Pour silica gel into chromatgc tube (plugged with glass wool) to ht of ca 4 cm. Tap side of tube lightly with tamping rod. Cover silica gel with pad of glass wool. Arrange columns so that eluate from Column I flows directly into Column II. Wet columns with 25 mL freshly prepd $H_2O$-satd $CHCl_3$.

### F. Cleanup

(Perform in subdued light.)

Accurately weigh prepd sample contg ca 200 mg *Rauwolfia serpentina* and transfer to 50 mL g-s centrf. tube. Add 10.0 mL DMSO-MeOH (1 + 1), stopper, and shake vigorously by hand until entire sample is thoroly wetted. Shake mech. 30 min at most vigorous setting. (Wrap tube with Al foil if shaker is not in dark place.) Centrf. 5 min at 1200 rpm. Pipet 5 mL aliquot of supernate into 125 mL separator contg 50 mL 0.5*N* $H_2SO_4$. (*Caution:* DMSO can be harmful. Do not use mouth suction to fill pipet.) (Do not pipet any undissolved residue.) Ext with four 25 mL portions $CHCl_3$.

In second separator contg 25 mL 0.5*N* $H_2SO_4$, shake each 25 mL $CHCl_3$ ext individually, and drain into Column I. Let each ext sink entirely into both columns before adding next 25 mL ext. After draining fourth ext into second separator, rinse tip of first separator into second with 1–2 mL $CHCl_3$. Then rinse tip of second separator into Column I, and tip of Column I into Column II. Discard all $CHCl_3$ eluted from Column II. Sep. columns and discard Column I.

Place 100 mL vol. flask under Column II, and elute with ca 90 mL $CHCl_3$-MeOH (1 + 1). Rinse tip of Column II into vol. flask with ca 5 mL $CHCl_3$-MeOH (1 + 1), and dil. eluate to vol. with $CHCl_3$-MeOH (1 + 1).

### G. Determination

Transfer duplicate 5.0 mL aliquots $CHCl_3$-MeOH eluate to sep. 25 mL vol. flasks. Transfer duplicate 5.0 mL aliquots working std soln to sep. 25 mL vol. flasks, each contg 5 mL $CHCl_3$-MeOH (1 + 1). Add 5.0 mL VP-PA working soln, (**g**)(*2*), to 1 flask of each set, and mix well. Add 5.0 mL acidic alcohol soln, (**a**), to other (blank) flasks, dil. to vol. with alcohol, and mix well. Det. fluorescence intensity of each soln within 15–60 min.

### H. Calculations

(**a**) *Tablets.*—Calc. % reserpine-rescinnamine group alkaloids in labeled amt *Rauwolfia serpentina*/tablet:

$$[(F - F_0)/(F' - F_0')] \times (C/W) \times (T/L) \times 100 \times D$$

where $F$ and $F'$ = fluorescence of sample and std, resp.; $F_0$ and $F_0'$ = fluorescence of sample and std blanks, resp.; $C$ = mg reserpine/mL in working std soln (0.002); $W$ = g sample; $T$ = av. tablet wt in g; $L$ = labeled mg *Rauwolfia serpentina*/tablet; and $D$ = sample diln factor (200 mL).

(**b**) *Powdered root.*—Calc. % reserpine-rescinnamine group alkaloids in *Rauwolfia serpentina* powd root:

$$[(F - F_0)/(F' - F_0')] \times (C/W) \times 100 \times D$$

where symbols are defined in (**a**).

Ref.: JAOAC **59,** 811(1976).

CAS-24815-24-5 (rescinnamine)
CAS-50-55-5 (reserpine)

## 956.09 Reserpine-Rescinnamine Group Alkaloids in *Rauwolfia serpentina* Drugs

**Spectrophotometric Method**

**Final Action 1965**

### A. Reagents

(**a**) *1,1,1-Trichloroethane.*—(*Caution:* Trichloroethane is toxic.) Redistil in all-glass app., collecting fraction boiling at 73–76°.

(**b**) *Reserpine std soln.*—20 μg/mL. Dissolve 20.0 mg USP Reserpine Ref. Std, previously dried 3 hr at 60°, in 25 mL hot alcohol, cool, and dil. to 50 mL with alcohol. Dil. 5 mL of this soln to 100 mL with alcohol.

(**c**) *Dilute sulfuric acid.*—0.5*N*. Dissolve ca 30 mL $H_2SO_4$ in 2 L $H_2O$.

(**d**) *Sulfamic acid soln.*—5% aq. soln. Prep. fresh every 2–3 days.

### B. Apparatus

*Soxhlet extraction apparatus.*—Medium size extractor with 250 mL flask and 35 × 80 mm thimble is most convenient, although smaller app. may be used.

### C. Determination

Ext 2–3 g finely powd *Rauwolfia serpentina* root, or equiv. in powd tablets, in Soxhlet extn app. 4 hr, using ca 100 mL vigorously boiling alcohol. Protect flask and thimble, and all solns of rauwolfia alkaloids, from strong or direct light.

Wash ext into 100 mL vol. flask with alcohol, cool, dil. to vol., and mix. Transfer 20 mL aliquot to separator contg 200 mL 0.5*N* $H_2SO_4$, mix, and ext with three 25 mL portions trichloroethane. Drain lower solv. phase as completely as possible. Wash each trichloroethane ext in second separator contg 50 mL 0.5*N* $H_2SO_4$, and discard.

Ext main aq. soln with 25, 15, 15, 10, 10, and 10 mL $CHCl_3$. Wash each $CHCl_3$ ext with the acid in second separator, and then with two 10 mL portions 2% $NaHCO_3$ soln in third and fourth separators. Filter $CHCl_3$ exts thru cotton into 100 mL vol. flask contg 10 mL alcohol. Dil. to 100 mL with $CHCl_3$ and mix.

Transfer duplicate 10.0 mL aliquots to 18 × 150 mm test tubes and mix each with 4 mL alcohol. Add two or three "20-mesh" SiC boiling chips, and heat to boiling in $H_2O$ bath at ca 70°. Gradually raise bath temp. to 100°, or until boiling in tube *just* stops (avoid prolonged heating in absence of solv.). Wipe outsides of warm tubes, place in vac. desiccator, and evap. to dryness under vac. Dissolve residues by agitating with 5.0 mL alcohol.

Take duplicate 5 mL aliquots reserpine std soln, and add 2.0 mL 0.5*N* $H_2SO_4$ to one sample tube and to one std tube (blanks). To other tubes add 1.0 mL 0.5*N* $H_2SO_4$ and 1.0 mL 0.3% $NaNO_2$ soln. Mix contents of each tube, and warm in $H_2O$ bath 20 min at 50–60°. Cool, add 0.5 mL sulfamic acid soln to each tube, and mix. Let stand 15 min and det. $A$ in matched 1 cm cells at 390 nm against alcohol-$H_2O$ (2 + 1).

$$\text{mg Reserpine-rescinnamine alkaloids in sample weighed} = 5 \times (A - A_0)/(A' - A_0')$$

where A and $A_0$ refer to nitrite-treated and untreated samples, resp., and $A'$ and $A_0'$ refer to std soln aliquots.

Refs.: J. Am. Pharm. Assoc. Sci. Ed. **45,** 708(1956). JAOAC **40,** 64(1957).

CAS-24815-24-5 (rescinnamine)
CAS-50-55-5 (reserpine)

## 961.21 Rescinnamine in Drugs

**Spectrophotometric Method**

**First Action 1961**
**Final Action 1970**

### A. Reagents

(**a**) *Ammonium sulfamate soln.*—2.5%. Prep. fresh every 2–3 days.

(**b**) *Alcoholic sodium nitrite soln.*—*See* **958.17A(b)**.

(**c**) *Rescinnamine std soln.*—40 μg/mL. Dissolve 20.0 mg USP Rescinnamine Ref. Std in 0.5 mL $CHCl_3$, transfer to 50 mL vol. flask, and dil. to vol. with alcohol. Protect all rescinnamine solns from direct or strong light. Alc. soln is stable several weeks in dark. Dil. 5.0 mL std soln to 50 mL with $CHCl_3$.

### B. Determination

(**a**) *Crystalline rescinnamine.*—Accurately weigh ca 20 mg sample, dissolve in 0.5 mL $CHCl_3$, transfer to 50 mL vol. flask, and dil. to vol. with alcohol. Pipet 5 mL aliquot into separator contg 50 mL 0.5*N* $H_2SO_4$, add 22 mL $CHCl_3$ and 3 mL alcohol, and shake vigorously 2 min. Transfer $CHCl_3$ layer to second separator contg 50 mL 1% $NaHCO_3$ soln, and shake again. Filter $CHCl_3$ layer thru cotton previously washed with $CHCl_3$ into 50 mL vol. flask contg 5.0 mL alcohol. Ext acid and alk. solns with 2 addnl 10 mL portions $CHCl_3$, filter into vol. flask, and dil. to vol. with $CHCl_3$.

Transfer duplicate 10 mL aliquots prepd sample soln and std soln, (**c**), to 25 mL vol. flasks, each contg 10 mL alcohol. Add 1 mL alc. $NaNO_2$ soln, **958.17A(b)**, to 1 flask of each set; to remaining flasks add 1 mL alcohol. Add 10 drops HCl to all flasks, swirl, and let stand 30 min. Add 1 mL 2.5% $NH_4$ sulfamate soln, dil. to vol. with alcohol, mix, and let stand 10 min.

Det. $A$ in matched 1 cm cells at 390 nm against mixt. of $CHCl_3$, alcohol, and $H_2O$ (9 + 15 + 1) as ref.

$$\text{mg Rescinnamine in sample weighed} = 20 \times (A - A_0)/(A' - A_0')$$

where $A$ and $A_0$ refer to nitrite-treated and untreated sample, resp., and $A'$ and $A_0'$ refer to corresponding std aliquots.

(**b**) *Tablets.*—Transfer accurately weighed portion powd tablets contg ca 2.5 mg rescinnamine to 50 mL beaker. Insert small glass rod and cover with watch glass. Add 10 mL alcohol, mark vol., and boil gently 20 min with occasional stirring, maintaining original vol. by adding alcohol when necessary. Cool to <50°, add 5 mL $CHCl_3$, and filter thru pledget of cotton previously washed with $CHCl_3$ into 25 mL vol. flask. Wash filter and solids with $CHCl_3$, cool, and dil. to vol. Mix, and let settle ca 10 min. (If soln is not clear, transfer to g-s graduate and let settle 10 min more.)

Pipet 20 mL aliquot into separator contg 50 mL 0.5*N* $H_2SO_4$, add 10 mL $CHCl_3$, and shake vigorously 2 min. Transfer $CHCl_3$ to second separator contg 50 mL 1% $NaHCO_3$ soln, and shake again. Filter $CHCl_3$ layer thru cotton previously washed with $CHCl_3$ into 50 mL vol. flask contg 5.0 mL alcohol. Ext acid and alk. solns with two 10 mL portions $CHCl_3$, filter into vol. flask, and dil. to vol. with $CHCl_3$.

Proceed as in (**a**), second par.

$$\text{mg Rescinnamine in sample weighed} = 2.5 \times (A - A_0)/(A' - A'_0)$$

### *C. Determination of Total Alkaloids*

Transfer 10 mL aliquot prepd sample soln and 10 mL std soln, (**c**), to sep. 25 mL vol. flasks, and dil. to vol. with alcohol. Det. spectrum of each soln in region 250–360 nm against blank of 9 mL $CHCl_3$ dild to 25 mL with alcohol.

$$\text{mg Total alkaloids in sample weighed} = 2.5 \times T/S$$

where $T$ and $S$ are $A$ of sample and std solns at max. near 304 nm, resp.

Presence of other alkaloids is indicated by difference between the 2 spectra; presence of reserpine in particular is indicated by difference between colorimetric and UV detns.

Ref.: JAOAC **44,** 303(1961).

CAS-24815-24-5 (rescinnamine)

## OTHER ALKALOIDS

## 932.24★ Aconitine in Aconite Root

**Qualitative Test**

**Procedure**
**Surplus 1965**

*See* **32.028**, 10th ed.

## Arecoline Hydrobromide in Drugs

*See* **960.53B**.

## Cocaine in Drugs

*See* **960.53B**.

## Pilocarpine Hydrochloride in Drugs

*See* **960.53B**.

## 984.39 Pilocarpine, Isopilocarpine, and Pilocarpic Acid in Drugs

**Liquid Chromatographic Method**

**First Action 1984**
**Final Action 1987**

### *A. Principle*

Pilocarpine is detd by LC, using acidified phosphate buffer–$CH_3CN$ (97 + 3) mobile phase, reverse phase phenyl bonded column, and detection at 220 nm.

### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Equipped with 7000 psi injection valve with 10 μL injection loop and printer-plotter; UV-visible detector set at 220 nm, 0.04 AUFS, time const set at 4 s; data integration system with peak width set to peak threshold ratio of 1:60 (Model 4200 or 4270, Spectra-Physics, Inc., 3333 N. First St, San Jose, CA 95134, or appropriate settings for equiv. chromatgc data system).

(**b**) *Liquid chromatographic column.*—10 μm reverse phase phenyl bonded column, 30 cm × 3.9 mm id (Waters Associates, Inc.).

### *C. Reagents*

(**a**) *Mobile phase.*—UV quality LC grade $H_2O$ and $CH_3CN$ (97 + 3). Add $KH_2PO_4$ to make 5% soln. Adjust pH to 2.5 ± 0.1, using 85% $H_3PO_4$. Filter soln thru 5 μm mixed cellulose acetate and nitrate filter.

(**b**) *Std soln.*—Dry USP Ref. Std Pilocarpine Nitrate Salt 2 h at 105°. Accurately weigh 10 mg into 100 mL vol. flask. Dissolve and dil. to vol. with mobile phase. Filter thru 5 μm mixed cellulose acetate and nitrate filter. Check std soln daily by LC injection; prep. new std soln if pilocarpine concn, as detd by peak area, has changed >2%.

(**c**) *System suitability std soln.*—Dil. 10 mg pilocarpine and 1 mg isopilocarpine to 100 mL with mobile phase.

### *D. Preparation of Sample*

Prep. soln contg 10 mg pilocarpine nitrate salt/100 mL (based on label concn) using mobile phase as diluent. Filter soln thru 5 μm mixed cellulose acetate and nitrate filters.

### *E. Liquid Chromatography Test*

Equilibrate overnight at 26 ± 1° with mobile phase at flow rate of 0.3 mL/min. Circulate solv. continuously thruout duration of analyses, without interruption.

*System suitability test.*—Inject 10 μL aliquots of system suitability std soln into chromatgc column. Retention time for pilocarpine should be 50–54 min, and isopilocarpine, 45–49 min. Resolution factor, $R_s$, for pilocarpine/isopilocarpine should be ≥1.13.

### *F. Determination*

Inject 10 μL aliquots of pilocarpine std soln and sample prepn in triplicate. Calc. quantity, in g/100 mL of pilocarpine, in each sample by formula:

$$\text{g pilocarpine (as nitrate)/100 mL} = PA/PA' \times C \times D$$

where $PA$ and $PA'$ = area of pilocarpine peak for sample and std solns, resp.; $C$ = g std/100 mL; $D$ = diln factor of sample prepn. Altho not necessarily quant., isopilocarpine (retention time 45–49 min) and pilocarpic acid (retention time 33–38 min) may be estd by use of stds and similar calcns. When using different pilocarpine salts, use correction factor for different mol. wts: F = MW pilocarpine salt/MW pilocarpine std (corrects for differences in extinction coefficients between pilocarpine and pilocarpic acid).

Ref.: JAOAC **67,** 924(1984).

## 920.211 Strychnine in Liquid Drug Preparations

**Final Action**

(Other alkaloids absent. *See* **38.069–38.074★**, 12th ed.)

Into evapg dish measure 50 mL sample, or enough to yield ≥0.065 g strychnine, and remove alcohol by evapn. Transfer to separator, add 1 mL $NH_4OH$, or enough to render soln alk., and proceed as in **961.18B**, beginning ". . . ext with four 25 mL portions $CHCl_3$ use . . ."

Refs.: JAOAC **3,** 379(1920); **4,** 572(1921).

CAS-57-24-9 (strychnine)

## Strychnine in Drug Tablets

(Other alkaloids absent)

*See* **968.18B**.

## 938.17★ Cinchophen in Drugs

**Final Action**
**Surplus 1975**

### A. In Presence of Salicylates

*See* **38.151–38.152**, 12th ed.

### B. In Presence of Sodium Bicarbonate

*See* **38.153–38.154**, 12th ed.

## 976.35 Nikethamide in Drugs

**Gas Chromatographic Method**
**First Action 1976**
**Final Action 1977**

### A. Principle

Nikethamide prepn is dild with acetone contg anthracene as internal std and detd by GC with flame ionization detector. Method is applicable to levels of nikethamide normally encountered in injectable prepns (25% w/v). Store all solns contg anthracene in low-actinic glassware and complete detn within 1 day.

### B. Apparatus and Reagents

(*Caution:* See safety notes on acetone.)

(**a**) *Gas chromatograph.*—Hewlett-Packard Model 838, or equiv., with flame ionization detector and 1.8 m (6′) × 4 mm id glass tube packed with 4% XE-60 on 80–100 mesh Gas-Chrom Q (Applied Science, precoated, or equiv., or prep. as in **976.35C**). Operating conditions: temps (°)—column 180, injection port 210, detector 210; gas flows (mL/min)—N carrier gas 56, air 200, H 25; sensitivity $10^{-9}$ amp full scale, attenuation 1×. Before use, condition column 24 hr at 240–250° with 100 ± 20 mL N/min. If necessary, vary column temp. or gas flow to attain retention times of ca 6 and 4–5 min for anthracene and nikethamide, resp. Also vary detector sensitivity or injection vol. (4–7 μL) to attain peak hts of 50–90% full scale.

(**b**) *Internal std soln.*—0.8 mg/mL. Accurately weigh ca 0.8 g anthracene, heat ca 15 min on steam bath with acetone to dissolve, and dil. to 100 mL with acetone.

(**c**) *Nikethamide std solns.*—(*1*) *Stock soln.*—Approx. 1.0 mg nikethamide (Sigma Chemical Co., No. D4378)/mL acetone, accurately prepd. (*2*) *Working soln.*— Approx. 0.5 mg nikethamide/mL acetone. Accurately measure equal vols (≥5.0 mL each) of (**b**) and (**c**)(*1*) and mix thoroly.

### C. Preparation of Column

Wash tube and small amt fine glass wool with 5% (v/v) dichlorodimethylsilane in toluene; rinse with acetone and dry thoroly at room temp. (*Caution:* Dichlorodimethylsilane is toxic. Avoid contact with skin and eyes. Use effective fume removal device.) Dissolve 3.0 g XE-60 in 100 mL $CHCl_3$. Transfer soln to 250 mL beaker and slowly add 20.0 g 80–100 mesh Gas-Chrom Q with const but gentle stirring. Continue stirring ca 30 sec after adding all support. Place beaker under bell jar and apply vac. Carefully increase vac. and hold at max. ca 1 min to degas.

Swirl slurry rapidly and transfer in small portions to buchner fitted with 9 cm Whatman No. 4 paper. Maintain vac. 5 min after last portion is added. Air dry coated support 1 hr by spreading on smooth surface, and oven-dry addnl hr at 105°.

Plug column exit with small wad of silanized fine glass wool and thru-hole septum. Apply vac. to exit and slowly add coated support thru injection end, tapping very gently to aid compaction. Pack to within *1 cm* of area heated by flash heater. Plug inlet with ≤3 mm wad of silanized fine glass wool and condition as in **976.35B(a)**.

### D. Determination

Pipet 5 mL sample (25% w/v com. prepn) into 250 mL vol. flask, dil. to vol. with acetone, and mix. Dil. 20.0 mL to 100 mL with acetone (dil. sample soln). Prep. assay soln by mixing equal vols of dil. sample soln and internal std soln. Inject 5 μL working std soln into gas chromatograph and record chromatogram; then inject 5 μL assay soln and record.

$$\% \text{ Nikethamide (w/v)} = (P \times I' \times C' \times 100)/(P' \times I \times C)$$

where $P$ and $P'$ = peak hts (or areas) of nikethamide in assay soln and working std soln, resp.; $I$ and $I'$ = peak hts (or areas) of anthracene (internal std) in assay soln and working std soln, resp.; $C'$ = g nikethamide/mL working std soln; and $C$ = mL sample/mL assay soln.

Ref.: JAOAC **59,** 93(1976).

CAS-59-26-7 (nikethamide)

# DIGITALIS

## 954.16 Digitoxin in Drugs

**Spectrophotometric Method**
**Final Action 1965**

### A. Reagents

(**a**) *Formamide.*—Shake 1 L $HCONH_2$ (99% grade) with ca 30 g anhyd. $K_2CO_3$ 15 min and filter. Distil under vac. in all-glass app. Reject first portion of distillate contg $H_2O$, and collect fraction boiling at ca 101°/12 mm Hg (1.6 kPa) (115°/25 mm Hg; 3.3 kPa). Store over $H_2SO_4$ until odor of $NH_3$ is no longer detected.

(**b**) *Alkaline picrate reagent.*—Mix 20 mL 1% aq. picric acid soln with 10 mL 5% NaOH soln, dil. to 100 mL with $H_2O$, and mix. Reagent is stable 2–3 days.

(**c**) *Digitoxin std soln.*—0.04 mg/mL. Dissolve 20.0 mg USP Digitoxin Ref. Std in alcohol, and dil. to 50 mL with alcohol. Dil. 10.0 mL of this stock soln to 100 mL with alcohol.

(**d**) *Diatomaceous earth.*—*See* **960.53B**.

### B. Preparation of Chromatographic Column

*Chromatographic tube.*—*See* **967.31A**.

*Wash layer.*—Add ca 2 g diat. earth to 1 mL $H_2O$ in 100 mL beaker. Mix thoroly with stirring rod or scoop until the mixt. appears fluffy and uniform, and transfer to chromatgc tube. Press down lightly with packing rod. (Wash layer should be 15–20 mm thick.)

*Trap layer.*—Add 3 g diat. earth to 3 mL formamide-$H_2O$

soln (2 + 1) in 150 mL beaker, mix thoroly, and transfer to tube containing wash layer. Press trap layer down lightly and evenly.

### C. Preparation of Sample

(**a**) *Crystalline digitoxin*.—Dissolve 20 mg digitoxin, accurately weighed, in 20 mL $CHCl_3$. Transfer to 100 mL vol. flask with several portions of benzene, dil. to vol. with benzene, and mix. Transfer 10.0 mL to chromatgc column, **954.16B**. When liq. has passed into column, proceed as in **954.16D**.

(**b**) *Tablets*.—Thoroly mix accurately weighed powd sample contg 2 mg digitoxin with 2 mL $H_2O$ in 250 mL beaker. Add 4 mL formamide, stir thoroly, and cover beaker with watch glass. Heat mixt. 20 min on steam bath, with frequent stirring. Cool; add 2 mL $H_2O$ and ca 8 g diat. earth. Stir thoroly until mass appears uniform and does not stick to beaker. Quant. transfer mixt. to prepd chromatgc tube, **954.16B**, thru powder funnel in several portions, pressing it down with stirring rod. Use rubber policeman to sweep adhering particles from beaker and funnel into tube. Scrub beaker and stirring rod with ca 1 g diat. earth, and add dry washings to tube thru funnel. Repeat washing with 2 addnl portions diat. earth. Place cotton pad in tube and press it down on column with packing rod, sweeping diat. earth on sides of tube before it. (Over-all ht of column should be 120–150 mm.)

### D. Separation of Digitoxin

Elute digitoxin with ca 240 mL benzene-$CHCl_3$ (3 + 1), collecting eluate in 250 mL vol. flask at rate $\leq$4 mL/min. Wash stem with stream of $CHCl_3$, dil. to 250 mL with $CHCl_3$, and mix.

Continue elution as in **954.16F**.

### E. Colorimetric Determination

Transfer 25 mL aliquot eluate to small erlenmeyer and evap. to dryness on steam bath with aid of air current. Moisten residue with ca 0.5 mL alcohol, and again evap. to dryness. Add 5.0 mL alcohol to cooled flask, stopper, and let stand 15 min with occasional shaking.

Transfer 5.0 mL aliquot dild std digitoxin soln to small flask and 5 mL alcohol to another flask as blank. Add 3.0 mL alk. picrate reagent to each flask, and mix by swirling. Protect soln from intense light. After 10 min, det. *A* of std and sample solns relative to blank at 495 nm, repeating measurements at 2 min intervals until max. values are attained. Calc. digitoxin content of sample.

### F. Tests for Other Digitoxosides

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

After digitoxin seps, elute other digitoxosides with 200 mL $CHCl_3$, collecting eluate in separator. Shake with 100 mL $H_2O$. Transfer lower layer to beaker, ext $H_2O$ with 30 mL $CHCl_3$, and add $CHCl_3$ washings to beaker. Evap. to dryness. Pipet 5 mL dild digitoxin std soln into second beaker and evap. to dryness. Add 4 mL Keller-Kiliani reagent, **959.17A(b)**, to each of the cooled residues and mix thoroly. After 15 min, filter thru glass wool if necessary, and det. *A* of *clear* sample and std relative to reagent blank, at 590 nm; repeat measurements at 5 min intervals until max. values are attained. Calc. content of other digitoxosides in sample as digitoxin.

Refs.: J. Am. Pharm. Assoc. Sci. Ed. **43,** 580(1954). JAOAC **41,** 487(1958).

CAS-71-63-6 (digitoxin)

## 975.58 Digoxin in Drugs

**Automated Method**

**First Action 1975**
**Final Action 1976**

### A. Principle

$KIO_4$ oxidizes *cis*-2-deoxy sugars to malonyldialdehydes which are condensed with 2-thiobarbituric acid to yield stable, intensely colored methine dyes. Glycoside moiety of digoxin consists of 3 molecules of digitoxose, 2,6-dideoxy-D-ribohexose, which yields colored compd with max. *A* at 530 nm.

### B. Apparatus

(**a**) *Automatic analyzer*.—AutoAnalyzer with following modules (Technicon Instruments Corp.): Sampler II with 40/hr (2:1) cam; proportioning pump I; manifold; const temp. bath (75°) with two 40′ × 1.6 mm id coils; Model 1 colorimeter, with 50 mm tubular flowcell, matched 530 nm filters; Bristol recorder linear in *T*, and paper printed in *A* units. (*See* Fig. **975.58**.)

(**b**) *Shaker*.—Model BT, wrist-action (Burrell Corp.).

(**c**) *Ultrasonic generator*.—150 watt.

### C. Reagents

(**a**) *Arsenic trioxide soln*.—Add 20.0 g $As_2O_3$ and 7.0 g NaOH pellets to 100 mL $H_2O$, and heat to bp to dissolve. (*Caution:* See safety notes on arsenic trioxide.) Add 800 mL $H_2O$ and 60 mL HCl, and dil. to 1 L.

(**b**) *Potassium metaperiodate soln*.—Add 3.6 g $KIO_4$ to 900 mL $H_2O$. Heat and stir to dissolve. Cool. Add 3.0 mL $H_2SO_4$ and dil. to 1 L.

(**c**) *Thiobarbituric acid (TBA) soln*.—Add 15.0 g TBA and 4.5 g NaOH pellets to 900 mL $H_2O$, and stir to dissolve. Add HCl slowly to pH 3.5–4.0. Filter and dil. to 1 L.

(**d**) *Digoxin std solns*.—(*1*) *Stock soln*.—0.05 mg/mL. Accurately weigh ca 25 mg USP Digoxin Ref. Std into 500 mL vol. flask and dil. to vol. with 50% alcohol. (*2*) *Working soln*.—5 μg/mL. Pipet 10 mL stock soln into 100 mL vol. flask and dil. to vol. with 50% alcohol. Prep. fresh daily.

### D. Preparation of Sample

Disintegrate individual tablet or disperse weighed composite in accurately measured vol. 50% alcohol to give digoxin concn of 5 μg/mL. Use ultrasonic generator $\geq$5 min to assure tablet disintegration. Shake mech. 1 hr. Let soln settle $\geq$2 hr.

### E. Analytical System

Sample is withdrawn, segmented with air, and oxidized with $KIO_4$ in mixing coil. $As_2O_3$ is added to remove excess $KIO_4$; then TBA is added. Color is developed in 75° heating bath, and *A* of soln at 530 nm is measured in 50 mm flowcell.

### F. Start-Up

Place all lines in their resp. solns, and let system equilibrate 30 min.

### G. Shut-Down

Place $KIO_4$, $As_2O_3$, and TBA lines in $H_2O$. Remove all other lines from their solns. After 10 min, remove remaining lines from $H_2O$ and pump system dry.

### H. Determination

Fill sample cups in following order: 4 cups std soln, 5 cups sample soln, 1 cup std soln, 5 cups sample soln, etc. Place 2 cups std soln at end of each run. (Extra cups of std solns at start and end of sampling pattern will eliminate carryover effect in transitions from wash soln to std soln and vice versa. Three extra cups at start and 1 extra cup at end should suffice, but det. number needed for equilibrium by experiment. System

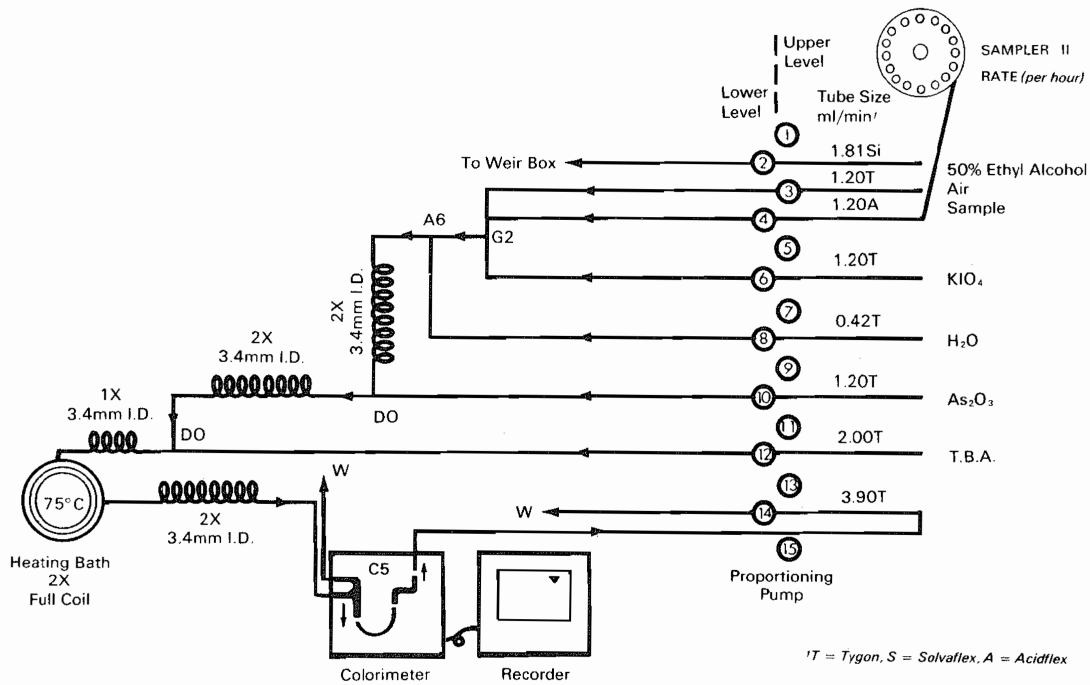



**FIG. 975.58—Flow diagram for automated analysis for digoxin**

should give uniform response for at least final pair of extra std cups before sample pattern is started.) Start Sampler II. After last cup has been sampled, let system operate until steady baseline is obtained. Draw tangent to initial and final baselines. Subtract baseline to det. $\Delta A$ and $\Delta A'$ for each sample and std peak, resp. Discard values for first 3 and last std peaks and calc. av. $\Delta A'$.

$$\text{mg Digoxin in portion taken} = (\Delta A/\Delta A') \times C \times D$$

where $C$ = concn of std in mg/mL and $D$ = diln factor.

Ref.: JAOAC **58,** 70(1975).

CAS-20830-75-5 (digoxin)

## 959.17 Digoxin and Total Digitoxosides in Drugs

### Spectrophotometric Method

### Final Action 1976

#### *A. Reagents*

(**a**) *Alkaline dinitrobenzene soln.*—(*1*) Prep. 5% soln *m*-dinitrobenzene in benzene, and store in g-s brown glass bottle. (*2*) Mix 1 mL 10% tetramethyl ammonium hydroxide soln with 140 mL absolute alcohol, titr. with 0.01*N* HCl, using Me red, and adjust to 0.008*N* with absolute alcohol. Just before use, mix 60 mL (*1*) with 40 mL (*2*).

(**b**) *Keller-Kiliani reagent.*—Mix 60 mL HOAc with 1 mL 9% $FeCl_3.6H_2O$ soln and 5 mL $H_2SO_4$, and cool.

(**c**) *Digoxin std soln.*—25.0 μg/mL. Dissolve 25.0 mg USP Digoxin Ref. Std, $C_{41}H_{64}O_{14}$, in hot alcohol, cool, dil. to 100 mL, and mix. Dil. 10.0 mL of this soln to 100 mL with alcohol and mix.

#### *B. Preparation of Sample*

(**a**) *Crystalline digoxin.*—Prep. alc. soln contg 125 μg digoxin/mL. Transfer 10.0 mL to separator, add 50 mL $H_2O$ and 1 mL 2*N* $H_2SO_4$, and ext with three 30 mL portions $CHCl_3$. Wash each $CHCl_3$ ext in second separator by shaking with 10 mL $H_2O$ and 1 g powd *anion-cation exchange resin* (Amberlite MB-1, anal. grade, indicator-free, has been found satisfactory; available as Mallinckrodt Cat. No. 5890), and filter thru pledget of cotton moistened with $CHCl_3$ into 100 mL vol. flask. Dil. to vol. with $CHCl_3$ and mix well. This soln is *Assay Soln.*

(**b**) *Elixirs and injections.*—Transfer aliquot contg 1.25 mg digoxin to separator, and proceed as in (**a**), beginning: ". . . add 50 mL $H_2O$ and 1 mL 2*N* $H_2SO_4$, . . ."

(**c**) *Tablets.*—Accurately weigh, into 100 mL beaker, portion of powd tablets contg 1.25 mg digoxin. Add 10 mL alcohol, cover with watch glass, and heat to bp on steam bath. Let simmer 20 min with frequent stirring. Cool, wash quant. into separator with 30 mL $CHCl_3$ and 50 mL $H_2O$, add 1 mL 2*N* $H_2SO_4$, and proceed as in (**a**), beginning: ". . . ext with three 30 mL portions $CHCl_3$."

#### *C. Determination*

(**a**) *Digoxin.*—Pipet 5.0 mL digoxin std soln and 10.0 mL assay soln into similar erlenmeyers, and evap. to dryness on steam bath with aid of air current. Cool, and to each flask add 5.0 mL freshly prepd alk. dinitrobenzene reagent. Let stand 5 min at ≤30°, with frequent mixing. Det. *A* of developing blue colors relative to reagent blank at 620 nm at 1 min intervals, using matched 1 cm cells and spectrophtr. Record max. *A* of aliquot of assay soln and that of digoxin std soln, *A'*. Digoxin (mg in assay soln) = 1.25 $A/A'$.

(**b**) *Other digitoxosides.*—Pipet 20 mL assay soln and 10 mL digoxin std soln into sep. beakers and evap. to dryness on steam bath with aid of air current. Cool, add 4.0 mL Keller-Kiliani reagent at ≤30° to each beaker, and mix thoroly. After 15 min, det. *A* of sample and std at 590 nm relative to reagent blank at 5 min intervals. Record max. *A* of sample and that of std, *A'*. Total digitoxosides calcd as digoxin (mg in sample soln) = 1.25 $A/A'$. Difference between this value and that obtained in (**a**) is amt of other digitoxosides in sample soln.

Ref.: JAOAC **42,** 453(1959).

CAS-20830-75-5 (digoxin)

## OTHER NATURAL PRODUCTS

### 926.18★ Camphor in Drugs

**Polarimetric Determination**

**Final Action**
**Surplus 1972**

(Not applicable to synthetic camphor)

*See* **39.061**, 12th ed.

### 945.100★ Camphor in Spirits

**Gravimetric Determination**

**Final Action**
**Surplus 1972**

*See* **39.062–39.063**, 12th ed.

### 972.55 Camphor in Drugs

**Gas Chromatographic Method**

**First Action 1972**
**Final Action 1974**

***A. Apparatus***

(**a**) *Gas chromatograph*.—With H flame ionization detector and strip chart recorder. Operate instrument in accordance with manufacturer's instructions. Operating conditions: temps (°)—column 180, detector 220, flash heater 250; N flow rate ca 60 mL/min adjusted to elute phenol in ca 9 min. Approx. retention time of camphor is 2.5 min. Adjust electrometer sensitivity so that 2.5 μg phenol gives ca 50% deflection.

(**b**) *GC column*.—(Material available from Applied Science has been found satisfactory.) Dissolve 1.2 g Carbowax 20M in ca 50 mL $CH_2Cl_2$ on steam bath. (*Caution:* Use effective fume removal device when heating or evapg $CH_2Cl_2$.) Add 10 g 100–140 mesh Gas-Chrom P and stir as solv. evaps. Dry 1 hr at 105°. Pour dry, coated packing material into 1.8 m (6′) × 4 mm glass tube, vibrating with hand vibrator. Place glass wool plugs at each end; then insert septums. Condition prepd column overnight at 200° with N flow.

***B. Reagents***

(**a**) *Camphor std soln*.—0.5 mg/mL. Dissolve and dil. 100 mg camphor to 200 mL with $CHCl_3$.

(**b**) *Phenol internal std soln*.—1 mg/mL. Dissolve and dil. 100 mg phenol to 100 mL with $CHCl_3$.

(**c**) *Menthol internal std soln*.—0.5 mg/mL. Dissolve and dil. 100 mg menthol to 200 mL with $CHCl_3$.

(**d**) *Methyl salicylate internal std soln*.—1 mg/mL. Dissolve and dil. 100 mg Me salicylate to 100 mL with $CHCl_3$.

(**e**) *Working std soln*.—Pipet equal vols of appropriate internal std soln, (**b**), (**c**), or (**d**), and camphor std soln, (**a**), into g-s flask. Phenol is preferred internal std but sample must not contain substance selected as internal std.

***C. Preparation of Sample***

(**a**) *Oily solns*.—Dil. aliquot of sample with $CHCl_3$ to ca 0.5 mg camphor/mL.

(**b**) *Ointments*.—Dissolve weighed sample in $CHCl_3$, warming very slightly if necessary. Dil. with $CHCl_3$ to ca 0.5 mg camphor/mL.

***D. Determination***

Pipet equal vols sample soln and internal std into g-s flask. Inject 5 μL into gas chromatograph and record chromatogram. Inject 5 μL working std soln and record chromatogram. Measure ht of each peak above baseline.

Concn (mg/mL or mg/g) in sample
$$= (P \times I' \times C')/(P' \times I \times C)$$

where $P'$ and $P$ = hts std and sample peaks, resp.; $I'$ and $I$ = hts internal std peak in std and sample, resp.; $C'$ = mg std/mL std soln used to prep. working std; and $C$ = mL or g sample/mL sample soln.

Ref.: JAOAC **55,** 610(1972).

CAS-76-22-2 (camphor)

### 922.14★ Camphor (Monobromated) in Drug Tablets

**Gravimetric Method**

**Final Action**
**Surplus 1975**

*See* **39.068–39.069**, 12th ed.

### 929.14★ Menthol in Drugs

**Saponification Method**

**Final Action**
**Surplus 1975**

*See* **39.070**, 12th ed.

### 939.18★ Cod Liver Oil in Emulsions

**Final Action**
**Surplus 1965**

*See* **32.299**, 10th ed.

### 932.25★ Aloin in Drugs

**Gravimetric Method**

**Final Action**
**Surplus 1975**

*See* **39.071**, 12th ed.

### 933.11★ Podophyllum in Drugs

**Gravimetric Method**

**Final Action**
**Surplus 1975**

*See* **39.072**, 12th ed.

### 931.14★ Chenopodium Oil in Drugs

**Titrimetric Method**

**Final Action**
**Surplus 1975**

*See* **39.073–39.074**, 12th ed.

## 952.28 Rutin in Drugs

### Spectrophotometric Method

### Final Action

### A. Reagents

(a) *Acid-alcohol reagent.*—Mix 550 mL alcohol with 50 mL HOAc and dil. to 1 L with $H_2O$.

(b) *Rutin std soln.*—0.02 mg/mL. Accurately weigh 100 mg rutin (obtainable from ICN–K&K Laboratories, Inc.) and dissolve in 50 mL acid-alcohol. Transfer to 250 mL vol. flask with small portions acid-alcohol. Dil. to vol. with reagent and mix well. Pipet 5 mL aliquot into 100 mL vol. flask and dil. to vol. with $H_2O$.

(c) *Quercetin std soln.*—0.01 mg/mL. Prep. as in (**b**), using 50 mg quercetin. Pure quercetin may be prepd as in J. Am. Pharm. Assoc. Sci. Ed. **42**, 66(1953).

### B. Apparatus

(a) *Spectrophotometer.*—Capable of isolating 338.5, 352.5, and 366.5 nm, with isolated spectrum $\leq 5$ nm.

(b) *Absorption cells.*—Matched 1 cm.

(c) *Glass stirring rods.*—Of small enough diam. to dislodge material from tips of 50 mL conical centrf. tubes.

### C. Preparation of Sample Solution

Weigh directly into 50 mL centrf. tube number of tablets required to give 0.05–0.50 g rutin ($\geq 5$ tablets). Record number and wt. (If tablets are coated, dissolve coating with distd $H_2O$ after weighing, discard aq. washings, and transfer rutin-contg core to centrf. tube.) Add 20 mL acid-alcohol reagent and break up tablets with stirring rod. After tablets are thoroly disintegrated, heat mixt. 10 min in $H_2O$ bath held at 70–80°, resuspending material occasionally by stirring. Remove stirring rod, rinse with acid-alcohol reagent, and centrf. 15 min. at ca 2000 rpm.

Decant supernate into 250 mL vol. flask, using funnel and decanting with one smooth motion, and let tube drain ca 10 sec. While still inverted, rinse mouth of tube with acid-alcohol reagent. Ext twice more, starting with "Add 20 mL acid-alcohol reagent . . ." After third extn, dil. combined supernates to 250 mL with acid-alcohol reagent. Any insol. material may be removed by filtration after diln if first 15–20 mL filtrate is discarded. Depending on original wt rutin taken, make diln with $H_2O$ to give final concn of 0.01–0.03 g rutin/L. Ppts forming during aq. diln may be removed by filtration if first portion of filtrate is discarded to guard against concn changes due to adsorption.

### D. Determination

Det. $A$ of sample soln against $H_2O$ blank at 338.5, 352.5, and 366.5 nm. Also det. $A$ of std rutin soln, $A'_R$, and std quercetin soln, $A'_Q$, against $H_2O$ blank at 352.5 and 366.5 nm. (In absence of std quercetin, values $A'_{Q,352.5} = 0.553$ and $A'_{Q,365.5} = 0.631$ may be used. Any error introduced by use of these predetd values should be of second order.) Calc. as follows:

$$R_1 = A_{338.5}/A_{352.5}$$

and

$$R_2 = A_{366.5}/A_{352.5}$$

If $R_1 = 0.914 \pm 0.009$ and $R_2 = 0.842 \pm 0.013$, extd material can be considered pure rutin and wt rutin/tablet can be calcd:

$$\text{mg Rutin/tablet} = A_{352.5} \times d \times W \times 0.02/A'_{R,352.5} \times w$$

where $d$ = sample diln factor; $W$ = av. wt/tablet; and $w$ = wt sample.

(Value of $R_1$ beyond its upper limit while $R_2$ remains within its range indicates interfering absorption which diminishes rapidly enough to be ineffective at 352.5 nm. Under this condition, $A$ observed at 352.5 nm is accepted as correct, and rutin content is calcd as for pure rutin. Increase in $R_2$ while $R_1$ remains within or below its limits usually indicates presence of quercetin. Simultaneous increase or decrease of both ratios beyond their respective limits indicates invalidating condition.) Amts of rutin and quercetin may be calcd by solution of following simultaneous equations:

$$A_{352.5} = (A'_{R,352.5} \times r/0.02) + (A'_{Q,352.5} \times q/0.01)$$
$$A_{366.5} = (A'_{R,366.5} \times r/0.02) + (A'_{Q,366.5} \times q/0.01)$$

where $r$ = mg rutin/mL in sample soln, and $q$ = mg quercetin/mL in sample soln.

Refs.: JAOAC **35,** 566(1952); **36,** 85, 699(1953).

CAS-117-39-5 (quercetin)
CAS-153-18-4 (rutin)

## 932.26 Santonin in Drug Mixtures

### Final Action

### A. Langer Method (Modified)★ —Surplus 1970

*See* **36.515**, 11th ed.

### *Dinitrophenylhydrazine Method*

### B. Reagent

*Dinitrophenylhydrazine sulfate soln.*—Dissolve 1 g 2,4-dinitrophenylhydrazine in mixt. of 90 mL $H_2O$ and 10 mL $H_2SO_4$ by warming; cool, and filter.

### C. Determination

(*Caution:* See safety notes on distillation, flammable solvents, toxic solvents, and benzene.)

Weigh 2.5 g ground sample into gooch and wash with ca 100 mL pet ether satd with santonin. Discard washings. Ext with ca 100 mL benzene, collecting filtrate in beaker. Evap. to dryness, warm residue with alcohol until dissolved, transfer to 100 mL vol. flask, cool, dil. to vol. at 20° with alcohol, and filter if necessary. To 25 mL of this soln add 50 mL dinitrophenylhydrazine sulfate soln and let stand 48 hr in dark. Collect ppt in gooch and wash with ca 150 mL alcohol (1 + 2). Dry residue 1 hr at 100°, cool, and weigh. Wt ppt × 0.5775 = wt santonin.

Refs.: J. Pharm. Chim. 8th ser. **16,** 49(1932). JAOAC **18,** 526(1935).

## 962.23 Santonin in Drug Mixtures

### Ultraviolet Absorption Method

### First Action 1962

(Applicable in presence of starch and calomel)

### A. Reagent

*Santonin std soln.*—10 μg/mL. Weigh 50 mg santonin NF XI, transfer to 50 mL vol. flask, dissolve in alcohol, and dil. to vol. with alcohol. Pipet 2 mL aliquot into 200 mL vol. flask and dil. to vol. with alcohol.

### *B. Determination*

Accurately weigh portion powd sample contg ca 35 mg santonin, transfer to 100 mL vol. flask, dil. to vol. with alcohol, and shake frequently during 15 min. Let settle ca 15 min, transfer 5 mL aliquot supernate to 200 mL vol. flask, dil. to vol. with alcohol, and mix. Det. $A$ of this soln and of std soln, $A'$, against alcohol at 240 nm.

$$\text{Grains santonin/tablet} = (\text{wt std, mg}) \times A \times 4000 \times \text{tablet wt (mg)}/A' \times \text{mg sample} \times 64.8$$

### *C. Identification*

Ext portion of powd tablets with alcohol or use alc. soln from detn and evap. to dryness. Santonin gives white tabular crystals, mp 170–173°.

Ref.: JAOAC **45,** 593(1962).

## 965.45 Santonin in Drug Mixtures

**Infrared Method**

**First Action 1965**
**Final Action 1966**

(Applicable to tablets in presence of calomel)

### *A. Apparatus*

*Infrared spectrophotometer.*—For operation in 2–15 μm region; equipped with 2 matched NaCl cells 1.0 mm thick, suitable for $CS_2$ solns. (Cells of shorter path length are not suitable because of low solubility of santonin.)

### *B. Determination*

(*Caution:* See safety notes on distillation, pipets, flammable solvents, toxic solvents, carbon disulfide, and chloroform.)

Transfer 25 mg Santonin NF XI, accurately weighed, to 125 mL separator contg ca 15 mL $H_2O$. Ext as for sample.

Transfer accurately weighed portion powd tablets, contg ca 25 mg santonin, to 125 mL separator contg ca 15 mL $H_2O$. Make just ammoniacal with $NH_4OH$ (1 + 9) (ca 1 drop) and ext with four 25 mL portions $CHCl_3$. Filter each ext thru cotton plug, moistened with $CHCl_3$, in long-stem glass funnel into 250 mL beaker. Evap. combined $CHCl_3$ exts to ca 5 mL on steam bath with aid of air current. Transfer quant. to 25 mL g-s erlenmeyer with ca 10 mL $CHCl_3$ in 2 mL portions, and evap. to dryness. Wash down sides of flask with few mL anhyd. ether, repeating if necessary to form dry residue. Use caution to avoid loss of sample by spattering. Add 10 mL $CS_2$ from pipet, stopper flask, and mix by swirling. Filter any insol. material thru cotton, and immediately det. baseline $A$ of sample and std ($A'$) solns relative to $CS_2$ at max. of 9.75 μm, drawing baseline between minima of 9.6 and 9.95 μm.

$$\% \text{ Santonin} = A \times \text{mg std} \times 100/A' \times \text{mg sample}$$

Record spectra of sample and std solns from 2 to 15 μm and compare for sample identity.

Ref.: JAOAC **48,** 592(1965).

CAS-481-06-1 (santonin)

## 932.27★ Santonin in Santonica (Levant Worm Seed)

**Dinitrodiphenylhydrazine Method**

**Final Action**
**Surplus 1975**

*See* **39.083**, 12th ed.

## 937.15★ Gums in Drugs

**Spot Test Identification**

**Final Action**
**Surplus 1972**

(*See* also **920.126**.)

*See* **39.088–39.090**, 12th ed.

## Ipomea in Drugs

**Final Action**

Proceed as in **932.28★**.

## 932.28 Jalap in Drugs

**Gravimetric Method**

**Final Action**

Place 10 g sample, as "60-mesh" powder, in 250 mL erlenmeyer and add 50 mL alcohol. Fit flask with stopper thru which is inserted glass tube ca 1 m long to act as condenser, and heat gently on simmering steam bath 30 min, shaking occasionally. Transfer contents to small percolator and percolate slowly with warm alcohol until ca 95 mL collects.

To test for complete extn, collect 10 mL more percolate and pour few drops into cold $H_2O$; if more than faint cloudiness appears, continue percolation with warm alcohol until test for resin fails. Conc. the addnl percolate by evapn and add to flask before dilg to vol. Cool percolate to room temp. and dil. to 100 mL with alcohol. Mix well.

Evap. 25 mL of the prepd tincture (representing 2.5 g drug) on $H_2O$ bath in beaker or flask and dry residue until alcohol-free. Add 15 mL $H_2O$, bring mixt. to bp, let cool ca 3 min, and stir well with flat-end rod 2 min to ensure thoro washing of resin. Cool mixt. by placing container in jar of ice-cold $H_2O$ and decant wash $H_2O$ onto 9 cm filter. Repeat washing of resin with another 15 mL portion $H_2O$, boiling and cooling mixt., kneading resin as before, and decanting washings into filter as before. Repeat washing and kneading process with hot $H_2O$ third time.

Dissolve residue in container in 10 mL warm alcohol and pour soln onto filter, collecting filtrate in weighed beaker or flask. Use enough hot alcohol in small portions to completely transfer soln of resin to filter and ensure thoro washing of filter. Evap. combined filtrate and washings to apparent dryness, add 1 mL absolute alcohol, and evap. solv., taking care to rotate container in inclined position as last portions of solv. are dissipated. Dry residue at 80° to const wt.

Refs.: JAOAC **15,** 448(1932); **16,** 375(1933).

## 912.03★ Acidity (Volatile) of Tragacanth Drugs

**Titrimetric Method**

**Final Action**
**Surplus 1972**

*See* **39.093**, 12th ed.

## 982.38 Allergenic Extracts in Drugs

**Protein Nitrogen Unit Precipitation Method**

**First Action 1982**
**Final Action 1985**

### *A. Principle*

Protein is pptd from allergenic ext by phosphotungstic acid, and N in ppt is detd by appropriate Kjeldahl procedure. Protein nitrogen unit (PNU) is equiv. to $1 \times 10^{-5}$ mg N.

### B. Reagents

(**a**) Phosphotungstic acid (PTA) *precipitating solution*.—15% PTA in 10% HCl. Dissolve 15.0 g PTA in ca 70 mL $H_2O$. Add 22.2 mL HCl (sp. gr. 1.19 g/mL, 37.8% HCl) and dil. to 100 mL with $H_2O$.

(**b**) *Sulfuric acid*.—Sp. gr. 1.84, N-free.

(**c**) *Copper sulfate*.—$CuSO_4.5H_2O$, N-free. Prep. satd aq. soln.

(**d**) *Acid soln*.—Add ca 40 mL satd aq. $CuSO_4$ to 9 lb bottle of $H_2SO_4$ in 10 mL portions with thoro mixing. After several days, excess anhyd. $CuSO_4$ crystallizes and supernatant acid is ready for use ($CuSO_4$–$H_2SO_4$ soln).

(**e**) *Potassium sulfate*.—N-free.

(**f**) *Sodium hydroxide soln*.—50%.

(**g**) Boric acid soln.—2%.

(**h**) *Indicator soln*.—Me red-bromocresol green soln. Mix 1 part 0.1% alc. Me red soln with 5 parts 0.1% alc. bromocresol green soln.

(**i**) *Hydrochloric acid*.—0.01N. Prep. as in **936.15**, or use 0.01000N HCl (purchased as std).

### C. Apparatus

(**a**) Digestion rack.—With either gas or elec. heaters which will supply enough heat to 30 mL flask to cause 15 mL $H_2O$ at 25° to come to rolling boil in ≥2 but <3 min.

(**b**) *Distillation apparatus*.—One-piece or Parnas-Wagner distn app. recommended by Committee on Microchemical Apparatus, ACS.

(**c**) *Digestion flasks*.—Use 30 mL regular Kjeldahl or Soltys-type flasks (Ref.: Anal. Chem. **23,** 523(1951)). For small samples, 10 mL Kjeldahl flasks may be used.

### D. Preparation of Sample

Combine vol. of allergenic ext indicated below with 0.25 mL HCl in 12 mL conical centrf. tube. Use 2 mL sample when approx. PNU value of ext is not known. When approx. PNU value of ext is known, analyze following vols:

| Allergenic ext, PNU/mL | Vol., mL |
|---|---|
| >35 500 | 1 |
| 15 500–35 500 | 2 |
| <15 500 | 3 |

Add 1 mL PTA pptg soln. Mix thoroly. Let stand 1 h at room temp. (22 ± 3°).

Centrf. mixt. at room temp. at 2700 rpm (rotor radius = 10.80 cm) for 10–15 min (rel. centrifugal force measured to tip of sample tube = $g$ = 880).

Test for completeness of pptn by adding 5 drops PTA soln. Check visually for turbidity in supernate. If turbidity develops, add addnl 0.5 mL PTA soln. Let mixt. stand 1 h at room temp. Recentrf. at 2700 rpm for 10–15 min (room temp.).

Pour off supernate. Invert centrf. tube to drain ppt. Do *not* wash ppt.

Dissolve ppt in 10 mL 2% NaOH by first adding 3 mL 2% NaOH with vol. pipet. Use vortex mixer to loosen ppt. Add 7 mL 2% NaOH (vol. pipet). Mix thoroly.

### E. Determination

Pipet 9 mL prepd sample into 30 mL digestion flask. Add ca 500 mg $K_2SO_4$, 3 boiling stones, and 2 mL $CuSO_4$–$H_2SO_4$ soln. Place flask in digestion rack. Heat carefully and digest sample until soln turns colorless. Continue digestion for addnl 1/2 h. Cool and place thin film of pet. jelly on rim of flask. Transfer digest and boiling chips to distn app. and rinse flask 5 or 6 times with 1–2 mL portions of $H_2O$. Place 125 mL erlenmeyer contg 5 mL 2% $H_3BO_3$ soln and 5 drops of indicator under condenser with tip extending below surface of soln. Add ca 6 mL NaOH (50% w/w) to still. If distn app. uses steam distn. distil at rate of 5 mL/min and collect ca 50 mL. If app. does not introduce steam into distg flask, collect 10–15 mL distillate and dil. to ca 50 mL with $H_2O$. Titr. distillate with 0.01N HCl to end point (pinkish purple). Perform blank detn in same manner, using $H_2O$ in place of sample.

### F. Calculation

$$\text{mg N/mL} = [(\text{mL HCl} - \text{mL HCl blank}) \times \text{normality} \times 14.007 \times 10/9]/\text{mL sample}$$

Calc. PNU/mL as follows:

$$\text{PNU/mL} = 10^5 \times \text{mg N/mL}$$

Ref.: JAOAC **64,** 1435(1981). **69,** 231,292(1986).

**Common and Chemical Names of Drugs in this Chapter**

| Common Name | Chemical Name |
|---|---|
| Acetanilide | *N*-Phenylacetamide |
| Aconitine | 16-Ethyl-1,16,19-trimethoxy-4-(methoxymethyl)aconitane-3,8,10,11,18-pentol 8-acetate 10-benzoate |
| Aloin | 10-Glucopyranosyl-1,8-dihydroxy-3-(hydroxymethyl)-9(10*H*)-anthracenone |
| Apomorphine (hydrochloride) | 5,6,6a,7-Tetrahydro-6-methyl-4*H*-dibenzo(*de,g*)quinoline-10,11-diol |
| Arecoline (hydrobromide) | 1,2,5,6-Tetrahydro-1-methyl-3-pyridinecarboxylic acid methyl ester |
| Atropine | α-(Hydroxymethyl) 8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester benzeneacetic acid |
| Benztropine mesylate | 3-(Diphenylmethoxy)-8-methyl-8-azabicyclo[3.2.1]octane methanesulfonate |
| Caffeine | 3,7-Dihydro-1,3,7-trimethyl-1*H*-purine-2,6-dione |
| Camphor | 1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one |
| Cephaeline | 7′,10,11-Trimethoxyemetan-6′-ol |
| Chlorpheniramine maleate | γ-(4-Chlorophenyl)-*N,N*-dimethyl-pyridinepropanamine-2-butenedioate |
| Cinchophen | 2-Phenyl-4-quinolinecarboxylic acid |
| Cocaine (hydrochloride) | 3-(Benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester |
| Codeine (phosphate, sulfate, monohydrate) | (5α,6α)-7,8-Didehydro-4,5-epoxy-3-methoxy-17-methymorphinan-6-ol |
| Colchicine | *N*-(5,6,7,9-Tetrahydro-1,2,3,10-tetramethoxy-9-oxobenzo[*a*]heptalen-7-yl)acetamide |
| Digitoxin | (3β,5β)-3[(*O*-2,6-Dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-*O*-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-14-hydroxycard-20(22)-enolide |
| Digoxin | (3β,5β,12β)-3-[(*O*-2,6-Dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-*O*-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12,14-dihydroxycard-20(22)-enolide |
| Emetine (hydrochloride) | 6′,7′,10,11-Tetramethoxyemetan |
| Ephedrine (hydrochloride, sulfate) | α-[1-(Methylamino)ethyl]benzenemethanol |
| Ergotamine (tartrate) | 12′-Hydroxy-2′-methyl-5′α-(phenylmethyl)ergotaman-3′,6′,18-trione |
| Ethylmorphine (hydrochloride) | (5α, 6α)-7,8-Didehydro-4,5-epoxy-3-ethoxy-17-methylmorphinan-6-ol |
| Homatropine (hydrobromide, hydrochloride) | α-Hydroxy-8-methyl-8-azabicyclo[3.2.1]oct-3-yl ester benzeneacetic acid |
| Hydrocodone (bitartrate, hydrochloride) | 5α-4,5-Epoxy-3-methoxy-17-methylmorphinan-6-one |
| Menthol | (1α,2β,5α)-5-Methyl-2-(1-methylethyl)cyclohexanol |
| Morphine (hydrochloride, sulfate, diacetate) | (5α,6α)-7,8-Didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol |
| Neostigmine (bromide, methylsulfate) | 3[[(Dimethylamino)carbonyl]oxy]-*N,N,N*-trimethylbenzeneaminium |
| Nikethamide | *N,N*-Diethyl-3-pyridinecarboxamide |
| Pamaquine | *N*′,*N*′-Diethyl-*N*⁴-(6-methoxy-8-quinolinyl)-1,4-pentanediamine |
| Phenacetin | *N*-(4-Ethoxyphenyl)acetamide |
| Physostigmine (salicylate, sulfate) | 1,2,3,3a,8,8a-Hexahydro-1,3a,8-trimethylpyrrolo(2,3-*b*)indol-5-ol methylcarbamate (ester) |
| Pilocarpine (hydrochloride, nitrate) | 3-Ethyldihydro-4-[(1-methyl-1*H*-imidazole-5-yl)methyl]-2(3*H*)-furanone |
| Procaine hydrochloride | 2-(Diethylamino)ethyl ester 4-aminobenzoic acid |
| Quercetin | 2-(3,4-Dihydroxyphenyl)3,5,7-trihydroxy-4*H*-1-benzopyran-4-one |
| Quinacrine hydrochloride | *N*⁴-(6-Chloro-2-methoxy-9-acridinyl)-*N*¹,*N*¹-diethyl-1,4-pentanediamine dihydrochloride |
| Quinine (ethylcarbonate) | 6′-Methoxycinchonan-9-ol |
| Rescinnamine | 11,17-Dimethoxy-18-[[1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]oxy]-3,20-yohimban-16-carboxylic acid methyl ester |
| Reserpine | 11,17-Dimethoxy-18-[(3,4,5-trimethoxybenzoyl)oxy]yohimban-16-carboxylic acid methyl ester |
| Rutin | 3-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one |
| Santonin | 3a,5,5a,9b-Tetrahydro-3,5a,9-trimethylnaphtho(1,2-b)furan-2,8(3*H*,4*H*)-dione |
| Strychnine (sulfate, nitrate) | Strychnidin-10-one |
| Terpin hydrate | 4-Hydroxy-α,α,4-trimethyl-cyclohexanemethanol monohydrate |
| Theobromine | 3,7-Dihydro-3,7-dimethyl-1*H*-purine-2,6-dione |
| Theophylline | 3,7-Dihydro-1,3-dimethyl-1*H*-purine-2,6-dione |
| Triprolidine (hydrochloride) | 2-[1-(4-Methylphenyl)-3-(1-pyrrolidinyl)-1-propenyl]pyridine |

Source: *USAN and the USP Dictionary of Drug Names* (1983; 1989) U.S. Pharmacopeial Convention, Rockville, MD.

# 21. Drugs: Part IV

**Thomas G. Alexander, *Associate Chapter Editor***
*Food and Drug Administration*

## NATURAL ESTROGENS

### 961.22★ Conjugated Estrogens in Drugs

**Spectrophotometric Method**

**First Action 1961**
**Final Action 1977**
**Surplus 1988**

*See* **39.001–39.005**, 14th ed.

### 955.55★ Ketosteroids in Drugs

**Colorimetric Methods**

**First Action**
**Surplus 1974**

*See* **39.006–39.012**, 12th ed.

### 946.05★ Beta-Estradiol in Drugs

**Colorimetric Method**

**First Action**
**Surplus 1970**

*See* **36.249–36.254**, 11th ed.

### 973.76 Estradiol Valerate in Drugs

**Spectrofluorometric Method**

**First Action 1973**
**Final Action 1975**

#### A. Principle

Oils are eluted with heptane from $CH_3NO_2$-diat. earth column. Estradiol valerate is eluted with addnl heptane, and detd by fluorometry at max. intensity, ca 328 nm.

#### B. Apparatus

(**a**) *Recording spectrophotofluorometer*.—With 1 cm cell path, excitation wavelength 285 nm, and sensitivity to produce 70% fluorescence for std soln at 328 nm.
(**b**) *Glass chromatographic tubes*.—250 × 25 mm id.

#### C. Reagents

(**a**) *Heptane*.—Redistd.
(**b**) *Nitromethane*.—Spectral grade, or equiv.
(**c**) *Diatomaceous earth*.—*See* **960.53B**.
(**d**) *Estradiol valerate std solns*.—(*1*) *Stock soln*.—0.4 mg/mL. Accurately weigh ca 40 mg USP Estradiol Valerate Ref. Std in 100 mL vol. flask and dil. to vol. with absolute alcohol. (*2*) *Working soln*.—16 μg/mL. Dil. 2 mL stock soln to 50 mL with absolute alcohol.

#### D. Preparation of Sample

Using "to contain" pipet (or hypodermic syringe fitted with $1^1/_2$", 18 gage needle), transfer accurately measured vol. sample contg ca 40 mg estradiol valerate to 100 mL vol. flask. Wash pipet with heptane and add wash to vol. flask. Dil. to vol. with heptane and mix.

#### E. Preparation of Column

Place glass wool plug in base of chromatgc tube. To 10 g diat. earth in 250 mL beaker, add 11 mL $CH_3NO_2$. (*Caution:* $CH_3NO_2$ is toxic and flammable. Wear resistant rubber gloves when using it. Use effective fume removal device.) Mix until fluffy and add to tube in portions, packing moderately after each addn. Top column with glass wool pad and prewash column with 50 mL heptane.

#### F. Determination

Transfer 2 mL sample soln to column. Wash with 5, 5, 10, 10, and 40 mL heptane (70 mL total), allowing each portion to pass thru column before adding next. Discard eluate. (*Caution:* See safety notes on distillation and flammable solvents.) Change receiver to 250 mL beaker and continue eluting with heptane, collecting ca 150 mL. Evap. eluate to dryness and quant. transfer residue to 50 mL vol. flask, using absolute alcohol. Dil. to vol. with absolute alcohol.

Adjust spectrophotofluorometer to ca 70% fluorescence intensity at 328 nm with working std soln. Scan sample and std solns from ca 280 to 450 nm, reading % fluorescence at max., ca 328 nm. Use absolute alcohol as blank.

$$\text{mg Estradiol valerate/mL} = 100 \times C \times (F/F') \times (1/V)$$

where $C$ = concn of std soln (mg/mL); $F$ and $F'$ = fluorescence of sample and std solns, resp., at 328 nm, each corrected for blank; and $V$ = vol. of sample taken.

Refs.: JAOAC **54,** 1192(1971); **56,** 86(1973).

CAS-979-32-8 (estradiol valerate)

### 974.43 Ethinyl Estradiol in Drugs

**Spectrophotometric Method**

**First Action 1974**
**Final Action 1976**

#### A. Reagents

(**a**) *Methanol-sulfuric acid*.—In ice bath, cautiously add chilled $H_2SO_4$ in small increments, with mixing, to 60 mL chilled anhyd. MeOH in 200 mL vol. flask. Cool to room temp., dil. to vol. with $H_2SO_4$, and mix. Reagent is stable at room temp. ca 1 month. (*Caution:* Wear face shield and heavy rubber gloves to protect against splashes.)
(**b**) *Washed chloroform*.—Vigorously shake ca 500 mL $CHCl_3$ with 30 mL $H_2SO_4$ in 1 L separator ca 2 min. Discard $H_2SO_4$ (bottom) layer. Wash $CHCl_3$ with 400 mL $H_2O$ by shaking vigorously 1 min; discard $H_2O$. Repeat $H_2O$ washing 3 times as above. Filter clear $CHCl_3$ layer thru funnel contg pad of glass wool covered with ca 50 g granular anhyd. $Na_2SO_4$. Prep. fresh daily. Use same batch of washed $CHCl_3$ for all samples and stds thruout series.
(**c**) *Ethinyl estradiol std solns*.—(*1*) *Stock soln*.—0.8 mg/

mL. Accurately weigh ca 40 mg USP Ref. Std Ethinyl Estradiol, dissolve in anhyd. MeOH in 50 mL vol. flask, dil. to vol. with MeOH, and mix. (*2*) *Intermediate soln.*—20 μg/mL. Pipet 5.0 mL stock soln into 200 mL vol. flask, dil. to vol. with isooctane, and mix. (*3*) *Working soln.*—4 μg/mL. Pipet 20 mL intermediate soln into 100 mL vol. flask, dil. to vol. with isooctane, and mix. (This soln is stable at room temp. ca 3 weeks.)

(**d**) *Diatomaceous earth.*—*See* **960.53B**.

### *B. Preparation of Column*

*Trap layer.*—Transfer ca 5 g granular anhyd. $Na_2SO_4$ to 25 × 250 mm chromatgc tube contg pad of glass wool in base. Thoroly mix 3 mL *10% NaOH soln* with 3 g diat. earth in 100 mL beaker. Transfer mixt. to tube in 1 portion and tamp moderately.

*Sample layer.*—Accurately weigh portion of ground tablet composite contg ca 40 μg ethinyl estradiol into 100 mL beaker. Add 3 mL $CHCl_3$ and 2 mL $H_2O$, and stir frequently 2 min to dissolve max. amt of sample. Mix with 4 g diat. earth 1 min, transfer quant. to tube in 1 portion, and tamp moderately. Dry-wash beaker with ca 0.5 g diat. earth and transfer wash to column. Wipe tamper, spatula, and beaker with glass wool and place glass wool on column.

### *C. Chromatography*

Rinse tamper, spatula, and beaker with 25 mL isooctane and add rinse to column. Discard eluate. Using total of 55 mL $CHCl_3$-isooctane (1 + 9), repeat rinsing as above and discard eluate. Wash column with 15 mL isooctane and discard eluate. Finally, elute ethinyl estradiol with 50 mL washed $CHCl_3$, followed by 25 mL isooctane, collecting eluate in 250 mL separator.

### *D. Determination*

Pipet 10 mL each of std soln and isooctane (reagent blank) into sep. dry 250 mL separators. To each add 50 mL washed $CHCl_3$ and 15 mL isooctane, and mix gently. Pipet 10 mL $MeOH$-$H_2SO_4$ into sample, blank, and std separators, letting pipet drain completely. (*Caution:* See safety notes on pipets and sulfuric acid.) Shake *vigorously* 4 min, and let layers sep. ca 15 min; protect from strong light. Within 30 min, scan spectra between 700 and 500 nm of pink (lower) phases of std and sample in 1 cm cells against reagent blank as ref., set at 0 at 700 nm.

$$\mu g \text{ Ethinyl estradiol in final soln} = (A/A') \times C \times 10 \text{ (mL)}$$

where $A$ and $A'$ refer to sample and std solns, resp., at max., ca 537 nm; and $C$ = μg/mL std soln.

Ref.: JAOAC **57**, 747(1974).

CAS-57-63-6 (ethinyl estradiol)

## 988.25 Cortisone Acetate in Bulk Drug and Dosage Forms

**Liquid Chromatographic Method**

**First Action 1988**

### *A. Principle*

Bulk drug or dosage form is dissolved in $CH_3CN$-0.025M acetate pH 4 buffer (1 + 1) and analyzed by exernal std method. Cortisone acetate is resolved from extaneous components by reverse phase liq. chromatgy and detected at 254 nm.

### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Equipped with isocratic pump system with UV detector (254 nm) and suitable recorder. Operate at ambient temp.

(**b**) *LC column.*—Reverse phase octadecylsilane, 10 μm.

(**c**) *Ultrasonic bath.*

(**d**) *Table top centrifuge.*

### *C. Reagents*

Use LC grade reagents.

(**a**) *Sodium acetate pH 4 buffer.*—Mix 20 mL 1N HCl, 150 mL 0.5N KCl, and 50 mL 0.5M NaOAc in 1 L vol. flask. Dil. to vol. with $H_2O$ (0.025M and 0.1 μ soln).

(**b**) *Diluent.*—Mix $CH_3CN$ and pH 4 buffer (1 + 1). Let mixt. equilibrate to room temp.

(**c**) *Mobile phase.*—Degas mixt. of 450 mL $CH_3CN$ and 550 mL $H_2O$. Adjust vol. of $CH_3CN$ as needed to obtain suitable retention time.

### *D. Preparation of Standard Solutions*

Accurately weigh ca 30 mg USP Ref. Std and transfer to 100 mL vol. flask. Add diluent, sonicate until std is dissolved, and dil. to vol. with diluent. Further dil. soln 10 mL to 50 mL, 10 mL to 25 mL, and 5 mL to 10 mL to prep. 3 std solns.

### *E. Preparation of Samples*

*Bulk drug.*—Accurately weigh ca 25 mg bulk drug and transfer to 250 mL vol. flask. Dissolve in and dil. to vol. with diluent. Use an aliquot, concn ca 0.1 mg/mL, for LC analysis.

*Tablets.*—Weigh and finely powder 10 tablets. Accurately weigh equiv. of ca 2 tablets or amt necessary to prep. soln not to exceed 1.0 mg/mL concn. Transfer sample to vol. flask, dissolve in diluent, and sonicate ca 5 min. Dil. as needed to concn of ca 0.1 mg/mL. Centrf. aliquot of prepd soln, and use portion of supernate for LC analysis. Filter supernate thru 0.45 μm filter before analysis if necessary.

*Suspension.*—Measure sample vol. of 1 or more vials as follows: Shake vial vigorously until product is homogeneous (but ≥15 s). Remove sample immediately by successive use of clean, dry hypodermic syringes of appropriate size. Deliver samples into same stdzd cylinder graduated to contain. Read vol. Transfer contents of cylinder to vol. flask. Rinse all glassware twice with diluent, and add rinses to vol. flask. Concn of soln should not exceed 1.0 mg/mL. Dil. soln as needed to concn of ca 0.1 mg/mL for LC analysis. Filter soln thru 0.45 μm filter before analysis if necessary.

### *F. System Suitability Tests*

Condition column with mobile phase until baseline is acceptable. Cortisone acetate peak should fulfill following performance specifications: column efficiency, ≥1500 theoretical plates; asymmetry or tailing factor (at 5% peak ht), ≤2; capacity factor, k', ≥2; relative std deviation <1% for 5 replicate 20 μL injections.

### *G. Determination*

Inject each of 3 std solns before and after all samples. Use peak area to calc. amt of each sample, μg/mL, with respect to stds. Curve-fit samples and calc. results mathematically or by calculator.

### *H. Calculations*

(*1*) Calc. concn, μg/mL, of cortisone acetate in each std soln ($C_{std}$) as follows:

$$C_{std} = (W_{std}/V_{std}) \times (V_d V_{df}) \times (P/100) \times 1000$$

where $W_{std}$ = wt of std, mg; $V_{std}$ = vol. of std, mL; $V_d$ = vol. of aliquot transferred for diln, mL; $V_{df}$ = vol. of flask used for diln, mL; $P$ = purity of std as %.

(*2*) Use linear regression procedure of $PA_{std}$ vs $C_{std}$ to prep. std curve mathematically or by computer. Correlation coefficient ($r$) should be ≥0.999 and intercept <±3.0.

Substitute calcd values for consts m and c, and variable $X$ in following equation to calc. individual $Y$ values:

$$Y = mX \text{ to } c$$

where $Y$ = std concn, μg/mL; and $X$ = av. of peak areas for stds injected before and after samples.

Enter $PA_{sam}$ (av. peak areas for samples = $X$) on std curve and obtain value for $C_{sam}$ (concn of sample, μg/mL = $Y$).

(*3*) Calc. cortisone acetate in bulk drug and dosage forms as follows:

Bulk drug:

$$\text{Cortisone acetate, mg} = (C_{sam} \times D)/1000$$

Suspension:

$$\text{Cortisone acetate, mg/mL} = [C_{sam} \times (D/N)]/1000$$

Tablets:

Cortisone acetate, mg/tablet

$$= C_{sam} \times D \times [W_{tab}/(W_{sam} \times 1000)]$$

where $D$ = diln factor; $N$ = vol. for vials sampled, mL; $W_{tab}$ = av. tablet wt, mg; $W_{sam}$ = sample wt, mg.

Ref.: JAOAC **71,** 534(1988).

CAS-50-04-4 (cortisone acetate)

## SYNTHETIC ESTROGENS

### 960.61 Diethylstilbestrol in Drugs

**Spectrophotometric Method**

**First Action 1960**
**Final Action 1965**

#### *A. Reagent and Apparatus*

(**a**) *Diethylstilbestrol std soln.*—Accurately weigh suitable amt of USP Diethylstilbestrol Ref. Std, dissolve in alcohol, and prep. soln contg 20.0 μg/mL by accurate stepwise diln with alcohol. Prep. working std soln by mixing 25 mL of this soln with 25 mL 1.8% $K_2HPO_4$ soln.

(**b**) *Irradiation containers.*—Quartz cells ≥4 mL capacity with clear sides, or 18 × 150 mm Vycor test tubes, held in rack that does not obstruct effective light beam of cylindrical 15 watt germicidal lamp, may be used conveniently.

#### *B. Preparation of Assay Solution*

(**a**) *Oil solns containing 2 mg or less diethylstilbestrol/mL.*—Using accurately calibrated hypodermic syringe, transfer vol. sample contg 2 mg diethylstilbestrol to separator contg 50 mL isooctane. Shake mixt. with 10 mL 1*N* NaOH and transfer well defined aq. layer as completely as possible to second separator contg 50 mL isooctane. Shake vigorously and transfer clear aq. layer to third separator. Repeat extn of the 2 isooctane layers successively with two 10 mL portions 1*N* NaOH, collect aq. layers in third separator, and discard extd isooctane layers.

Acidify combined aq. exts with 3 mL $H_2SO_4$ (1 + 1), cool, and ext diethylstilbestrol with three 30 mL portions $CHCl_3$. Wash $CHCl_3$ exts successively in 2 separators, first contg 20 mL 1% $NaHCO_3$ soln and second, 20 mL $H_2O$.

Filter washed $CHCl_3$ exts thru cotton pledget moistened with $CHCl_3$ into 100 mL vol. flask, dil. to vol. with $CHCl_3$, and mix.

Transfer 10.0 mL $CHCl_3$ soln, contg 200 μg diethylstilbestrol, to small erlenmeyer and evap. just to dryness on steam bath with aid of air current. Cool in vac. desiccator 10 min. Add 10.0 mL alcohol, stopper, and dissolve residue by swirling. After 15 min, mix with 10.0 mL *1.8%* $K_2HPO_4$ to prep. assay soln.

(**b**) *Oil solns containing more than 2 mg diethylstilbestrol/mL.*—Dil. convenient accurately measured vol. oil soln with $CHCl_3$ to obtain soln contg 0.5 mg diethylstilbestrol/mL. Transfer 4 mL aliquot to separator contg 50 mL isooctane and proceed as in (**a**), beginning "Shake mixt. with 10 mL 1*N* NaOH . . ."

(**c**) *Tablets.*—Transfer accurately weighed portion powd material contg 2 mg diethylstilbestrol to separator contg 30 mL $CHCl_3$. Add 10 mL $H_2O$ and 1 mL $H_2SO_4$ (1 + 1) and shake vigorously. Drain $CHCl_3$ layer into second separator, wash with 5 mL $H_2O$, and filter thru cotton pledget moistened with $CHCl_3$ into 100 mL vol. flask. Repeat extn with three 20 mL portions $CHCl_3$, dil. combined exts to 100 mL, and mix.

Proceed as in (**a**), fourth par.

#### *C. Irradiation*

(*Caution:* Protect eyes from direct rays of UV light.)

Test transparency of several irradiation containers as follows: Transfer convenient vols of working std soln to tubes, place them ca 7 cm from 15 watt germicidal lamp, and irradiate soln transversely ca 10 min. Measure $A$ of yellow solns at 418 nm in suitable spectrophtr in matched 1 cm cells, against $H_2O$. Re-irradiate for 1–3 min intervals, and note irradiation time required for max. $A$. Repeat irradiation process, varying distance of tubes from lamp, and det. most convenient conditions for developing stable, repeatable colors of max. $A$ (ca 0.7 at 418 nm).

Transfer portions of working std soln and assay soln to clean, dry irradiation containers, and irradiate under optimum conditions previously detd. Calc. wt diethylstilbestrol in sample.

#### *D. Total Phenols*

Transfer 20 mL $CHCl_3$ ext, **960.61B**, contg 400 μg diethylstilbestrol to beaker. Transfer alc. soln contg 400.0 μg USP Diethylstilbestrol Ref. Std to similar beaker, and treat both solns as follows: Evap. to dryness on steam bath with aid of air current. Dissolve residues in 2.0 mL HOAc with gentle warming. Cool to room temp., add 10 drops $H_2SO_4$ (1 + 1), and mix. Cool, add 5 drops *10%* $NaNO_2$ *soln*, and let stand 45 min with occasional mixing. Wash quant. into 25 mL vol. flask with ca 20 mL alc. $NH_4OH$ soln, prepd by mixing equal vols alcohol and dil. $NH_4OH$ (4 + 6). Cool in ice bath, and let stand at room temp. 1 hr. Dil. to vol. with the alc. $NH_4OH$ soln, and mix. If ppt forms, filter thru dry paper, rejecting first few mL filtrate. Det. $A$ of clear, yellow alk. solns at 420 nm in tightly stoppered 1 cm cells, in suitable spectrophtr, against alcohol (1 + 2). Calc. % total phenols, as diethylstilbestrol, in sample.

Ref.: JAOAC **43,** 248(1960).

CAS-56-53-1 (diethylstilbestrol)

### 973.77 Dienestrol in Drugs

**Spectrophotometric Method**

**First Action 1973**
**Final Action 1977**

(*Caution:* See safety notes on distillation, pipets, benzene, diethyl ether, isooctane, and methanol.)

#### *A. Reagents*

(**a**) *Dienestrol std soln.*—Approx. 15 μg/mL. Accurately weigh USP Dienestrol Ref. Std, dissolve in MeOH, and serially dil. to concn. Store in low-actinic vol. flask.

(**b**) *Methanolic sulfuric acid.*—Carefully add, with swirling, 50 mL $H_2SO_4$ to 50 mL cold MeOH, while continuously chilling mixt. in ice-$H_2O$. Use reagent at room temp. Reagent is stable 3–4 days in g-s flask.

(**c**) *Ethyl ether.*—Test as follows on day of use: Evap., with gentle heat and air stream, mixt. of 10.0 mL dienestrol std soln in ca 200 mL $H_2O$-washed ether. Dissolve residue in 10.0 mL MeOH. Proceed as in **973.77D**, using this soln and 5.0 mL dienestrol std soln. Resulting solns should be clear and exhibit single max. at ca 303 nm, and corrected $A$, **973.77E**, should differ ≤3%. If necessary, wash 750 mL ether with three 50 mL portions 10*N* KOH in 1 L separator. Percolate upper ether layer thru 300 × 22 mm glass chromatgc tube contg glass wool plug and 20 g diat. earth thoroly mixed with 15 mL 10*N* KOH and tamped moderately tight. Discard first 30 mL eluate and collect remainder for use. Column will only purify max. of 1 L ether.

(**d**) *Diatomaceous earth.*—*See* **960.53B**.

### B. Preparation of Columns

*Trap column.*—Mix 4 g diat. earth and 3 mL *0.25M KOH* and transfer to 200 × 22 mm glass chromatgc tube contg glass wool plug. Tamp mixt. tightly and top with glass wool pad. Prewash column with 25 mL $H_2O$-washed ether, followed by 25 mL benzene.

*Sample column.*—Accurately weigh freshly ground sample contg ca 400 μg dienestrol into 150 mL beaker. Add 3 mL *0.3M $K_3PO_4$* and wet sample completely. Add 5 g diat. earth and mix thoroly with spatula. Transfer quant. in 2 equal portions to 200 × 22 mm glass chromatgc tube contg glass wool pad, tamping each portion moderately tight. Dry-rinse beaker with 1–2 g diat. earth and add rinse to column. Wipe tamper, spatula, and beaker with glass wool pad and add pad to top of column.

### C. Chromatography

Arrange columns so that eluate from sample column passes into trap column. Add 25 mL benzene to trap column; then add 175 mL benzene-isooctane (9 + 1) to sample column, using several portions to rinse sample beaker. Maintain layer of eluant over trap column. (To maintain this reservoir in trap column, connect the 2 columns with air-tight stopper, i.e., hollow No. 4 Nalgene stopper with hole drilled to accommodate stem of sample column.) Discard sample column when elution is complete. Wash trap column with addnl 25 mL benzene-isooctane (9 + 1) and discard eluates.

Elute dienestrol from trap column with 225 mL $H_2O$-washed ether into 250 mL g-s conical flask contg 10 mL absolute alcohol. Without delay, evap. to near dryness, using air stream and gentle heat. Rinse flask walls with small amt of absolute alcohol and evap. soln to dryness. Pipet 25 mL MeOH into flask, stopper tightly, and let stand several min with frequent vigorous swirling.

### D. Isomerization

Into sep. 25 mL g-s conical flasks, pipet 5 mL dienestrol std soln, 5 mL sample prepn, and 5 mL MeOH as reagent blank. Add 5.0 mL methanolic $H_2SO_4$ to each flask with swirling (solns will become warm). Stopper flasks tightly and shake vigorously; then let cool ≥25 min at room temp.

### E. Determination

Det. $A$ of sample and std solns between 400 and 240 nm in 1 cm cells against reagent blank. Correct $A$ at ca 303 nm by subtracting $A$ at 360 nm.

$$\text{mg Dienestrol/tablet} = [(A/A') \times C \times V \times W]/Q$$

where $A$ and $A'$ refer to sample and std solns, resp.; $C$ = exact concn of std in mg/mL; $V$ = mL sample diln (25 mL); $W$ = av. tablet wt (g); and $Q$ = sample wt (g).

Refs.: JAOAC **55,** 190(1972); **56,** 674(1973).

CAS-87-17-3 (dienestrol)

## 965.46 Hexestrol in Drugs

**Spectrophotometric Method**

**First Action 1965**

**Final Action 1967**

### A. Determination

Grind tablets to fine powder. Weigh amt powder contg ca 5 mg hexestrol into 125 mL separator contg 25 mL $H_2O$ and 1 mL HCl (1 + 9). Ext with 25, 15, 10, and 10 mL $CHCl_3$. Drain each ext thru $CHCl_3$-satd cotton pledget into 100 mL beaker. Evap. combined exts to ca 25 mL on steam bath in air current. Check for completeness of extn by evapg addnl 10 mL ext to dryness.

Quant. transfer concd $CHCl_3$ exts to 125 mL separator contg 10 mL isooctane. Ext with 25, 15, 15, and 10 mL ca 0.1*N* NaOH, rolling or shaking separator gently 90 sec each time; emulsions may form. Drain lower org. layer into second 125 mL separator, each time including any small emulsion layer present. Continue alk. extn of org. phase, draining it alternately into two 125 mL separators and combining alk. exts by pouring each time into original separator. Discard org. phase.

Make combined alk. exts acid with HCl. Ext with 25, 15, 15, and 10 mL $CHCl_3$, collecting combined exts in 125 mL separator. Wash $CHCl_3$ exts with two 15 mL portions $H_2O$. Discard $H_2O$ washes.

Pass combined $CHCl_3$ exts thru 1 cm column of granular anhyd. $Na_2SO_4$ in coarse fritted glass funnel, ca 3.5 cm id, into 100 mL vol. flask. Rinse column and stem tip with small portions $CHCl_3$. Dil. to vol. with $CHCl_3$. Place 50.0 mL aliquot in g-s flask and evap. just to dryness on steam bath, with aid of air current. Remove last traces of $CHCl_3$ with air current and without heat. Pipet 50 mL alcohol onto dry residue; shake 1 min to dissolve. This is sample soln.

Prep. std soln by dissolving pure hexestrol in enough alcohol to make concn ca 2.5 mg/50.0 mL. Use alcohol as ref. blank with sample and std solns.

Det. baseline $A$ of sample and std solns at 280 nm with spectrophtr. If recording UV spectrophtr is used, record spectra between 320 and 240 nm. Adjust instrument to begin at 320 nm with zero $A$, and record spectra to 240 nm.

$$\text{mg Hexestrol in assay sample} = (A/A') \times (\text{mg/mL std soln}) \times \text{total mL sample soln}$$

where $A$ refers to sample and $A'$ refers to std soln at 280 nm.

### B. Qualitative Identification

(**a**) *Ultraviolet spectra.*—Dil. alc. soln of sample and std previously used for quant. assay to ca 20 μg/mL with alcohol. Compare UV spectrum from 215 to 320 nm with similar spectrum from authentic hexestrol.

(**b**) *Infrared spectra.*—Prep. KBr disk contg 0.3–0.6% hexestrol from residue obtained by evapg portion of remaining $CHCl_3$ sample soln from assay. Compare IR spectrum from 2 to 16 μm with similar spectrum from authentic hexestrol. (Extraneous peak at 5.85 μm appears in spectra of tablet prepns that does not appear in std.)

Ref.: JAOAC **48,** 613(1965).

CAS-5635-50-7 (hexestrol)

## 971.42 Mestranol in Drugs

### Spectrophotometric Method

**First Action 1971**
**Final Action 1973**

(Applicable in presence of norethindrone and norethynodrel; not applicable in presence of ethynodiol diacetate or chlormadinone acetate)

### *A. Reagents*

**(a)** *Diatomaceous earth.—See* **960.53B**.

**(b)** *Immobile solvent.*—Mix equal vols DMF and formamide (either redistd or stabilized formamide may be used).

**(c)** *n-Heptane.*—Redistd (may be prepd by fractionating thru all-glass column). *A* against alcohol in 1 cm cells should be <0.500 in range 250–360 nm (limit of aromatic content). Residue from evapn of 25 mL distillate, dissolved in 10 mL alcohol, should have *A* ≤0.01 in range 230–360 nm (nonvolatile residue limit).

**(d)** *Spectrophotometric solvent.*—Transfer 10.0 mL $CHCl_3$ to 100 mL vol. flask, add ca 80 mL *n*-heptane, warm to room temp., and dil. to vol. with *n*-heptane.

**(e)** *Mestranol std soln.*—0.06 mg/mL. Dissolve accurately weighed amt USP Mestranol Ref. Std in $CHCl_3$ and dil. quant. to ca 0.6 mg mestranol/mL. Transfer 10.0 mL aliquot to 100 mL vol. flask, add ca 80 mL *n*-heptane, warm to room temp., and dil. to vol. with *n*-heptane.

### *B. Preparation of Assay Mixture*

Finely powder tablets. Transfer accurately weighed portion contg ca 0.6 mg mestranol to 100 mL beaker. Add 2.0 mL immobile solv., mix, and warm 5 min on steam bath with occasional stirring with spatula to ensure that powder is thoroly wetted. Cool, add 4 g diat. earth, and mix with spatula until fluffy.

### *C. Column Chromatography*

Pack pledget of fine glass wool in base of 25 × 250 mm chromatgc tube. Transfer 3.0 mL immobile solv. to 100 mL beaker, add 1 g anhyd. $Na_2SO_4$, and mix by swirling. Add 5 g diat. earth and mix until fluffy. Transfer to tube and tamp gently to compress to uniform mass. Quant. transfer prepd sample to column, scrub beaker with 0.5 g diat. earth, and tamp as before. Wipe beaker, spatula, and funnel with pad of glass wool. Place pad on top of column and tamp lightly.

Add *n*-heptane to column. Discard first 20 mL eluate and then collect ca 99 mL eluate in 100 mL vol. flask. Wash tip of column with heptane, dil. eluate to vol. with heptane, and mix. Transfer 50.0 mL aliquot to 125 mL g-s conical flask and evap. on steam bath with aid of air current to ca 1 mL. (*Caution:* Use effective fume removal device when evapg heptane.) Remove last traces of solv. without heat. Wash sides of flask with ca 2 mL alcohol and evap. solv. on steam bath as before, removing last traces of solv. without heat. Add 5.0 mL spectrophtric solv. to flask, stopper, let stand ca 5 min, and swirl to ensure soln of residue.

### *D. Determination*

Record *A* of sample and std solns in 1 cm cells against spectrophtric solv. Construct baseline by extending line passing between points on spectrum at 302 and 315 nm. Det. baseline-corrected *A* of sample ($\Delta A$) and std ($\Delta A'$) at max., ca 287 nm.

$$\text{mg Mestranol in portion of tablets taken} = 10C \times (\Delta A/\Delta A')$$

where $C$ = exact concn, mg/mL, of mestranol std.

Ref.: JAOAC **54**, 590(1971).

CAS-72-33-3 (mestranol)

## 975.59 Mestranol with Ethynodiol Diacetate in Drugs

### Spectrophotometric Method

**First Action 1975**
**Final Action 1976**

### *A. Principle*

Mestranol is sepd on partition column, eluted with *n*-heptane, and extd into MeOH-$H_2SO_4$ reagent to form colored steroid complex with max. *A* at ca 540 nm.

### *B. Apparatus and Reagents*

(*Caution:* Dimethyl sulfoxide (DMSO) and formamide can be harmful. Avoid skin contact by wearing heavy rubber gloves. Use effective fume removal device.)

**(a)** *Chromatographic tubes and tamping rod.*—Glass, 25 (od) × 300 mm. *See* **967.31A**.

**(b)** *Formamide.*—Reagent grade contg no stabilizing agent or $H_2O$. Use recently opened bottle.

**(c)** *Washed n-heptane.*—Vigorously shake *n*-heptane (bp 98–99°) with ca 10% of its vol. of $H_2SO_4$ in separator ≥5 min. Discard $H_2SO_4$ (lower) layer and wash heptane with $H_2O$ until washings are neut. to pH test paper. Filter thru firm plug of absorbent cotton covered with ca 50 g anhyd. $Na_2SO_4$, discarding first 5 mL. Use same batch of washed heptane for all samples and stds thruout series.

**(d)** *Methanol-sulfuric acid reagent.*—Cautiously add in small increments, with mixing, chilled $H_2SO_4$ (min. 95%) to 60 mL chilled anhyd. MeOH in 200 mL vol. flask in ice bath. Adjust to room temp., dil. to vol. with $H_2SO_4$, and mix. Reagent is stable at room temp. ca 1 month. (*Caution:* See safety notes on sulfuric acid and methanol.)

**(e)** *Mestranol std solns.*—(*1*) *Stock soln.*—1 mg/mL. Dissolve ca 25 mg USP Ref. Std Mestranol, accurately weighed, with 3 mL $CHCl_3$ in 25 mL vol. flask, dil. to vol. with *n*-heptane, and mix well. (*2*) *Intermediate soln.*—30 μg/mL. Pipet 3 mL stock soln into 100 mL vol. flask, dil. to vol. with *n*-heptane, and mix. (*3*) *Working soln.*—0.75 μg/mL. Pipet 5 mL intermediate soln into 200 mL vol. flask, dil. to vol. with *n*-heptane, and mix. Prep. fresh daily.

### *C. Preparation of Column*

Thoroly mix 3 g diat. earth, **960.53B**, and 1 mL $H_2O$ in 100 mL beaker, transfer to chromatgc tube contg pledget of glass wool at base, and tamp moderately tight.

Thoroly mix 7 g diat. earth and 3.5 mL DMSO (spectral grade)-formamide (10 + 9) in 150 mL beaker, transfer to tube in 2 portions, and tamp each moderately tight.

Accurately weigh portion of ground tablets contg ca 150 μg mestranol into 100 mL beaker. Add 2 mL formamide and stir continuously 2 min to wet and disperse sample completely. Mix thoroly with 4 g diat. earth, transfer quant. to column in 1 portion, and tamp moderately tight. Scrub beaker with ca 0.5 g diat. earth and transfer to column. Wipe tamper, spatula, and beaker with glass wool, and place as pad above column contents.

### *D. Determination*

Rinse tamper, spatula, and beaker with 65 mL *n*-heptane, and pour rinse into column. Discard eluate contg ethynodiol diacetate. Elute mestranol with total of 135 mL *n*-heptane, collecting eluate in 200 mL vol. flask. Dil. to vol. with *n*-heptane and mix.

Pipet 50 mL each of mestranol working std soln and sample eluate in sep. dry 250 mL separators. Pipet 10 mL MeOH-$H_2SO_4$ (*Caution:* See safety notes on pipets.) reagent into each, draining pipet completely. Shake vigorously 4 min and let stand 45 min, protected from light. Within ≤25 min, scan pink (lower) phase in each separator between 700 and 500 nm in 10 mm cells against MeOH-$H_2SO_4$ reagent as ref., setting instrument to 0 $A$ at 700 nm for each scan.

$$\mu g \text{ Mestranol in sample taken} = 200 \times C \times (A/A')$$

where 200 = mL sample diln, $C$ = μg mestranol std/mL std soln, and $A$ and $A'$ refer to sample and std, resp., at max., ca 540 nm.

Ref.: JAOAC **58,** 75(1975).

CAS-72-33-3 (mestranol)

## PROGESTATIONAL STEROIDS

### 971.43 Progestational Steroids in Drugs

**Spectrophotometric Method**
**First Action 1971**
**Final Action 1973**

#### A. Principle

$CHCl_3$ ext of norethindrone, norethindrone acetate, dimethisterone, or medroxyprogesterone acetate is treated directly with isonicotinic acid hydrazide to produce stable color measured at 380 nm. Norethynodrel in $CHCl_3$ ext is isomerized with HCl prior to same reaction.

#### B. Reagents

(**a**) *Isonicotinic acid hydrazide (INH) soln.*—Transfer 100 mg INH (mp 171–173°) to 200 mL vol. flask. Add ca 150 mL MeOH and 0.1 mL HCl. Shake to dissolve, and dil. to vol. with MeOH.

(**b**) *Washed cotton.*—Wash absorbent cotton with $CHCl_3$ and air dry.

(**c**) *Washed chloroform.*—Shake $CHCl_3$ with equal vol. $H_2O$ in separator. After $CHCl_3$ layer clears, filter thru pledget of washed cotton covered with bed of ca 50 g anhyd. $Na_2SO_4$. Use thruout method.

(**d**) *Methanolic HCl soln.*—Dil. 3.0 mL HCl to 50 mL with MeOH.

(**e**) *Std soln.*—Dissolve accurately weighed amt std drug in $CHCl_3$ and dil., if necessary, with $CHCl_3$ to ca 10 mg/100 mL.

#### C. Preparation of Sample

Finely powder tablets. Transfer accurately weighed portion of powder contg ca 10 mg steroid to 125 mL separator contg 10 mL $H_2O$. Add 25 mL $CHCl_3$, shake continuously 5 min, and filter ext thru pledget of cotton and ca 30 g anhyd. $Na_2SO_4$ into 100 mL vol. flask. Repeat extn with two 25 mL portions $CHCl_3$, combine exts, rinse filter with $CHCl_3$, and dil. filtrate to vol. with $CHCl_3$.

#### D. Determination

(**a**) *Norethindrone, norethindrone acetate, dimethisterone, and medroxyprogesterone acetate.*—To sep. 50 mL g-s conical flasks, transfer 5.0 mL sample ext, 5.0 mL std soln, and 5.0 mL $CHCl_3$ as blank. To each flask add 25.0 mL INH soln, stopper, mix, and let stand 30 min. Record spectra from 500 to 350 nm against reagent blank.

$$\text{mg Steroid/tablet} = (A/A') \times W' \times (T/W)$$

where $A$ and $A'$ refer to sample and std, resp., at max., ca 380 nm; and $W$, $W'$, and $T$ = mg sample, mg std/100 mL, and av. tablet wt in mg, resp.

(**b**) *Norethynodrel.*—Add 1.0 mL methanolic HCl to sep. flasks contg 100 mL sample ext, 100 mL norethynodrel std, and 100 mL $CHCl_3$ as blank. Shake vigorously 3 min (mixts may be hazy) and let stand 70 min. Add 1.0 mL MeOH to each flask and mix thoroly (mixts become clear).

Transfer 5.0 mL each soln to sep. 50 mL g-s conical flasks and continue as in (**a**).

Refs.: JAOAC **53,** 831(1970); **54,** 617(1971).

CAS-79-64-1 (dimethisterone)
CAS-71-58-9 (medroxyprogesterone acetate)
CAS-68-22-4 (norethindrone)
CAS-51-98-9 (norethindrone acetate)
CAS-68-23-5 (norethynodrel)

### 977.32 Progestational Steroids in Drugs

**Single Tablet Assay**
**First Action 1977**
**Final Action 1979**

#### A. Principle

Principle is same as in **971.43A**, except that before colorimetric detn, sample is eluted from $H_2O$-diat. earth column with $CHCl_3$. Elution vols and vols of isonicotinic acid hydrazide used vary with individual tablet dosage levels ranging from 0.35 to 10 mg.

#### B. Apparatus and Reagents

(**a**) *Glass chromatographic tube and tamping rod.* —250 × 25 (od) mm. *See* **967.31A**.

(**b**) *Isonicotinic acid hydrazide (INH) soln.*—Prep. 500 mL as in **971.43B(a)**, using 500 mL vol. flask and 2.5-fold amts of all reagents.

(**c**) *Washed chloroform.—See* **971.43B(c)**.

(**d**) *Methanolic hydrochloric acid soln.*—10%. Dil. 5.0 mL HCl to 50 mL with MeOH.

(**e**) *Std solns.*—Dissolve in individual vol. flasks accurately weighed amt of each std material in $CHCl_3$, and dil. with $CHCl_3$ to ca 0.035 mg norethindrone/mL, 0.025 mg norgestrel/mL, and 0.25 mg norethynodrel/mL. Prep. fresh daily.

#### C. Preparation of Column

Soak 1 accurately weighed tablet with 1 mL $H_2O$ in 100 mL beaker. Thoroly mix 1 g diat. earth, **960.53B**, with 0.5 mL $H_2O$ in another 100 mL beaker with small metal spatula, transfer to chromatgc tube contg small pledget of glass wool at base, and tamp tight. Carefully triturate tablet with spatula, add 3 mL $CHCl_3$, and mix gently to dissolve as much as possible of tablet. Mix sample thoroly with 3 g diat. earth, transfer to tube in 1 portion, and tamp tight. Scrub beaker with ca 0.5 g diat. earth and transfer to tube. Wipe tamper, spatula, and beaker with small pledget of glass wool and add to column.

#### D. Elution

(**a**) *Norethindrone (0.35 mg/tablet).*—Pipet 15 mL INH soln into 25 mL vol. flask and place to collect eluate from column. Rinse tamper, spatula, and beaker with 10 mL $CHCl_3$, and pour rinse into column. When elution stops, continue elution by adding 1 mL portions $CHCl_3$ until eluate fills flask to within ca 0.5 mL of mark. Gently swirl flask occasionally during elution without detaching it from column. Detach flask from column, add $CHCl_3$ to vol., mix, and let stand 45 min before colorimetric detn.

For >0.35 mg norethindrone, increase elution vol. and/or dil. eluate.

(**b**) *Norgestrel (0.5 mg/tablet)*.—Pipet 30 mL INH soln into 50 mL vol. flask and place it to collect eluate from column. Rinse tamper, spatula, and beaker with 10 mL $CHCl_3$, and pour rinse into column. When elution stops, continue elution by adding 2 mL portions $CHCl_3$ until eluate fills flask to within ca 0.5 mL of mark. Continue as in (**a**).

(**c**) *Norethynodrel (2.5–5.0 mg/tablet)*.—Place 100 mL vol. flask marked to indicate ca 75 mL vol. to collect eluate from column. Rinse tamper, spatula, and beaker with 10 mL $CHCl_3$, and pour rinse into column. When elution stops, continue elution by adding 5 mL portions $CHCl_3$ until eluate fills flask to 75 mL mark. Pipet 10 mL norethynodrel std soln into another 100 mL vol. flask and mix with 65 mL $CHCl_3$. Add 75 mL $CHCl_3$ into third 100 mL vol. flask as blank. Add ca 5 small boiling chips and 1.0 mL methanolic 10% HCl into each flask. Stopper, and shake vigorously 1–2 min. Remove stoppers and heat soln to bp on steam bath. Continue heating 15 min with occasional swirling. Remove flasks and cool to room temp. Dil. each to vol. with $CHCl_3$ and mix. ($CHCl_3$ solns should be completely clear.)

### E. Determination

(**a**) *Norethindrone*.—Into 25 mL vol. flask, pipet 10 mL norethindrone std soln and 15.0 mL INH soln. Stopper, and shake ca 1 min. Into another 25 mL vol. flask, pipet 10 mL $CHCl_3$ and 15 mL INH soln as blank for both norethindrone and norgestrel. Stopper flask and mix. Let all flasks stand 1 hr.

(**b**) *Norgestrel*.—Pipet 20 mL norgestrel std soln into 50 mL vol. flask and add 30.0 mL INH soln. Stopper, and shake ca 1 min. Let soln and blank, (**a**), stand 1 hr.

(**c**) *Norethynodrel*.—Into sep. 25 mL vol. flasks, pipet 10 mL each of isomerized sample, std, and blank. Add 15.0 mL INH soln to each. Stopper, shake ca 1 min, and let stand 1 hr.

Record spectra of samples and stds from 550 to 350 nm within next hr, against corresponding ref. blank, setting instrument to 0 $A$ at 550 nm for each scan.

$$\mu g \text{ Progestin/tablet} = (A/A') \times C \times (W/W)$$

where $A$ and $A'$ refer to sample and std, resp., at ca 380 nm; $C$ = μg of corresponding std in 10.0 mL norethindrone or norgestrel std soln, or 20.0 mL norethynodrel std soln; $W$ = av. tablet wt; and $W$ = individual tablet wt.

Ref.: JAOAC **60,** 922(1977).

CAS-68-22-4 (norethindrone)
CAS-68-23-5 (norethynodrel)
CAS-797-63-7 (norgestrel)

## 963.31★ Ethisterone in Drugs

**Infrared Spectroscopic Method**

**First Action 1963**
**Surplus 1974**

*See* **39.043–39.046**, 12th ed.

# ADRENOCORTICO STEROIDS

## 984.40 Hydrocortisone in Drugs

**Liquid Chromatographic, Infrared Spectroscopic, and Thin Layer Chromatographic Methods**

**First Action 1984**
**Final Action 1988**

### A. Principle

Sample is dissolved in MeOH and $CH_2Cl_2$, and hydrocortisone is detd by liq. chromatography using acetaminophen internal std. Identity is confirmed by IR or TLC.

### B. Apparatus and Reagents

(**a**) *Liquid chromatograph*.—Equipped with sampling valve capable of introducing 10 μL injections, 25 cm × 4.6 mm id stainless steel column packed with spherical 5–6 μm diam. porous silica particles, and 254 nm UV detector set at sensitivity to produce ca ½ full scale peak ht on suitable recorder for 10 μL injection of std soln. Mobile solv. flow rate 1.5 mL/min, ambient temp.

(**b**) *Water-washed 1,2-dichloroethane*.—Shake 500 mL LC grade 1,2-dichloroethane with 250 mL $H_2O$ for 1 min, let layers sep., and filter lower layer thru 0.5–1 μm porosity polytetrafluoroethylene (PTFE) membrane.

(**c**) *Mobile solvent*.—Mix 55 mL $H_2O$-LC grade MeOH (5 + 95) soln with 1.0 mL glacial HOAc and dil. to 1 L with $H_2O$-washed 1,2-dichloroethane. Degas mixt. Adjust $H_2O$ and MeOH content to obtain suitable retention times.

(**d**) *Internal std soln*.—Dissolve 200 mg acetaminophen (100% pure, Aldrich Chemical Co.) in 4 mL MeOH and dil. to 200 mL with $H_2O$-washed 1,2-dichloroethane.

(**e**) *Std soln*.—Accurately weigh ca 10 mg USP Ref. Std Hydrocortisone, add 2 mL MeOH and 4.0 mL internal std soln, and dil. to 50 mL with LC grade $CH_2Cl_2$.

### C. Chromatographic System Suitability Test

Equilibrate LC system and inject 10 μL portions of std soln. Retention times for hydrocortisone and acetaminophen should be ca 8 and 10.5 min, resp. Column efficiency, $n$, calcd using hydrocortisone peak, should be ≥5000 theoretical plates; resolution, $R_s$, between hydrocortisone and acetaminophen peaks should be ≥2.5. Inject 6 replicate 10 μL portions of std soln and calc. response ratios, $R$, of hydrocortisone peak relative to acetaminophen peak. Relative std deviation for six $R$ values should be ≤1.0%.

### D. Sample Preparation

(**a**) *Bulk material*.—Accurately weigh ca 50 mg previously dried (3 h at 105°) sample, add 10 mL MeOH and 20.0 mL internal std soln, and dil. to 250.0 mL with $CH_2Cl_2$.

(**b**) *Tablet composite*.—Weigh and finely powder ≥20 tablets. Accurately weigh portion of powder equiv. to 1 tablet and transfer to vol. flask of a size to yield final hydrocortisone concn of 0.2 mg/mL. Add 2 mL MeOH for each 10 mg of labeled hdyrocortisone content. Place flask in ultrasonic bath 2 min. Add $CH_2Cl_2$ until flask is ca ½-full and return to ultrasonic bath 1 min. Add 4.0 mL internal std soln for each 10 mg labeled hydrocortisone content. Dil. to vol. with $CH_2Cl_2$, mix, and filter thru 0.5–1.0 μm porosity Teflon membrane.

(**c**) *Individual tablets*.—Place 1 tablet in g-s or vol. flask of a size to yield final hydrocortisone concn of ca 0.2 mg/mL. Place 100 μL $H_2O$ for each 10 mg hydrocortisone directly on tablet and let soak 30 min. Add 2 mL MeOH for each 10 mg of labeled hydrocortisone content and place flask in ultrasonic bath 10 min or until tablet disintegrates. Proceed as in *Tablet composite*, starting with "Add $CH_2Cl_2$ until . . .".

### E. Determination

Equilibrate LC system by passing mobile phase thru column ca $1/2$ h. Inject 10 μL portions of std soln until $R$ values for 3 consecutive std chromatograms agree within 1%, then inject 10 μL sample prepn.

$$\text{mg Hydrocortisone in sample } (R/R') \times (V/V') \times W$$

where $R$ and $R'$ = response ratios of hydrocortisone peak relative to internal std peak for sample and std, resp.; $V'$ and $V$ = mL internal std soln in std and sample solns, resp.; $W$ = mg hydrocortisone in std soln.

### F. Infrared Spectroscopic Identification

**(a)** *Bulk material.*—IR spectrum of KBr dispersion of sample, previously dried 3 h at 105°, exhibits max. at same wavelengths as that of similar prepn of USP Ref. Std Hydrocortisone.

**(b)** *Tablets.*—Powder tablets equiv. to ca 50 mg hydrocortisone and digest 5 min with 15 mL hexane. Decant hexane, ext residue with 15 mL peroxide-free ether in same manner as before, and discard ext. Digest residue with 25 mL dehydrated alcohol for 5 min, filter, and evap. alcohol ext on steam bath to dryness. Add 10 mL $H_2O$, mix, let residue settle, and decant $H_2O$. Dry residue at 105°. Dissolve USP Ref. Std Hydrocortisone in alcohol and treat as sample prepn, beginning with "evap. alcohol ext . . .". The IR spectrum of KBr dispersion of sample residue exhibits absorbance maxima at the same wavelengths as that of similar USP Ref. Std Hydrocortisone prepn.

## Thin Layer Chromatographic Identification

### G. Apparatus and Reagents

**(a)** *Thin layer plate.*—20 × 20 cm, coated with 0.25 mm thick layer of chromatographic silica gel with fluorescent indicator.

**(b)** *Developing solvent.*—$CHCl_3$-MeOH-$H_2O$ (180 + 15 + 1).

**(c)** *Spray reagent.*—$H_2SO_4$-EtOH (1 + 4).

**(d)** *Std soln.*—0.2 mg USP Ref. Std Hydrocortisone/mL $CH_2Cl_2$.

### H. Procedure

Apply 10 μL portions of sample prepn from LC *Determination* and std soln (**d**) to TLC plate 2 cm from bottom edge. Develop plate in suitable tank equilibrated with developing solv. Let plate dry and examine under shortwave UV light. Spray plate with $H_2SO_4$-EtOH (1 + 4), heat 5 min at 120°, and examine under longwave UV light. Hydrocortisone has $R_f$ of ca 0.2, appearing as dark spot under UV before spraying and as bright yellow spot in final step.

Ref.: JAOAC **67,** 218(1984).

CAS-50-23-7 (hydrocortisone)

## 974.44★ Dexamethasone Phosphate in Drugs

**Spectrophotometric Method**

**First Action 1974**
**Surplus 1988**

*See* **39.056–39.060,** 14th ed.

## 988.27 Dexamethasone in Drug Substance and Elixirs

**Quantitative and Identification Methods**

**First Action 1988**

### A. Principle

Dexamethasone content in drug substance and elixir is detd by normal phase LC using quaternary mobile phase with controlled $H_2O$ content, UV detection at 254 nm, and cortisone as internal std. Identity is confirmed in bulk drug substance and elixir by TLC and in drug substance by IR spectroscopy and relative LC retention time ratios. Alcohol content in elixir is detd by GC on porous polymer column using internal std and flame ionization detector.

## Liquid Chromatographic Method

### B. Apparatus

**(a)** *Liquid chromatograph.*—Model 8100 (Spectra-Physics, 3333 N First St, San Jose, CA 95134-1995) equipped with Model 100-10 photometric detector (Hitachi/NSA, 460 E Middlefield Rd, Mountain View, CA 94043), 15–30 μL injection valve (Valco Instruments Co., Inc., PO Box 55603, Houston, TX 77255), and Model CR1A integrator (replacement Model C-R6A) (Shimadzu Scientific Instruments, Inc., 7102 Riverwood Rd, Columbia, MD 21046). Equiv. LC system, UV detector, auto-sampler, and strip chart recorder may be used. LC pumping system in which bubbles develop in mobile phase is unsuitable. 1 μg dexamethasone should produce 50% full scale response with appropriate detector and recorder or integration settings at 254 nm. Mobile phase flow rate 1.2 mL/min at ambient temp.

**(b)** *Chromatographic column.*—Stainless steel, 25 cm × 4.6 mm id, packed with 5 μm Zorbax-Sil (E.I. du Pont de Nemours and Co.), or equiv. meeting appropriate LC system suitability requirements. Stainless steel guard column, 3 cm × 4.6 mm id, packed with 10 μm silica particles may be used. If necessary, dry silica column by eluting with 20 mL $CH_2Cl_2$-HOAc-2,2-dimethoxypropane (90 + 2 + 2 v/v/v).

### C. Reagents

**(a)** *Solvents.*—Glacial HOAc (J.T. Baker, Inc., No. 9508, or equiv.), UV grade MeOH and $CH_2Cl_2$ (Burdick and Jackson Laboratories, Inc., or equiv.), and distd-in-glass $H_2O$.

**(b)** *Methanol soln.*—Pipet 5.0 mL $H_2O$ into 100 mL vol. flask and dil. to vol. with MeOH.

**(c)** *Mobile phase.*—Pipet 1.0 mL glacial HOAc and 45.0 mL MeOH soln into 1 L vol. flask, and dil. to vol. with $CH_2Cl_2$. Degas mixt. Adjust MeOH content to obtain retention times of approx. 6 and 9 min for cortisone and dexamethasone, resp. Cortisone retention time should be used for mobile phase composition adjustments; increased MeOH content decreases retention time.

**(d)** *Sodium bicarbonate soln.*—1M. Dissolve 8.4 g $NaHCO_3$ in 100 mL $H_2O$.

**(e)** *Internal std soln.*—Dissolve 30 mg cortisone (Sigma Chemical Co. or equiv.) in 4.0 mL MeOH and dil. to 100.0 mL with $CH_2Cl_2$.

**(f)** *Dexamethasone std soln.*—4.0 mg/100 mL. Transfer ca 25 mg accurately weighed USP Ref. Std Dexamethasone (previously dried 30 min at 105°) to 25 mL vol. flask, and dissolve in and dil. to vol. with MeOH. Transfer 2.0 mL aliquot of this soln to 50 mL vol. flask contg 6.0 mL internal std soln, and dil. to vol. with $CH_2Cl_2$. Do not filter thru membrane filter.

### D. Sample Preparation

(**a**) *Drug substance*.—Prep. as directed for *Dexamethasone std soln*, using 25 mg dexamethasone. Do not filter thru membrane filter.

(**b**) *Elixir*.—Transfer accurately measured 10 mL portion of Dexamethasone Elixir, contg 1 mg dexamethasone, to 125 mL separatory funnel, add 5 mL 1M $NaHCO_3$ soln, and ext with four 20 mL portions of $CH_2Cl_2$. Collect exts in 250 mL separatory funnel contg 5 mL $H_2O$. Back-wash combined exts and filter thru cotton wet with $CH_2Cl_2$ into suitable beaker. Rinse $H_2O$ back-wash and 125 mL separatory funnel consecutively with 10 mL $CH_2Cl_2$. Filter this rinse into beaker. Evap. filtrate on steam bath under jet of air to approx. 10 mL and quant. transfer with $CH_2Cl_2$ to 25 mL vol. flask contg 1.0 mL MeOH and 3.0 mL internal std soln. Dil. to vol. with $CH_2Cl_2$. Do not filter thru membrane filter.

### E. Determination

Equilibrate column with mobile phase at 1.2 mL/min. Monitor response at 254 nm. Make 3 replicate injections of dexamethasone std soln. Using either peak area or peak ht measurements for each injection, calc. coefficient of variation (CV) of peak response ratios of dexamethasone to internal std. In suitable system, CV should be $\leq 2.5\%$ and resolution factor, $R_s$, for dexamethasone peak and internal std peak should be $\geq 3$. Make duplicate injections of std and sample solns and det. response ratio for each. Relative retention ratios of dexamethasone to internal std should agree within $\pm 2.0\%$. If relative retention ratios differ by $>2.0\%$, then dry silica column as described in *Apparatus* (**b**).

### F. Calculations

Calc. content of dexamethasone as follows:

Drug substance:

$$\text{Dexamethasone, mg} = 625 \times C \times (RR/RR')$$

Elixirs:

$$\text{Dexamethasone, mg/5 mL} = 12.5 \times C \times (RR/RR')$$

where $C$ = final concn of std soln (mg/mL), and $RR$ and $RR'$ = av. response ratio for peak ht or area of analyte to that of internal std for sample and std solns, resp.

## Thin Layer Chromatographic Identification

### G. Apparatus, Reagents, and Test Solutions

(**a**) *Thin layer plates*.—Glass, 20 × 20 cm, coated with 250 μm layer of silica gel G with fluorescent indicator (Analtech, Inc., Cat. No. 02011 or equiv.).

(**b**) *Developing solns*.—(*1*) *Drug substance*.—$CHCl_3$-diethylamine (2 + 1). (*2*) *Elixir*.—$CHCl_3$-acetone-glacial HOAc (80 + 40 + 1).

(**c**) *TLC std test solns*.—(*1*) *Drug substance*.—Prep. 1 mg/mL soln of USP Ref. Std Dexamethasone in $CH_2Cl_2$-MeOH (1 + 1). (*2*) *Elixir*.—Evap. 10 mL of *Dexamethasone std soln* (**f**) just to dryness on steam bath. Dissolve residue in 1 mL $CH_2Cl_2$-MeOH (1 + 1). Prep. individual 400 μg solns of dexamethasone (Sigma Chemical Co., or equiv.) and cortisone in $CH_2Cl_2$-MeOH (1 + 1) to serve as chromatgc identification stds.

(**d**) *TLC sample test solns*.—(*1*) *Drug substance*.—Prep. as directed for *TLC std test soln*. (*2*) *Elixir*.—Evap. 10 mL of elixir sample prepn (**b**) just to dryness on steam bath. Dissolve residue in 1 mL $CH_2Cl_2$-MeOH (1 + 1).

### H. Chromatography

Equilibrate suitable chromatgc tank with appropriate developing solv. Spot 5 μL of each test soln ca 2.5 cm from bottom of coated plate. Let spots dry and develop chromatogram until solv. front has moved 10 cm from origin. Remove plate, mark solv. front, air-dry plate, and locate spots under shortwave UV light. For drug substance, $R_f$ of major spot in sample test soln corresponds to that for std test soln. For elixir, relative $R_f$ of dexamethasone to cortisone for TLC sample test soln corresponds to that for TLC std test soln.

### I. Infrared Spectroscopic Identification

*Drug substance*.—Prep. KBr dispersions from previously dried sample and std material. Scan spectra between 2.5 and 15.0 μm. Compare sample and std spectra. If difference appears, dissolve portions of both sample and std in $CH_3CN$, evap. solns to dryness, and repeat test on residues. Sample and std prepns exhibit maxima at same wavelength.

### J. Identification by Relative Retention Times

*Drug substance*.—Compare retention ratios of main peak to internal std peak obtained for dexamethasone std soln and for assay sample prepn as directed in LC assay. Ratios that do not differ by $>2.0\%$ confirm identity.

## Alcohol in Elixir
## Gas Chromatographic Method

### K. Apparatus and Reagents

(**a**) *Gas chromatograph*.—Model 5830A, with flame ionization detector and electronic integrator (Hewlett-Packard), or equiv. Operating conditions: column temp. 165° and N gas flow adjusted so that 2-propanol elutes in 3–5 min.

(**b**) *Chromatographic column*.—Glass, 6 ft × 4 mm id, packed with 80–100 mesh copolymer of ethylvinylbenzene and divinylbenzene that has nominal surface area of 500–600 sq m/g and av. pore diam. of 0.0075 μm. This material has been washed with org. solvs and acids and then preconditioned in bulk in O-free atm. Super-Q (Alltech Associates Cat. No. 2735) has been found to be suitable.

(**c**) *Internal std soln*.—Dil. 5.0 mL 2-propanol with $H_2O$ to 250 mL.

(**d**) *Alcohol std soln*.—Dil. 5.0 mL absolute alcohol with $H_2O$ to 250 mL. Pipet 10 mL of this soln and 10 mL internal std soln into 100 mL vol. flask and dil. to vol. with $H_2O$.

### L. Preparation of GC Column

With small plug of silanized glass wool in end of column, apply vac. to exit of column and add packing in small amts to inlet end. With aid of gentle vibration, pack column firmly. Condition column overnight at 235° with slow N flow. Check column for voids after conditioning. Gently vibrate column to remove voids. Check column performance by injecting alcohol std soln and calcg following: resolution $\geq 3$; RSD $<1.5\%$ for alcohol peak area relative to 2-propanol peak area with 6 replicate injections; and tailing factor $\leq 2.0$ for alcohol.

### M. Sample Preparation

Pipet 4 mL elixir and 10 mL std soln into 100 mL vol. flask and dil. to vol. with $H_2O$.

### N. Determination and Calculation

Inject ca 5 μL each of sample and std solns in duplicate, Calc. % alcohol in elixir as follows:

$$\%\ \text{alcohol (v/v)} = (RR/RR') \times C \times D$$

where $RR$ and $RR'$ = av. response ratio for peak area of analyte to that of internal std for sample and std, resp.; $C$ = % alcohol in std soln; and $D$ = sample diln factor.

Ref.: JAOAC **70,** 967(1987).

CAS-50-02-2 (dexamethasone)

## 988.26 Dexamethasone Acetate in Bulk Drug and Suspensions

### Liquid Chromatographic Method

### First Action 1988

#### *A. Principle*

Bulk drug or suspension is dissolved in $CH_3CN$-0.025M phosphate pH 6 buffer (1 + 1) and analyzed by external std method. Dexamethasone acetate is resolved from extraneous components by reverse phase liq. chromatgy and detected at 254 nm.

#### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Equipped with isocratic pump system with UV detector (254 nm) and suitable recorder. Operate at ambient temp.

(**b**) *Column.*—Reverse phase octadecylsilane, 10 μm.

(**c**) *Ultrasonic bath.*

#### *C. Reagents*

Use LC grade reagents.

(**a**) *Potassium phosphate pH 6 buffer.*—Mix 3 mL 1N NaOH, 138 mL 0.5N KCl, and 50 mL 0.5M $KH_2PO_4$ in 1 L vol. flask. Dil. vol. with $H_2O$ (0.025M and 0.1 μ soln).

(**b**) *Diluent.*—Mix $CH_3CN$ and 0.025M phosphate pH 6 buffer (1 + 1). Let mixt. equilibrate to room temp.

(**c**) *Mobile phase.*—Degas mixt. of 450 mL $CH_3CN$ and 550 mL $H_2O$. Adjust vol. of $CH_3CN$ as needed to obtain suitable retention time.

#### *D. Preparation of Standard Solutions*

Dry USP Ref. Std 2 h in 105° oven. Accurately weigh ca 30 mg dried std and transfer to 100 mL vol. flask. Add diluent, sonicate until std is dissolved, and dil. to vol. with diluent. Further dil. soln 10 mL to 50 mL, 10 mL to 25 mL, and 5 mL to 10 mL to prep. 3 std solns.

#### *E. Preparation of Samples*

*Bulk drug.*—Accurately weigh ca 25 mg bulk drug that has been dried 2 h in 105° oven and transfer to 250 mL vol. flask. Dissolve in and dil. to vol. with diluent. Use an aliquot, concn ca 0.1 mg/mL, for LC analysis.

*Suspension.*—Measure sample vol. of 1 or more vials as follows: Shake vial vigorously until product is homogeneous (but ≥15 s). Remove sample immediately by successive use of clean, dry hypodermic syringes of appropriate size. Deliver samples into same stdzd cylinder graduated to contain. Read vol. Transfer contents of cylinder to vol. flask. Rinse all glassware twice with diluent, and add rinses to vol. flask. Concn of soln should not exceed 0.8 mg/mL. Dil. soln as needed to concn of ca 0.1 mg/mL. Filter soln thru 0.45 μm filter before LC analysis if necessary.

#### *F. System Suitability Tests*

Condition column with mobile phase until baseline is acceptable. Dexamethasone acetate peak should fulfill following performance specifications: column efficiency, ≥1500 theoretical plates; asymmetry or tailing factor (at 5% peak height), ≤2; capacity factor, k′ ≥2; relative std deviation <1% for 5 replicate 20 μL injections.

#### *G. Determination*

Inject each of 3 std solns before and after all samples. Use peak area to calc. amt of each sample, μg/mL, with respect to stds. Curve-fit samples and calc. results mathematically or by calculator.

#### *H. Calculations*

(*1*) Calc. concn, μg/mL, of dexamethasone acetate in each std soln ($C_{std}$) as follows:

$$C_{std} = (W_{std}/V_{std}) \times (V_d/V_{df}) \times (P/100) \times 1000$$

where $W_{std}$ = wt of std, mg; $V_{std}$ = vol. of std, mL; $V_d$ = vol. of aliquot transferred for diln, mL; $V_{df}$ = vol. of flask used for diln, mL; $P$ = purity of std as %.

(*2*) Use linear regression procedure of $PA_{std}$ vs $C_{std}$ to prep. std curve mathematically or by computer. Correlation coefficient ($r$) should be ≥0.999 and intercept <±3.0.

Substitute calcd values for consts m and c, and variable $X$ in following equation to calc. individual $Y$ values:

$$Y = \mathrm{m}X + \mathrm{c}$$

where $Y$ = std concn, μg/mL; and $X$ = av. of peak areas for stds injected before and after samples.

Enter $PA_{sam}$ (av. peak areas for samples = $X$) on std curve and obtain value for $C_{sam}$ (concn of sample, μg/mL = $Y$)

(*3*) Calc. dexamethasone acetate and dexamethasone equiv. in bulk drug and suspension, resp. as follows:

Bulk drug:

$$\text{Dexamethasone acetate, mg} = (C_{sam} \times D)/1000$$

Suspension:

$$\text{Dexamethasone equiv., mg/mL} = [C_{sam} \times (D/N) \times F]/1000$$

where $D$ = diln factor; $N$ = vol. for vials sampled, mL; $F$ = factor to convert acetate to free base = 0.903.

Ref.: JAOAC **71,** 534(1988).

CAS-55812-90-3 (dexamethasone acetate monohydrate)
CAS-50-02-2 (dexamethasone)

## 977.33 Prednisolone or Prednisone in Drugs

### Semiautomated Method

### First Action 1977
### Final Action 1979

#### *A. Principle*

Alcoholic soln of drug is extd with $CHCl_3$ and reacted with tetramethylammonium hydroxide and blue tetrazolium. $A$ of resulting complex is read in flowcell at 525 nm.

#### *B. Apparatus*

(**a**) *Automatic analyzer.*—Include following modules: Sampler with 30/hr (3:1) cam; proportioning pump; colorimeter, equipped with 15 mm tubular flowcell and matched 525 nm filters; recorder compatible with colorimeter; manifold (*see* Fig. **977.33**, or equiv.).

(**b**) *Shaker.*—Wrist-action

(**c**) *Ultrasonic generator.*—150 watt.

#### *C. Reagents*

(**a**) *Blue tetrazolium (BT) reagent.*—0.15%. Dissolve 1.5 g BT in 50 mL MeOH and dil to 1 L with alcohol. Store in light-resistant bottle.

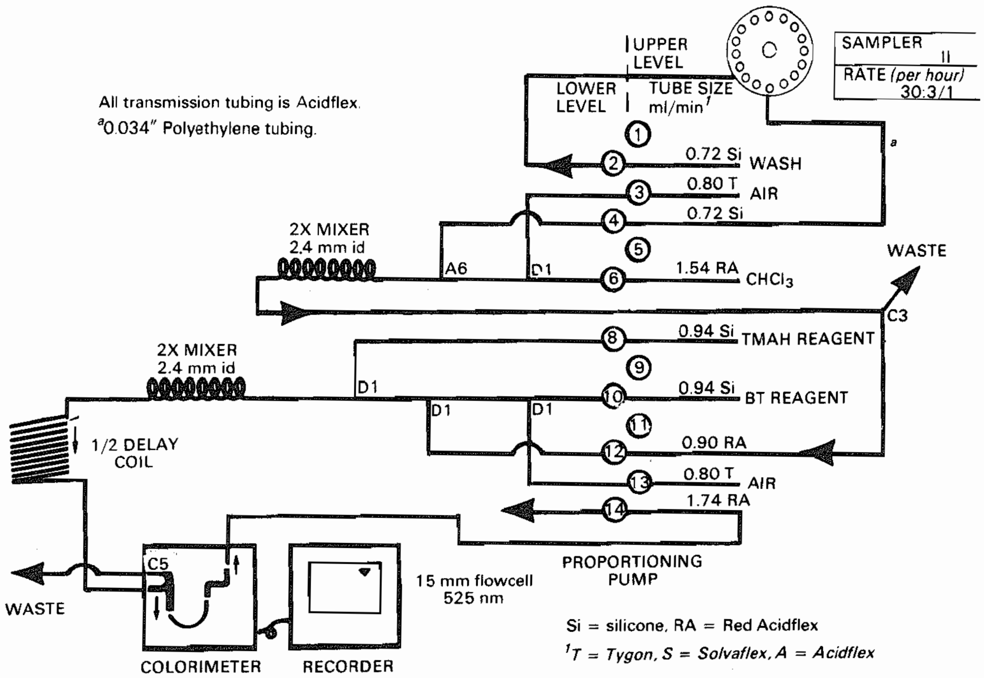



**FIG. 977.33—Flow diagram for semiautomated analysis for prednisolone or prednisone**

(**b**) *Tetramethylammonium hydroxide* (*TMAH*) *reagent*.—0.15%. Dil. 15.0 mL 10% TMAH soln to 1 L with alcohol.

(**c**) *Prednisolone std soln*.—(*1*) *Stock soln*.—0.4 mg/mL. Accurately weigh ca 40 mg USP Prednisolone Ref. Std into 100 mL vol. flask, dissolve in 50% alcohol, and dil to vol. (*2*) *Working soln*.—0.10 mg/mL. Pipet 25 mL stock soln into 100 mL vol. flask and dil. to vol. with 50% alcohol.

(**d**) *Prednisone std soln*.—(*1*) *Stock soln*.—0.2 mg/mL. Accurately weigh ca 20 mg USP Prednisone Ref. Std into 100 mL vol. flask, dissolve in 50% alcohol, and dil. to vol. (*2*) *Working soln*.—0.05 mg/mL. Pipet 25 mL stock soln into 100 mL vol. flask and dil. to vol. with 50% alcohol.

### *D. Preparation of Sample*

Disintegrate individual tablet or disperse weighed composite in accurately measured vol. 50% alcohol to give prednisolone concn of 0.1 mg/mL or prednisone concn of 0.05 mg/mL. Use ultrasonic generator until tablet is disintegrated and shake mech. 15 min. Let soln settle ≥2 hr.

### *E. Analytical System*

Sample is withdrawn and extd with air-segmented stream of $CHCl_3$ in double mixer, and org. phase is sepd in BO fitting. BT and TMAH reagents are added to org. phase and mixed. Soln is passed thru delay coil and $A$ is detd at 525 nm in 15 mm flowcell.

### *F. Start-Up and Shut-Down Operations*

Pump alcohol thru $CHCl_3$ line 10 min; then pump $CHCl_3$ thru line 5 min. Place remaining tubes in their resp. solns and let system equilibrate 20–30 min or until steady baseline is obtained. To shut down system, place $CHCl_3$, BT, and TMAH lines in alcohol and remove all other lines from their solns. After 15 min, remove remaining lines from alcohol soln and pump system dry.

### *G. Determination*

Fill sample cups in following order: 3 cups std soln, 5 cups sample soln, 1 cup std soln, 5 cups sample soln, etc. Place 2 cups std soln at end of each series. (First 2 cups of std solns are used to equilibrate system, but are not included in calcns.) Start Sampler II. After last cup has been sampled, let system operate until steady baseline is obtained. Draw tangent to initial and final baselines. Subtract baseline to det. net $A$ and $A'$ for each sample and std peak, resp. Discard values for first 2 and last std peaks and calc. av. std $A'$.

$$\text{mg Drug in portion taken} = (A/A') \times C \times D$$

where $C$ = concn of std in mg/mL and $D$ = diln factor.

Ref.: JAOAC **60**, 27(1977).

CAS-50-24-8 (prednisolone)
CAS-53-03-2 (prednisone)

## 986.38 Prednisolone in Tablets and Bulk Drugs

**Liquid Chromatographic Method**

**First Action 1986**
**Final Action 1989**

### *A. Principle*

Prednisolone is detd by normal phase liq. chromatgy, using silica column and UV detection.

### *B. Apparatus*

(**a**) *Liquid chromatograph*.—Equipped with solv. delivery system, UV detector, and data module. Operating conditions: flow rate 1.5 mL/min; 254 nm detector, 0.20 AUFS; temp. ambient; 10–15 μL injection.

(**b**) *LC column*.—25 cm × 4.6 mm id, packed with 5–6 μm porous spherical silica particles, that passes system suitability tests.

(**c**) *Filters*.—Polytetrafluoroethylene membrane filters pore sizes 0.5 and 5.0 μm, resp.

### C. Reagents

(a) *Solvents.*—Distd in glass, LC grade.

(b) *LC mobile phase.*—Mix 60 mL 95% MeOH with 1.0 mL acetic acid and dil. to 1 L with $H_2O$-washed 1,2-dichloroethane. Filter thru Type FH filter and degas 5 min.

(c) *Internal std soln.*—1.0 mg/mL. Transfer 100 mg fluoxymesterone to 100 mL vol. flask. Dissolve in 5 mL MeOH and dil. to vol. with $CH_2Cl_2$.

(d) *Prednisolone std soln.*—(*1*) *Stock std soln.*—0.5 mg/mL. Accurately weigh ca 25 mg USP Ref. Std Prednisolone into 50 mL vol. flask. Add 2 mL MeOH and dil. to vol. with $CH_2Cl_2$. (*2*) *Working std soln.*—0.05 mg/mL. Pipet 10.0 mL stock std soln and 5.0 mL internal std soln into 100 mL vol. flask. Add 6 mL MeOH and dil. to vol. with $CH_2Cl_2$.

### D. Preparation of Samples

*Bulk drugs.*—Accurately weigh ca 50 mg sample (dried in vac. 3 h at 105°) into 100 mL vol. flask. Add 4 mL MeOH and dil. to vol. with $CH_2Cl_2$. Pipet 10.0 mL sample soln and 5.0 mL internal std soln into 100 mL vol. flask. Add 6 mL MeOH and dil. to vol. with $CH_2Cl_2$.

*Tablets.*—Grind tablets to pass No. 60 sieve. Transfer accurately weighed portion of powder contg 5 mg prednisolone to 100 mL vol. flask. Add 6 mL MeOH and place flask in ultrasonic bath 2 min. Add ca 50 m $CH_2Cl_2$ and return to ultrasonic bath 1 min. Add 5.0 mL internal std soln and dil. to vol. with $CH_2Cl_2$. Shake flask vigorously and filter portion of soln thru Type LS filter into 25 mL g-s flask.

*Individual tablet assay (content uniformity).*—Place tablet in 125 mL g-s flask and add 200 μL $H_2O$. Let stand until tablet disintegrates and add 1 mL MeOH for each mg prednisolone declared. Place flask in ultrasonic bath until tablet is dispersed. Add 1.0 mL internal std soln for each mg prednisolone declared and dil. with $CH_2Cl_2$ to ca 0.05 mg/mL. Shake flask vigorously and filter portion of soln thru Type LS filter into 25 mL g-s flask.

### E. Determination

Let LC system equilibrate with 1.5 mL/min flow rate. Inject 10–15 μL prednisolone working std soln. Retention times of fluoxymesterone and prednisolone should be ca 6 and 9 min, resp., with $R$ (resolution) value ≥6. Inject 5 replicate aliquots and prednisolone working std soln and calc. response ratios. CV will be ≤2.0% in suitable system. Proceed with sample analysis, injecting same amt of sample soln.

### F. Calculations

Calc. results, using response ratios ($A$ and $A'$) relative to internal std:

Bulk drugs:

$$\text{Prednisolone, \%} = (A/A') \times (C/S) \times 100$$

Tablets (composite):

$$\text{Prednisolone, mg/tab.} = (A/A') \times C \times (W/S) \times 100$$

Tablet (individual):

$$\text{Prednisolone, mg/tab.} = (A/A') \times C \times (T/D)$$

where $A$ and $A'$ = response ratios for sample and std solns, resp.; $C$ = mg prednisolone/mL working std soln.; $W$ = av. tablet wt (g); $S$ = sample wt (g); $T$ = labeled amt (mg) of prednisolone in tablet; and $D$ = concn (mg/mL) of prednisolone in tablet soln, based on labeled amt/tab. and diln.

Ref.: JAOAC **67**, 674(1984).

CAS-50-24-8 (prednisolone)

## THYROID

## 982.39 Iodine in Thyroid Drug Tablets

**Differential Pulse Polarographic Method**

**First Action 1982**
**Final Action 1984**

### A. Apparatus

*Polarograph.*—With dropping Hg electrode. Typical operating parameters: scan rate 5 mV/s; scan direction "−"; potential scan range 1.5 V; initial potential −0.9 V; modulation amplitude 50 mV; differential pulse operating mode; display direction "+"; drop time, 1 s; low pass filter off; push-button, initial; offset, off; current range 1–10 μamp, or as needed.

### B. Reagents

Use anal. reagents and glass-distd $H_2O$ thruout.

(a) *Bromine water.*—Br-satd $H_2O$. Prep. fresh daily.

(b) *Potassium carbonate.*—If reagent grade $K_2CO_3$ gives high blank, purify as follows: Dissolve ca 200 g $K_2CO_3$ in 400 mL $H_2O$, add 50 g 20–50 mesh Amberlite IRA-400 ion exchange resin (Mallinckrodt Chemical Works), and agitate 30 min. Filter thru glass wool plug into porcelain crucible, evap. to dryness on hot plate, and heat at 675° in muffle 25 min. Cool to room temp., and grind to fine powder with mortar and pestle.

(c) *Reagent blank.*—Dissolve 8 g $K_2CO_3$ in ca 70 mL $H_2O$ in 100 mL vol. flask. Add 1 mL Br-satd $H_2O$ and 20 mg $Na_2SO_3$. Mix, dil. to vol. with $H_2O$, and mix.

(d) *Standard solns.*—(*1*) 1 mg I/mL: Dissolve 1.686 g $KIO_3$ in ca 200 mL $H_2O$ in 1 L vol. flask. Dil. to vol. and mix. (*2*) 32 μg I/mL: Pipet 8 mL std soln (*1*) into 250 mL vol. flask, dil. to vol., and mix.

(e) *Working soln.*—Pipet aliquot ($V$) of std soln (2) contg same amt of I contained in one tablet (see below) into 100 mL vol. flask contg 8 g $K_2CO_3$ dissolved in 70 mL $H_2O$.

| Tab. strength, gr. thyroid | I content, μg | Std soln 2, mL |
|---|---|---|
| 1/4 | 32.4 | 1 |
| 1 | 128.6 | 4 |
| 2 | 259.2 | 8 |
| 5 | 643.0 | 20 |

Add 1 mL Br-satd $H_2O$ and mix. Add $Na_2SO_3$ (ca 20 mg) until soln becomes colorless; mix. Dil. to vol. with $H_2O$ and mix.

### C. Sample Preparation

(a) *Composite assay.*—Weigh and finely powder tablets. Weigh portion of powder equiv. to 1 tablet into porcelain crucible that has been washed with $HNO_3$ (1 + 1), rinsed with $H_2O$, and wiped dry. Mix with 4 g $K_2CO_3$ and overlay with addnl 4 g $K_2CO_3$. Place crucible in preheated 675° muffle 25 min. Cool, add 30 mL $H_2O$, carefully heat on hot plate to dissolve residue, and filter thru funnel with glass wool plug into 100 mL vol. flask. Repeat heating with 2 addnl 30 mL portions of $H_2O$, and add these exts to vol. flask. Add 1 mL Br-satd $H_2O$, mix, add $Na_2SO_3$ (ca 20 mg) until soln becomes colorless. Dil. to vol. with $H_2O$ and mix.

(b) *Individual tablet assay.*—Crush 1 tablet in porcelain crucible with glass rod. Remove any sample adhering to glass rod with spatula, and add to crucible. Proceed as in Composite Assay, (**a**), beginning "Mix with 4 g $K_2CO_3$ . . ."

### D. Determination

Add ca 10 mL working soln to dry polarographic cell. Bubble N thru cell 5 min; then direct stream of N above soln. Using typical operating parameters as guide, switch selector to external cell and wait until pen becomes stationary; then depress scan button. Similarly, using same settings, analyze sample soln followed by reagent blank. From baseline established by reagent blank, measure peak hts of std and sample solns at ca −1.18 V vs SCE. Calc. as follows:

I as % of declared thyroid

$$= (PH \times V \times W_t \times 3.2)/(PH' \times W_s \times TH)$$

where $PH$ and $PH'$ = peak ht of sample and std, resp.; $V$ = mL of 32 μg/mL std used to prep. working std soln; $W_t$ and $W_s$ = av. wt of tablet and wt of sample, g, resp.; and $TH$ = declared thyroid per tablet, mg.

Ref.: JAOAC **65,** 1059(1982).

CAS-7553-56-2 (iodine)

**Common and Chemical Names of Drugs in this Chapter**

| Common Name | Chemical Name |
|---|---|
| Beta-Estradiol | (17β)-Estra-1,3,5(10)-triene-3,17-diol |
| Cortisone acetate | 17,21-Dihydroxypregn-4-ene-3,11,20-trione 21-acetate |
| Dexamethasone (acetate, phosphate) | 9-Fluoro-11β,17,21-trihydroxy-16α-methylpregna-1,4-diene-3,20-dione |
| Dienestrol | 4,4′-(1,2-Diethylidene-1,2-ethanediyl)-bisphenol |
| Diethylstilbestrol | 4,4′-(1,2-Diethyl-1,2-ethenediyl)-bisphenol |
| Dimethisterone | 17β-Hydroxy-6α-methyl-17-(1-propynyl)-androst-4-en-3-one |
| Equilin | 3-Hydroxyestra-1,3,5(10),7-tetraen-17-one |
| Estradiol valerate | (17β)-Estra-1,3,5(10)-triene-3,17-diol-17-pentanoate |
| Estrone (sodium sulfate) | 3-Hydroxyestra-1,3,5(10)-trien-17-one |
| Ethinyl estradiol | (17α)-19-Norpregna-1,3,5(10)-trien-20-yne-3,17-diol |
| Ethisterone | 17-Ethynyl-17β-hydroxyandrost-4-en-3-one |
| Hexestrol | 4,4′-(1,2-Diethylethylene)diphenol |
| Hydrocortisone | (11,17,21-Trihydroxy-pregn-4-ene-3,20-dione |
| Medroxyprogesterone (acetate) | 6α-17-(Acetyloxy)-6-methyl-pregn-4-ene-3,20-dione |
| Mestranol | 3-Methoxy-19-norpregna-1,3,5(10)-triene-20-yn-17-ol |
| Norethindrone (acetate) | 17α-Hydroxy-19-norpregn-4-en-20-yn-3-one acetate |
| Norethynodrel | 17-Hydroxy-19-nor-17α-pregn-5(10)-en-20-yn-3-one |
| Norgestrel | 13-Ethyl-17α-hydroxy-18,19-dinorpregn-4-en-20-yn-3-one |
| Prednisolone | 11,17,21-Trihydroxypregna-1,4-diene-3,20-dione |
| Prednisone | 17,21-Dihydroxypregna-1,4-diene-3,11,20-trione |

Source: *USAN and the USP Dictionary of Drug Names* (1983; 1989), U.S. Pharmacopeial Convention, Rockville, MD.

# 22. Drugs: Part V

**Linda L. Ng, *Associate Chapter Editor***
*Merck Sharp & Dohme Research Laboratories*

### Diacetylmorphine (Heroin) in Drug Powder
**Microchemical Tests**
**Final Action**

*See* **930.40**.

### Lysergic Acid Diethylamide
**Optical Crystallographic Tests**
**Final Action**

*See* **960.57** (lysergic acid diethylamide (LSD) tartrate, 4-methyl-2,5-dimethoxyamphetamine.HCl (STP.HCl, DOM), and psilocybin).

### 978.29 Cocaine Hydrochloride in Drug Powders
**Gas Chromatographic Method**
**First Action 1978**
**Final Action 1983**

***A. Principle***

Cocaine is extd from weakly basic, aq. soln with $CHCl_3$ contg internal std, and then sepd by GC from other amines, org.-sol. neutrals, and internal std.

***B. Apparatus and Reagents***

(**a**) *Gas chromatograph*.—With H flame detector and 1.8 m (6′) × 4 (id) mm glass column packed with 3% OV-1 on 100–120 mesh Chromosorb W(HP) (Applied Science). Typical operating conditions: temps (°): column 225, detector and injector 240; N carrier gas flow rate, 60 mL/min. Adjust column temp. to elute cocaine in 3 ± 0.5 min. Adjust H and air flow rates and electrometer sensitivity so that 4 μL cocaine std soln gives 40–60% full scale deflection. Retention time of cocaine relative to internal std is ca 0.65.

(**b**) *Dibasic potassium phosphate*.—10%. Dissolve 5 g $K_2HPO_4$ in 50 mL $H_2O$, and mix well.

(**c**) *Dilute hydrochloric acid*.—0.1*N*. Dil. 7.0 mL HCl to 1 L with $H_2O$, and mix well.

(**d**) *Internal std soln*.—0.8 mg/mL. Dissolve 80 mg tetracosane in 100 mL $CHCl_3$, and mix well.

(**e**) *Cocaine hydrochloride std soln*.—1 mg/mL. Accurately weigh ca 10 mg cocaine.HCl into 10 mL vol. flask, and dil. to vol. with internal std soln. Prep. fresh every 3 months. Store in refrigerator.

***C. Preparation of GC Column***

Plug column exit with silanized glass wool. Apply vac. to exit end, and slowly add packing material thru column inlet while gently tapping column. Fill to within 1 cm of column inlet, and plug with silanized glass wool. Condition column overnight at 260° with slow stream of N. Sat. column with cocaine by making successive 4 μL injections of cocaine std soln until cocaine: internal std ratio differs by <3% from preceding std injection.

***D. Determination***

Accurately weigh ca 250 mg finely ground sample and dil. quant. with 0.1*N* HCl to estd cocaine.HCl concn of 1 mg/mL. Mix well and let any insol. material settle. Pipet 2 mL sample prepn, 2 mL internal std soln, and 1 mL 10% $K_2HPO_4$ into test tube; stopper, and shake vigorously. Let layers sep. Pipet 2 mL cocaine std soln, 2 mL 0.1*N* HCl, and 1 mL 10% $K_2HPO_4$ soln into another test tube, stopper, and shake vigorously. Let layers sep. Inject 4 μL $CHCl_3$ (bottom) layer of sample and std solns, each in duplicate, using 10 μL syringe.

$$\%\ \text{Cocaine.HCl} = (P/P') \times (B'/B) \times (C/W) \times D \times 100$$

where $P$ and $P'$ = av. areas or peak hts of sample and std cocaine.HCl, resp.; $B$ and $B'$ = av. areas or peak hts of internal std in sample and std, resp.; $C$ = mg cocaine.HCl/mL in std soln; $W$ = mg sample; and $D$ = diln factor for sample.

$$\text{mg Cocaine.HCl/tablet} = \%\ \text{cocaine.HCl} \times \text{av. tablet wt in mg}/100$$

Refs.: JAOAC **61,** 473, 683(1978).

CAS-53-21-4 (cocaine hydrochloride)

### 921.13★ Diacetylmorphine (Heroin) in Drug Tablets
**Titrimetric Method**
**First Action**
**Surplus 1970**

*See* **36.022**, 11th ed.

### 955.56★ Diacetylmorphine and Quinine in Drug Powders
**Spectrophotometric Method**
**Final Action 1965**
**Surplus 1973**

*See* **40.003–40.005**, 12th ed.

### 985.50 Diazepam in Drug Tablets
**Liquid Chromatographic Method**
**First Action 1985**
**Final Action 1988**

***A. Principle***

Diazepam content of tablets is detd by reverse phase liq. chromatgy using $MeOH\text{-}H_2O$ mobile phase, UV detection at 254 nm, and *p*-tolualdehyde as internal std.

***B. Apparatus***

(**a**) *Liquid chromatograph*.—Tracor Model 950 solv. pump with Model 970A variable wavelength detector capable of monitoring elution at 254 nm (Tracor Instruments Inc. (replacement models 951 and 971, resp.)), injection valve with

20 μL sample loop (Valco Instruments, Inc., Houston, TX 77055), and suitable strip chart recorder, or equiv. LC system. Operating conditions: mobile phase flow rate ca 1.2 mL/min, temp. ambient, sensitivity adjusted to give 60–90% FSD for sample and std injections. Retention times for *p*-tolualdehyde and diazepam ca 5 and 10 min, resp.

(**b**) *Chromatographic column*.—Stainless steel, 30 cm × 3.9 mm id, packed with $C_{18}$ μBondapak, 10 μm (Waters Associates, Inc.) or equiv. column meeting LC system suitability requirements.

### C. Reagents

(**a**) *p-Tolualdehyde*.—98% (cat. no. 89850, Fluka Chemical Corp., 980 S. Second St, Ronkonkoma, NY 11779, or equiv.).

(**b**) *Solvents*.—LC grade MeOH.

(**c**) *Mobile phase*.—MeOH-$H_2O$ (65 + 35), degassed before use.

(**d**) *Internal std soln*.—Prep. fresh daily as follows: Pipet 1 mL *p*-tolualdehyde into 50 mL vol. flask, dil. to vol. with MeOH, and mix. Pipet 4 mL of this soln into 250 mL vol. flask, dil. to vol. with MeOH, and mix.

(**e**) *Diazepam std soln*.—USP Diazepam Ref. Std (RS), previously dried in vac. over $P_2O_5$ 4 h at 60°. Dissolve accurately weighed amt in MeOH, and dil. quant. with MeOH to ca 1 mg/mL. Pipet 5.0 mL of this soln and 5.0 mL internal std soln into 25 mL vol. flask, dil. to vol. with MeOH, and mix.

### D. LC System Suitability Test

Make 5 replicate injections of std soln and record peak ht or peak area responses. System is suitable if relative std dev. ($S_r$) is ≤2.0%, using the equation:

$$S_r, \% = \frac{100}{\bar{x}}\left[\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}\right]^{1/2}$$

where $\bar{x}$ = mean of set of $n$ measurements, and $x_i$ = individual measurement.

Resolution factor, $R$, between *p*-tolualdehyde and diazepam should be ≥3.5, using the equation:

$$R = [2(t' - t)]/(PW + PW')$$

where $t$ and $t'$ = mm retention of diazepam and *p*-tolualdehyde, resp.; and $PW$ and $PW'$ = mm peak widths measured at baseline obtained by extrapolating rel. straight sides of peaks to baseline of diazepam and *p*-tolualdehyde, resp.

Tailing factor ratio, $T$, should be ≤2.5, using the equation:

$$T = W_{0.05}/2f$$

where $W_{0.05}$ = distance from leading edge to trailing edge of peak; and $f$ = distance from peak max. to leading edge of peak, both measured at point 5% of peak ht from baseline.

### E. Sample Preparation

Weigh and finely powder ≥20 tablets. Transfer accurately weighed portion of powder, equiv. to ca 10 mg diazepam, into 50 mL vol. flask. Pipet 10 mL internal std soln into flask, add ca 25 mL MeOH, mech. shake 30 min, dil. to vol. with MeOH, and mix. Filter soln thru 0.5 μm membrane filter, discarding first 10 mL filtrate.

### F. Determination

Introduce equal vols (10–20 μL) of sample prepn and std soln into liq. chromatograph by means of suitable syringe or sampling device. Measure responses and det. response ratios (diazepam/internal std peaks) for sample and std solns.

$$\text{Diazepam, mg/tablet} = 50C \times (R/R') \times (T/W)$$

where $C$ = concn diazepam in std soln, mg/mL; $R$ and $R'$ = ratios of peak responses of diazepam to *p*-tolualdehyde for sample prepn and std soln, resp.; $T$ = av. tablet wt, mg; and $W$ = sample wt taken for assay, mg.

Ref.: JAOAC **68**, 545(1985).

CAS-439-14-5 (diazepam)

## 969.52★ Lysergic Acid Diethylamide in Drug Powders

**Paper Chromatographic-Spectrophotometric Method**

**First Action 1969**
**Surplus 1977**

*See* **40.008–40.011**, 13th ed.

## 962.24 Cannabinol (Marihuana) in Drug Powders

**Duquenois-Levine Qualitative Test**

**First Action 1962**
**Final Action 1965**

### A. Reagent

*Duquenois reagent*.—Dissolve 12 drops acetaldehyde (fresh) and 1 g vanillin in 50 mL alcohol.

### B. Test

Ext ca 100 mg sample with 25 mL pet ether, filter into white porcelain dish, and evap. to dryness on steam bath. Add 2 mL Duquenois reagent and stir to dissolve residue. Add 2 mL HCl, stir, and let stand 10 min. Note color, transfer soln to test tube, add 2 mL $CHCl_3$, and shake. Let sep. and note color in $CHCl_3$ layer; purple color is pos. test.

Ref.: JAOAC **45**, 597(1962).

CAS-521-35-7 (cannabinol)

## 977.34 Methaqualone in Drug Powders

**Gas Chromatographic Method**

**First Action 1977**
**Final Action 1980**

### A. Apparatus and Reagents

(**a**) *Gas chromatograph*.—With flame ionization detector and 1.8 m × 4 (id) mm glass column packed with 3% OV-1 on 100–120 mesh Chromosorb W(HP). Typical operating conditions: temps (°): column 235, detector and injector 260; flow rates (mL/min): N carrier gas 60, H 30, air 300; set column temp. and flow rate to give methaqualone retention time of 2.5 ± 0.5 min.

(**b**) *Sodium bicarbonate soln*.—1*M*. Dissolve 7 g $NaHCO_3$ in 100 mL $H_2O$.

(**c**) *Internal std soln*.—Dissolve tetraphenylethylene (Eastman Kodak Co.) in $CHCl_3$ to give concn of 4 mg/mL. Each analysis requires 25 mL.

(**d**) *Methaqualone hydrochloride std soln*.—4 mg/mL. Accurately weigh methaqualone.HCl and dil. with internal std soln to give concn of 4 mg/mL.

### B. Determination

Condition new column overnight at 270° with slow stream of N. Sat. column immediately before analysis by making three 3 μL injections of methaqualone.HCl std soln.

Accurately weigh sample contg ca 100 mg methaqualone.HCl and transfer quant. to 50 mL erlenmeyer. Pipet in 25 mL internal std soln and add ca 10 mL 1*M* $NaHCO_3$ soln. Heat on steam bath 5–8 min, cool to room temp., stopper, and shake. Let sep. and inject 1–2 μL of $CHCl_3$ (bottom) layer into gas chromatograph.

$$\% \text{ Methaqualone.HCl} = (P/P') \times (B'/B) \times (C/W) \times 2500$$

where $P$ and $P'$ = areas or peak hts of sample and std methaqualone.HCl, resp.; $B$ and $B'$ = areas or peak hts of sample and std internal std, resp.; $C$ = mg methaqualone.HCl/mL in std soln; and $W$ = mg sample.

Ref.: JAOAC **60,** 935(1977).

CAS-72-44-6 (methaqualone)

## 983.30 Oxazepam in Drug Tablets and Capsules

### Liquid Chromatographic Method

### First Action 1983

#### *A. Principle*

Oxazepam is extd into MeOH and detd by liq. chromatgy with UV (254 nm) detector.

#### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Model 204 equipped with 2 Model 6000A pumps, Model 660 solv. programmer, Model 440 UV (254 nm) detector, Model U6K injector (all Waters Associates, Inc.), and Model 3380A integrator (Hewlett-Packard), or equiv.

(**b**) *LC column.*—Bondapak $C_{18}$, 3.9 mm id × 30 cm long (Waters Associates, Inc.) at ambient temp., or equiv.

(**c**) *Filter.*—Millipore type EG (replacement Model GV) (pore size 0.2 μm) (Millipore Corp., Bedford, MA 01730) or 0.45 μm MeOH compatible equiv.

#### *C. Reagents*

(**a**) *Methanol.*—UV quality, LC grade.

(**b**) *Mobile phase.*—MeOH–$H_2O$–glac. HOAc (60 + 40 + 1) at flow rate of 1.0 mL/min. MeOH concn and flow rate may be varied to give approx. retention time of 6–8 min for oxazepam.

(**c**) *System suitability std soln.*—Dissolve 10 mg USP Oxazepam and 15 mg USP 2-Amino-5-chlorobenzophenone in 250.0 mL MeOH.

(**d**) *Oxazepam std soln.*—Transfer 25 mg USP Oxazepam, accurately weighed, to 250 mL vol. flask. Add 5 mL $H_2O$ and dil. to vol. with MeOH. Soln is stable 90 min.

#### *D. Sample Preparation*

(**a**) *Tablets.*—Det. av. wt/tablet and grind tablets to pass No. 60 mesh sieve. Transfer accurately weighed portion of powder contg 25 mg oxazepam to 250 mL vol. flask. Add 5 mL $H_2O$ and 25 mL MeOH. Mix thoroly. Add 75 mL MeOH and place in ultrasonic bath 10 min. Dil. to vol. with MeOH. Stir 30 min. Filter portion of soln thru type EG filter into small g-s flask. Soln is stable 90 min.

(**b**) *Capsules.*—Det. av. wt of capsule contents. Transfer accurately weighed portion of capsule contents contg 25 mg oxazepam to 250 mL vol. flask. Proceed as in (**a**).

#### *E. System Suitability Check*

(**a**) *Resolution.*—Inject 10.0 μL system suitability std soln. Retention times for oxazepam and 2-amino-5-chlorobenzophenone should be ca 6 and 14 min, resp. Resolution factor, $R$, for the 2 peaks should be ≥5.0, using following formula:

$$R = 2(t' - t)/(PW + PW')$$

where $t$ and $t'$ = mm retention of oxazepam and 2-amino-5-chlorobenzophenone peaks, resp.; and $PW$ and $PW'$ = mm peak widths measured at baseline of oxazepam and 2-amino-5-chlorobenzophenone, resp. Adjust MeOH concn if resolution is unsatisfactory.

(**b**) *Repeatability.*—Make five 10.0 μL injections of oxazepam std soln and measure peak areas. In suitable system, coefficient of variation is not >2.0%.

#### *F. Determination*

Make duplicate 10 μL injections each of sample soln and std soln, alternating sample and std solns. Calc. results by using peak areas:

$$\text{mg Oxazepam/tablet or capsule} = (PA/PA') \times C \times (T/S)$$

where $PA$ and $PA'$ = peak area for sample and std solns, resp.; $C$ = mg oxazepam/250 mL std soln; $T$ = av. wt, g, of tablet or capsule contents; $S$ = sample wt, g.

Ref.: JAOAC **66,** 864(1983).

CAS-604-75-1 (oxazepam)

## 988.28 Enantiomers of Amphetamine in Bulk Drugs, Syrups, and Capsules

### Liquid Chromatographic Method

### First Action 1988

#### *A. Principle*

Samples are dissolved in $CH_2Cl_2$, and 2-naphthoyl amide derivatives are formed by adding 2-naphthoyl chloride. Isomers are detd by liq. chromatgy on chiral stationary phase LC column, with hexane-isopropyl alcohol-$CH_3CN$ (97 + 3 + 0.5) mobile phase and detection at 254 nm.

#### *B. Apparatus*

(**a**) *Liquid chromatograph.*—Spectra-Physics Model 8000 equipped with Valco 7000 psi injection valve with 10 μL injection loop, temp.-controlled oven or column $H_2O$ jacket, and printer plotter. Equiv. app may be used.

(**b**) *Detector.*—Spectra-Physics Model 770 UV-vis, or equiv., set at 254 nm, 0.04 AUFS, and time constant at 4 s.

(**c**) *Data integration system.*—Set peak width to peak threshold ratio at 1:60 for Spectra-Physics Model 8000 or at appropriate settings for equiv. chromatgc data system.

(**d**) *LC column.*—Pirkle covalent D-phenyl glycine analytical column (J.T. Baker, Inc. (Bakerbond Chiral Phase [DNBPG] column No. 7113) or Regis Chemical Co., 8210 N Austin Ave, PO Box 519, Morton Grove, IL 60053), or equiv.

#### *C. Reagents*

(**a**) *Solvents.*—Use UV quality, LC grade $H_2O$, hexane, isopropyl alcohol, $CH_3CN$, and $CH_2Cl_2$.

(**b**) *LC mobile phase.*—Hexane-isopropyl alcohol-$CH_3CN$ (97 + 3 + 0.5 v/v/v).

(**c**) *2-Naphthoyl chloride.*—98% (Aldrich Chemical Co.).

(**d**) *Reagent solns.*—20% NaOH soln; 0.01M soln of 2-naphthoyl chloride in $CH_2Cl_2$; 0.01M $H_2SO_4$ soln.

#### *D. Preparation of LC Standard and Sample Solutions*

(**a**) *Standard.*—Dissolve 10 mg USP Ref. Std in 5 mL $CH_2Cl_2$, and add 5 mL 20% NaOH soln. Continue with (**e**).

(**b**) *Bulk drug.*—Dissolve 10 mg bulk drug in 5 mL $CH_2Cl_2$, and add 5 mL 20% NaOH soln. Continue with (**e**).

(**c**) *Syrup.*—Mix 10 mL syrup with 5 mL $CH_2Cl_2$, and add 5 mL 20% NaOH soln. Continue with (**e**).


**FIG. 988.28—Chromatogram of system suitability standard composed of 50:50 mixture of *d*:*l*-amphetamine: 1, *l*-amphetamine; 2, *d*-amphetamine**

(**d**) *Capsules*.—Place 1 capsule in 5 mL 20% NaOH soln, ultrasonicate 45 min or until dissolved. Filter resulting soln. Add 5 mL $CH_2Cl_2$ to filtrate. Continue with (**e**).

(**e**) *Working solns*.—Transfer soln from (**a**), (**b**), (**c**), or (**d**) to 30 mL separatory funnel. Add 10 mL 0.01M soln of 2-naphthoyl chloride in $CH_2Cl_2$, and shake mixt. 1 min. Transfer org. phase to another 30 mL separatory funnel. Wash aq. phase with 5 mL $CH_2Cl_2$, and combine org. layers. Wash combined org. layers with 5 mL 0.01M $H_2SO_4$, and filter washed org. layer thru syringe (10 mL plastic syringe with plunger removed) contg glass cotton plug and anhyd. $Na_2SO_4$. Discard aq. layers.

### *E. Liquid Chromatography*

(**a**) *Column preparation*.—Equilibrate overnight chiral stationary phase LC column with mobile phase at flow rate of 0.25 mL/min with temp. controlled at 20 ± 1°. Circulate solv. during analysis without interruption. Use flow rate of 2 mL/min during analysis.

(**b**) *System suitability test*.—After derivatization, inject 10 μL aliquots of 50:50 mixt. of *d*:*l*-amphetamine (system suitability std; available from Sigma Chemical Co.) into chromatgc column. Fig. **988.28** shows LC resolution of this mixt. Repeat analysis in triplicate. Calc. mean and coefficient of variation as follows:

$$\text{mean, } \bar{X} = (X_1 + X_2 + \ldots X_n)/n$$

$$\text{Coeff. of var., \%} = (\text{std dev./mean}) \times 100$$

Efficiency is optimum when resolution is ≥1.2 and max. relative std dev. is ≤2.0.

(**c**) *Procedure*.—Inject 10 μL aliquots of working solns of std, bulk drug, syrup, or capsules. Make each injection in duplicate.

### *F. Calculations*

Calc. % area of *l*-amphetamine (percent *l*-amphetamine) and *d*-amphetamine (percent *d*-amphetamine) in std and samples as follows:

$$l\text{-Amphetamine, \%} = [PA_l/(PA_l + PA_d)] \times 100$$

$$d\text{-Amphetamine, \%} = [PA_d/(PA_l + PA_d)] \times 100$$

where $PA_l$ and $PA_d$ = peak areas for *l*- and *d*-amphetamines, resp.

Ref.: JAOAC **71,** 530(1988).

CAS-156-34-3 (*l*-amphetamine)
CAS-51-64-9 (*d*-amphetamine)

## 979.27 Phencyclidine in Drug Powders
### Gas Chromatographic Method
**First Action 1979**
**Final Action 1983**

### *A. Principle*

Phencyclidine is extd from weakly basic, aq. soln with $CHCl_3$ contg internal std. Phencyclidine is sepd by GC from other amines, org. sol. neutral compds, and internal std.

### *B. Reagents and Apparatus*

(**a**) *Internal std soln*.—Dissolve 80 mg eicosane ($C_{20}H_{42}$) in 100 mL $CHCl_3$.

(**b**) *Phencyclidine hydrochloride std soln*.—Weigh 10.0 mg phencyclidine.HCl (USP Authentic Substance) and dissolve in 10.0 mL internal std soln. Store in refrigerator and replace every 3 months.

(**c**) *Gas chromatograph*.—With H flame detector. Typical operating conditions: temps (°): column 190, detector 240, injection port 240; N carrier gas flow rate 60 mL/min. Adjust column temp. to elute phencyclidine in 3 ± 0.5 min. Adjust H and air flow rates and electrometer sensitivity so that 4 μL phencyclidine std soln gives 40–60% full scale deflection. Retention time of phencyclidine relative to internal std is ca 0.75.

(**d**) *Column*.—1.8 m (6′) × 4 mm (id) glass column, packed with 3% OV-1 on 100–120 mesh Chromosorb W HP (Applied Science Laboratories, Inc.). Plug column exit with plug of silanized glass wool. Apply vac. to exit end and slowly add packing thru inlet end while tapping gently. Fill to within 1 cm of inlet and plug with silanized glass wool. Condition column overnight at 260° with slow flow of N while disconnected from detector. Sat. column by making successive phencyclidine injections until phencyclidine/internal std ratio differs by <3% from that of preceding injection.

### *C. Determination*

Accurately weigh ca 250 mg finely ground sample, dissolve in 0.1*N* HCl, and quant. dil. to estimated phencyclidine.HCl concn of 1 mg/mL. Let any insol. material settle. Pipet 2 mL aliquots sample soln and 2 mL std soln into sep. test tubes. Add 2.0 mL internal std soln and 1.0 mL 10% $K_2HPO_4$ soln to sample tube, stopper, and shake vigorously. Add 2.0 mL 0.1*N* HCl and 1.0 mL 10% $K_2HPO_4$ to std tube, stopper, and shake vigorously. Let sep. Inject duplicate 4 μL aliquots of lower $CHCl_3$ layers into gas chromatograph from 10 μL syringe.

$$\text{\% Phencyclidine.HCl} = (H/H')\ (C'/W)\ (B'/B) \times DF \times 100$$

where $H$ and $H'$ = av. peak ht or area of sample and std, resp.; $C'$ = mg std phencyclidine.HCl/mL in std soln; $W$ = mg sample; $B$ and $B'$ = av. peak ht or area of internal std in sample and std, resp.; and $DF$ = diln factor for sample.

$$\text{mg Phencyclidine.HCl/tablet} = \text{\% Phencyclidine.HCl} \times \text{av. tablet wt (mg)}/100$$

Ref.: JAOAC **62,** 560(1979).

CAS-956-90-1 (phencyclidine hydrochloride)

**Common and Chemical Names for Drugs in this Chapter**

| Common Name | Chemical Name |
|---|---|
| Amphetamine | (±)-α-Methylphenethylamine |
| Cannabinol | 6,6,9-Trimethyl-3-pentyl-6*H*-dibenzo(*b,d*)pyran-1-ol |
| Cocaine (hydrochloride) | 3-(Benzoyloxy)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylic acid methyl ester |
| Heroin | Diacetylmorphine |
| Diazepam | 7-Chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one |
| Lysergic acid diethylamide | 9,10-Didehydro-*N,N*-diethyl-6-methylergoline-8β-carboxamide |
| Methaqualone | 2-Methyl-3-(2-methylphenyl)-4(3*H*)-quinazolinone |
| Morphine (hydrochloride, sulfate, diacetate) | 7,8-Didehydro-4,5-epoxy-17-methylmorphinan-3,6-diol |
| Oxazepam | 7-Chloro-1,3-dihydro-3-hydroxy-5-phenyl-2*H*-1,4-benzodiazepin-2-one |
| Phencyclidine (hydrochloride) | 1-(1-Phenylcyclohexyl)piperidine |
| Quinine ethylcarbonate | 6′-Methoxycinchonan-9-ol ethyl carbonate (ester) |

Source: *USAN and the USP Dictionary of Drug Names* (1983; 1989) U.S. Pharmacopeial Convention, Rockville, MD.

# 23. Drugs and Feed Additives in Animal Tissues

**Charlie J. Barnes, *Associate Chapter Editor***
*Food and Drug Administration*

**961.23** **ANOT Residues in Animal Tissues**
**Spectrophotometric Method**
**Final Action**

### *A. Principle*

ANOT, metabolite of zoalene, is liberated from ground tissue by enzymatic digestion with ficin. Digest is treated with $NaHCO_3$ and extd with acetone. $CHCl_3$ is added to sep. soln into 2 layers. Org. layer is concd and passed thru alumina column. Adsorbed ANOT is washed with $CHCl_3$ and eluted with 80% alcohol. Alcohol soln is passed thru cation exchange resin and ANOT is eluted with 4*N* HCl. Colored compd formed by diazotization and coupling with *N*-1-naphthylethylenediamine is measured at 540 nm.

### *B. Apparatus*

(**a**) *Chromatographic tube.*—600 × 16 mm id.
(**b**) *Ion exchange columns for Dowex resin.*—180 × 11 mm id.
(**c**) *Mixer.*—High speed, high shear mixer with explosion-proof motor, and ca 1 L container.
(**d**) *Spectrophotometer.*—Beckman Model DU, 24, or 25 (replaced by Models DU-64), or equiv.

### *C. Reagents*

(**a**) *Alumina.*—Activated, Alcoa grade F-20, 80–200 mesh.
(**b**) *3-Amino-5-nitro-o-toluamide.*—ANOT, anal. std. Available from Dow Chemical Co.
(**c**) *Ammonium sulfamate soln.*—1.0%. Prep. fresh weekly.
(**d**) *Coupling reagent.*—0.25% aq. soln of *N*-1-naphthylethylenediamine.2HCl. Prep. fresh weekly and store in dark bottle.
(**e**) *Dowex 50W-X8 cation exchange resin.*—Hydrogen form, 200–400 mesh (Bio-Rad Laboratories).
(**f**) *Ficin.*—ICN Pharmaceuticals, Inc., Life Sciences Group. (*Caution:* Ficin is potent proteolytic enzyme which attacks living tissues. Avoid contact with skin and eyes and breathing dust.)
(**g**) *Sodium nitrite soln.*—0.25%. Prep. fresh daily.

### *D. Preparation of Alumina Column*

Insert small plug of glass wool into chromatgc tube and compress in lower end of tube. Add 60 g alumina and pack by gently tapping tube on rubber stopper to ht of ca 30 cm. Add 100 mL $CHCl_3$ and drain to just above level of alumina. Do not drain $CHCl_3$ below level of alumina.

### *E. Preparation of Ion Exchange Column*

Heat 100 g Dowex 50W-X8 on steam bath with 400 mL 6*N* HCl 2–3 hr. Filter on buchner and wash with $H_2O$ until washings are acid-free. Wash resin with 100 mL 80% alcohol. Then mix resin with 250 mL 80% alcohol. Pour enough resin slurry into ion exchange column to give bed ht of ca 5 cm after settling. Wash resin with 25 mL 80% alcohol. Slight air pressure can be used to increase flow of liq. thru resin. Do not let liq. level drain below top of resin bed.

### *F. Preparation of Standard Curve*

Accurately weigh 100 mg ANOT into 1 L vol. flask, dissolve in 50 mL acetone, and dil. to vol. with $H_2O$. Dil. 10 mL of this stock soln to 100 mL with $H_2O$ to give working soln of 10 μg/mL. Pipet 0, 2, 4, 6, 8, and 10 mL aliquots of this soln into sep. 50 mL vol. flasks. Dil. each to ca 40 mL with 4*N* HCl. Proceed as in **961.23G**, beginning "Add 1 mL 0.25% $NaNO_2$ . . .". Plot *A* at 540 nm against μg ANOT.

### *G. Determination*

(*Caution:* See safety notes on distillation, toxic solvents, and chloroform.)

Collect tissue, freeze with solid $CO_2$, and keep frozen until analyzed. Grind tissue while at least partially frozen and weigh 50 g into 1 qt (1 L) Mason jar. Add 125 mL $H_2O$, 15 mL 1*N* HCl, and 5 g ficin, and mix with mixer ca 5 min. Cover jar loosely and keep 24 hr at 30°. Then keep 30 min in bath at 70–80°, remove, and cool.

Weigh ca 10 g $NaHCO_3$ and slowly add to jar with stirring, taking care that sample does not foam over top of jar. When foaming has subsided, add 500 mL acetone and mix 5 min with mixer.

Filter on buchner into 1 L filter flask, using 11 cm paper and ca 5 g Super-Cel as filter pad. Wash residue with 200 mL acetone, collecting washings in same flask. Transfer filtrate to 2 L separator and add 1 L $CHCl_3$. Shake ext in separator vigorously and let stand until layers sep. Drain $CHCl_3$ layer into 2 L beaker. Ext aq. layer with 200 mL $CHCl_3$ and combine $CHCl_3$ washing with original ext. Evap. $CHCl_3$ ext to ca 50 mL under heat lamp with air current. Add 100 mL $CHCl_3$ and again evap. to 50 mL. If soln is not clear, repeat addn and evapn of $CHCl_3$ to remove $H_2O$.

Add clear $CHCl_3$ soln to alumina column and drain to level of alumina. Wash with four 50 mL portions $CHCl_3$. Discard washings. Add 90 mL 80% alcohol to column to elute ANOT. Discard first 30 mL effluent and collect 60 mL in 100 mL beaker. Transfer this soln to ion exchange column. Slight air pressure may be used to increase flow of soln. After soln has drained to top of resin bed, wash with 50 mL 80% alcohol followed by 50 mL $H_2O$. Discard washings. Add 45 mL 4*N* HCl and collect effluent in 50 mL vol. flask.

Add 1 mL 0.25% $NaNO_2$, mix, and let stand 5 min. Add 1 mL 1% $NH_4$ sulfamate soln, mix, and let stand 5 min. Add 1 mL coupling reagent, mix, dil. to vol. with 4*N* HCl, and mix thoroly. Let stand 15 min and read *A* at 540 nm, using 1 cm cells, against $H_2O$ as ref.

### *H. Calculations*

Obtain μg of ANOT corresponding to *A* from std curve.

$$\text{ppm ANOT in sample} = \mu g \text{ ANOT/g sample}$$

Refs.: J. Agric. Food Chem. **9**, 201(1961). JAOAC **49**, 708 (1966).

CAS-3572-44-9 (ANOT)

## 973.78 Arsenic (Total) Residues in Animal Tissues

### Spectrophotometric Method

**First Action 1973**
**Final Action 1975**

(Complete analysis in 1 day; otherwise stop after ashing step.)

### A. Reagents and Apparatus

(**a**) *Silver diethyldithiocarbamate*.—Chill 200 mL 0.1*M* $AgNO_3$ soln (3.4 g/200 mL) and 200 mL 0.1*M* Na diethyldithiocarbamate soln (4.5 g/200 mL) to 10° or lower. Add carbamate soln to $AgNO_3$ soln slowly with stirring. Filter thru buchner, wash with chilled $H_2O$, and dry under reduced pressure at room temp. Dissolve salt in pyridine (reagent grade) with stirring, chill, and add cold $H_2O$ slowly until completely pptd. Filter thru buchner, and wash with $H_2O$ to remove all pyridine. Dry pale yellow crystals under reduced pressure (mp 185–187°; recovery 85–90%). Store in amber bottle in refrigerator. (Second recrystn may be necessary to obtain correct mp.)

(**b**) *Silver diethyldithiocarbamate soln*.—Dissolve 0.5000 g salt, (**a**), in colorless pyridine in 100 mL vol. flask, and dil. to vol. with pyridine. Mix, and store in amber bottle. Reagent is stable several months at room temp.

(**c**) *Arsenic std solns*.—(*1*) *Stock soln*.—500 μg/mL. Accurately weigh 0.660 NIST SRM $As_2O_3$, or equiv., dissolve in 25 mL 2*N* NaOH, and dil. to 1 L with $H_2O$. (*2*) *Working solns*.—0–2 ppm. Just before use, prep. by dilg stock soln with $H_2O$.

(**d**) *Zinc*.—Shot, contg ≤0.00001% As (Fisher Scientific Co., No. Z-12, or granules of equiv. purity).

(**e**) *Cellulose powder*.—Whatman CF-11 fibrous.

(**f**) *Distillation apparatus*.—(*1*) *Flask*.—250 mL erlenmeyer. (*2*) *Connecting tube*.—L-shaped 8 mm od glass tube with 11 and 7 cm sides. Plug shorter end with 2 pieces of glass wool satd with 10% $Pb(OAc)_2$ soln and dried (replace plugs when discolored). (*3*) *Delivery tube*.—L-shaped 6 mm od glass tube with 22 and 5 cm sides. Constrict end of longer side to 1 mm opening. (*4*) *Receiver*.—8 cm length of 15 mm glass tube sealed to open end of 100 × 10 mm od test tube.

Connect flask thru 1-hole rubber stopper with 11 cm side of connecting tube. Attach connecting tube to 5 cm side of delivery tube with rubber tube sleeve. Fit constricted end of delivery tube into bottom of receiver.

### B. Dry Ashing

Blend liver and kidney in high-speed blender. Pass fibrous tissues such as muscle and skin thru meat grinder, and divide and quarter. Weigh 10 g tissue into 100 mL Coors crucible. Add 3 g MgO and 20 mL cellulose powder (10 mL beaker is convenient measure) to liver, kidney, and skin samples and 10 mL cellulose powder to muscle samples. Mix thoroly and char cautiously over open flame until evolution of smoke ceases. (*Caution:* Rapid rise in temp. will cause crucibles to crack; avoid overheating samples to prevent loss of As.)

Cool, add 3 g $Mg(NO_3)_2.6H_2O$, and place in cold furnace preset at 555°. After furnace reaches operating temp., ash 2 hr. Cool, moisten ash with 10 mL $H_2O$, and transfer quant. to 250 mL erlenmeyer with 90 mL 6*N* HCl. Dil. to 175 mL with $H_2O$. (Presence of black carbonaceous particles does not interfere.)

### C. Distillation

Add 2 mL 15% KI soln, and swirl. Add 1 mL *$SnCl_2$ soln*, **963.21A(g)**, and swirl. Cool in freezer or ice bath 45 min or until sample reaches 4°. Prep. blank contg 90 mL 6*N* HCl and 85 mL $H_2O$, and treat similarly.

Prep. trapping soln by pipetting 3 mL AgDDC reagent into receiving tube and place in ice bath. Attach delivery tube to connecting tube and insert delivery tube into AgDDC soln. Add 10 g Zn shot or granules to cooled erlenmeyer, immediately connect flask to connecting tube, and let distn proceed 1 hr at room temp. Det. *A* against reagent blank in 1 cm cell at 540 nm; det. As content from std curve.

Calc. As concn in sample by multiplying *A* at 540 nm by reciprocal slope of std curve, disregarding *y* intercept term.

### D. Preparation of Standard Curve

Add As working std solns (but <2 mL soln) to 10 g tissue to provide curve over desired range (usually 0–2 ppm As). Carry these samples thru ashing and distn. Det. best fitting straight line from ≥4 sets of detns for each tissue by method of least sqs, *Definitions of Terms and Explanatory Notes.*

Ref.: JAOAC **56,** 793(1973).

CAS-7440-38-2 (arsenic)

## 974.45 Clopidol Residues in Animal Tissues

### Gas Chromatographic Method

**First Action 1974**
**Final Action 1977**

(Diazomethane is toxic, can cause specific sensitivity, and is potentially explosive. Prep. diazomethane reagent, methylate, and evap. in hood. Avoid metal, ground glass joints, etched or scratched glassware, and sharp edges. Store diazomethane solns in freezer; do not expose to direct sunlight or strong artificial light.)

### A. Principle

Tissues and eggs are extd with MeOH; ext is filtered and cleaned up on alumina and anion exchange columns. Eluate is methylated with diazomethane, producing Me ether of clopidol (3,5-dichloro-4-methoxy-2,6-lutidine), which is detd by electron capture GC. Applicable to ≥0.1 ppm in chicken tissues and ≥0.05 ppm in eggs.

### B. Apparatus

(**a**) *Centrifuge*.—Clinical (Model CL, International Equipment Co.), or equiv., with head and cups to accommodate 13 × 100 mm tubes.

(**b**) *Flask*.—500 mL r-b ₮ 29/42 neck. Make 6 irregularly spaced 6 mm projections into flask by heating spot ca 2 cm diam. with torch and pushing spot in with blunt instrument.

(**c**) *Gas chromatograph*.—With electron capture detector. Operating conditions: temps (°)—column 155, injection port 220, detector 220; flow rates—N carrier gas 120 mL/min; sensitivity $3 \times 10^{-10}$ amp; and chart speed 20″/hr.

(**d**) *Gas chromatographic column*.—25% DC-200 silicone oil (Dow Corning Corp.) on 80–100 mesh Chromosorb W (AW) (Applied Science). Prepd column available from Applied Science, or prep. as follows: Weigh 12 g Chromosorb W (AW), from which fines have been removed on No. 100 sieve, into specially modified r-b flask, (**b**), contg 100 mL $CHCl_3$ and 3 g DC-200 fluid. Dry on rotary evaporator under vac. Use heat lamp or hot $H_2O$ to aid evapn. Sieve and discard fines passing No. 100 sieve. Pack 1.9 m (74″) × 3 mm id U-shaped borosilicate glass column and condition ≥18 hr at 200° with N flow of 75–100 mL/min before use. Add packing to column, tapping on floor to settle. Insert glass wool plug at effluent end. Level of packing in injection arm should be few mm below depth of needle point at inlet.

(**e**) *Liquid chromatographic columns.*—(*1*) *Alumina column.*—Add 6 g (1 heaping 5 mL beaker) alumina, **974.45C(a)**, to 300 × 18 mm id column, with coarse fritted disk and 30 × 5 mm id stem. (*2*) *Anion exchange column.*—Place 1 cm (after settling) AG1-X8 resin, **974.45C(b)**, in 170 × 10 mm id column, with coarse fritted disk and 30 × 5 mm id stem, using MeOH to transfer resin. Rinse column with 2 mL MeOH, applying air pressure from squeeze bulb.

(**f**) *Homogenizer.*—*See* **961.23B(c)**, or equiv., for use with Brockway 4 oz (125 mL) sq powder jar (No. 72 G1333, with 38 mm Polyseal caps, Brockway Plastics, Inc., 9211 Forest Hill Ave, PO Box 35110, Richmond, VA 23235-0110) and pt (500 mL) or qt (1 L) Mason jars.

(**g**) *Meat grinder.*—With stainless steel attachment.

(**h**) *Shaker.*—Wrist-action (Model BT, Burrell Corp., or equiv.).

(**i**) *Culture tubes.*—13 × 100 mm (Corning Glass Co., No. 9825), with 13 mm rubber-lined plastic screw caps.

### C. Reagents

(**a**) *Alumina.*—Alcoa F-20, 80–200 mesh (Fisher Scientific Co.).

(**b**) *Anion exchange resin.*—Bio-Rad AG1-X8, 100–200 mesh, acetate form (Bio-Rad Laboratories).

(**c**) *Diazomethane reagent.*—Approx. 18 mg/mL in Et ether. Add 35 mL 2-(2-ethoxy-ethoxy) ethanol (Aldrich Chemical Co., Inc.) and 10 mL ether to soln of 6 g KOH in 10 mL $H_2O$ in 125 mL long-neck distg flask. Place mag. stirring bar in flask and mount above $H_2O$ bath on top of hot plate mag. stirrer. Attach dropping funnel and efficient condenser connected in series to 250 and 50 mL erlenmeyers. Place 25 mL ether in second flask and place inlet tubing below surface of ether. Cool both receiving flasks in ice. Place soln of 21.5 g *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald; Aldrich Chemical Co., Inc.) dissolved in 140 mL ether in dropping funnel. Heat $H_2O$ bath to 55° and raise it to heat distn flask. Stir contents of flask while adding Diazald soln over 20 min. Interrupt distn when distillate is nearly colorless. Combine contents of the 2 receivers and store at 0° in culture tubes with screw caps or as in JAOAC **65,** 273–274(1982). Reagent is stable several weeks if kept in freezer in full, closed tubes. Diazomethane reagent may also be prepd as in Anal Chem. **32,** 1412–1414(1960).

(**d**) *Methanolic hydrochloric acid.*—5%. Add 25 mL HCl to 475 mL MeOH.

### D. Standard Solutions

(*Caution:* See safety note on benzene.)

(**a**) *Clopidol std solns.*—(*1*) *Stock soln.*—100 μg/mL. Accurately weigh ca 100 mg Clopidol Anal. Std (available from Dow Chemical Co.) in weighing bottle, transfer to 1 L vol. flask with MeOH, to total vol. of ca 950 mL. Stir mag. to dissolve clopidol (may take 2–3 hr). Dil. to vol. at room temp., and mix well. (*2*) *Intermediate soln.*—10 μg/mL. Pipet 10 mL stock soln into 100 mL vol. flask, dil. to vol. with MeOH, and mix. (*3*) *Working soln.*—1 μg/mL. Pipet 10 mL intermediate soln into 100 mL vol. flask, dil. to vol. with MeOH, and mix.

(**b**) *3,5-Dichloro-4-methoxy-2,6-lutidine* (*clopidol methyl ether*) *std solns.*—(*1*) *Stock soln.*—1 mg clopidol equiv./mL. Weigh 107.3 mg 3,5-dichloro-4-methoxy-2,6-lutidine anal. std (available from Sampling Coordinator, Ag-Organics Dept, Dow Chemical USA) in weighing bottle. Transfer to 100 mL vol. flask with benzene, dil. to vol. with benzene, and mix well. (*2*) *Intermediate soln I.*—10 μg clopidol equiv./mL. Pipet 1 mL stock soln into 100 mL vol. flask, dil. to vol. with benzene, and mix. (*3*) *Intermediate soln II.*—1.0 μg clopidol equiv./mL. Pipet 10 mL intermediate soln *I* into 100 mL vol. flask, dil. to vol. with benzene, and mix. (*4*) *Working solns.*—Prep. series of std solns contg 0.01 to 0.20 μg clopidol equiv./mL by dilg portions of intermediate soln *II* with benzene.

### E. Chromatography of Standards

Fill syringe needle with benzene, avoiding entrapped air, draw 3 μL sample aliquots of clopidol methyl ether working soln into syringe, and inject onto column. Measure peak hts (*PH*) in terms of % full-scale deflection, and plot *PH* against μg/mL. Prep. std curve daily and check by injecting std soln after every 1 or 2 samples.

### F. Determination

(**a**) *Muscle, liver, and kidney.*—Homogenize by grinding thru meat grinder. Accurately weigh ca 20 g tissue into 4 oz (125 mL) jar. Add 50 mL MeOH and 3 g HyFlo Super-Cel filter aid for muscle; use 12 g filter aid for liver and kidney. Attach jar to homogenizer and blend 3 min at max. speed. Filter thru 2 g pad of filter aid in 60 mL coarse fritted glass buchner mounted on filter assembly. Collect filtrate in 100 mL graduate, and wash jar and filter cake with MeOH to nearly 100 mL. If filter cake goes dry, break up with spatula during addn of more MeOH to prevent channeling. Dil. to 100 mL at room temp., stopper, and mix well.

Place anion exchange column under alumina column. Pipet 20 mL ext onto alumina column and let elute thru both columns into beaker. Wash columns with 10 mL MeOH added to alumina column, rinsing sides. Remove alumina column and beaker. Place 25 mL vol. flask under anion exchange column and elute clopidol with two 10 mL portions 5% HCl in MeOH. Dil. eluate to vol. with MeOH, and mix well. Pipet 1 mL aliquot into 13 × 100 mm tube and evap. to dryness (4–5 min) by mounting tube in 70° $H_2O$ bath so $H_2O$ level is at same ht as soln in tube. Direct very small jet of air down tube using rubber tubing and medicine dropper tip, or equiv. Remove tube, add 0.2 mL 80% MeOH, and heat briefly to redissolve residue. Add 1 mL diazomethane reagent, seal with screw cap, and heat gently 2 min by mounting tube as before in 70° $H_2O$ bath. Remove tube, and let cool 5 min before removing cap. Add small SiC boiling chip and evap. reagents gently by mounting tube with only extreme rounded bottom portion touching $H_2O$ of 70° bath. Continue heating 2–3 min until ether is evapd. Add 0.1 mL 1*N* NaOH, 5 mL $H_2O$, and 1.0 mL benzene, cap tube, and vigorously shake mixt. 1 min. Centrf. 3 min. Dil. further by adding more benzene, if necessary. Inject 3 μL of benzene layer as in **974.45E**. Det. *PH* and interpolate μg/mL clopidol in benzene ext from std curve.

(**b**) *Eggs.*—Accurately weigh ca 20.0 g sample into 4 oz. (125 mL) jar, and add ca 12 g Hyflo Super-Cel. Shake mech. 15 min. Proceed as in (**a**), beginning "Filter thru 2 g pad . . ." Elute with 9.5 mL 5% HCl in MeOH and collect eluate in 10 mL vol. flask.

(**c**) *Recovery factor.*—Accurately weigh 20.0 g samples of homogenized clopidol-free tissue, and add equiv. of 0.0, 0.1, and 0.5 ppm clopidol working std soln (0.0, 2.0, and 10.0 mL, resp.). Proceed as in (**a**) and calc. av. recovery factor, *R* = (ppm found from std curve)/(ppm added). For eggs, use 20 g samples, add equiv. of 0.00, 0.05, and 0.2 ppm (0.0, 1.0, and 4.0 mL working std soln, resp.), proceed as in (**b**), and calc. av. recovery factor, *R*.

$$\text{ppm Clopidol in tissues} = 6.25\,(G - G')/R$$
$$\text{ppm Clopidol in eggs} = 2.5\,(G - G')/R$$

where G = μg/mL from std curve of sample, and G′ = μg/mL from std curve of blank.

Refs.: JAOAC **57,** 914(1974); **59,** 476(1976).

CAS-2971-90-6 (clopidol)

## 973.79 Decoquinate Residues in Animal Tissues

### Fluorometric Method

**First Action 1973**
**Final Action 1974**

(Applicable to chicken tissues at ≤2.5 ppm level)

#### *A. Principle*

Tissue is homogenized in MeOH-$CHCl_3$. After addn of metaphosphoric acid, decoquinate is extd into $CHCl_3$ and sepd from interfering materials by chromatgy on Florisil. Decoquinate is eluted from column with $CaCl_2$-MeOH and detd by fluorometry against std treated similarly. Range 0.1–2.2 ppm; sensitivity 0.1 ppm.

#### *B. Apparatus*

(**a**) *Fluorometer.*—Aminco-Bowman SPF, or equiv.

(**b**) *Chromatographic columns.*—Draw 30 cm length of 9 mm tubing (7 mm id) to drip tip. Insert small glass wool plug to support adsorbent. Close drip end with short piece of tubing and pinch clamp. Add 5 mL $CHCl_3$ to column, then 0.4 ± 0.02 g Florisil. Add 2 mL addnl $CHCl_3$ and stir with thin glass rod to settle adsorbent. Remove tubing and wash down sides of tube with $CHCl_3$. Prep. just before use.

#### *C. Reagents*

(**a**) *Methanol-chloroform soln.*—Mix 4 parts MeOH, redistd in all-glass app., and 1 part $CHCl_3$, spectral grade.

(**b**) *Decoquinate std solns.*—(*1*) *Stock soln.*—200 μg/mL. Weigh 20 mg Decoquinate Ref. Std (available from Hess & Clark Laboratories). Dissolve and dil. to 100 mL with $CHCl_3$. (*2*) *Working soln.*—10 μg/mL. Pipet 5 mL stock soln into 100 mL vol. flask and dil. to vol. with $CHCl_3$. (*3*) *Fluorescence reference soln.*—0.2 μg/mL. Pipet 2 mL working soln into 100 mL vol. flask and dil. to vol. with elution solv., (**c**). Solns are stable ≥1 month.

(**c**) *Elution solvent.*—Dissolve 10 g anhyd. $CaCl_2$ in 1 L redistd MeOH. Let stand 24 hr. Decant from any insol. residue.

(**d**) *Metaphosphoric acid soln.*—5%. Dissolve 50 g metaphosphoric acid (J. T. Baker, Inc., No. 0252) in 1 L $H_2O$. Refrigerate at 5° and use cold.

(**e**) *Florisil.*—100–200 mesh (Fisher Scientific Co., No. F-101).

#### *D. Preparation of Standard Curve*

Add 55 mL (50 g) MeOH-$CHCl_3$ (4 + 1) soln to each of four 250 mL separators. Add 0.0, 0.1, 0.3, and 0.5 mL working soln contg 0, 1, 3, and 5 μg decoquinate, resp. Proceed with *Determination,* beginning "Add 100 mL 5% metaphosphoric acid, . . .", adding entire $CHCl_3$ ext to column. Construct std curve by plotting fluorescence against μg decoquinate/mL.

#### *E. Determination*

Weigh 20 g tissue into high-speed blender. Add 80 ± 1 g MeOH-$CHCl_3$ (4 + 1) (weigh on top-loading balance). Blend 1 min. Transfer to centrf. bottle and centrf. 5 min at ca 2000 rpm. Decant and weigh 50 g supernate (equiv. to 10 g tissue) into 250 mL separator. Add 100 mL 5% metaphosphoric acid, invert 50 times, let phases sep. 10 min, and drain and retain $CHCl_3$ layer. Add 10 mL addnl $CHCl_3$ to separator, shake, and let sep. as before. Combine $CHCl_3$ exts, add 2 mL MeOH, and dil. to 25 mL with $CHCl_3$.

Depending on expected decoquinate content, add 5, 10, or 25 mL ext (2, 4, or 10 g tissue, resp.) to chromatgc column. Normally use 10 mL for liver, kidney, skin, and fat, and 25 mL for muscle samples. Wash column with 10 mL MeOH. Elute with 15 mL elution solv., (**c**), collecting in tube marked at 15 mL. Mix and transfer to fluorometer cell. Set activation wavelength at 270 nm and emission wavelength at 390 nm. With fluorescence ref. std in cell, adjust microphotometer controls to give reading of 80 on rel. intensity scale. Det. fluorescence of samples, and calc. μg decoquinate from std curve.

Ref.: JAOAC **56,** 71(1973).

CAS-18507-89-6 (decoquinate)

## 968.48 Ethoxyquin Residues in Animal Tissues

### Photofluorometric Method

**First Action 1968**

(Applicable to chicken tissues and eggs)

#### *A. Apparatus*

(**a**) *Photofluorometer.*—(*Caution:* See safety notes on photofluorometers.) Instrument with primary filter passing only 365 nm Hg line and secondary filter passing light between 410 and 580 nm (but not below 410 nm).

(**b**) *Separators.*—250 mL with Teflon stopcocks.

#### *B. Reagents*

(**a**) *Isooctane.*—Fluorescence <2% that of soln contg 0.020 μg quinine sulfate/mL 0.1*N* $H_2SO_4$. If necessary, purify isooctane by passing thru 30 × 2 cm activated alumina column.

(**b**) *Sulfuric acid-sodium sulfate soln.*—0.3*N* $H_2SO_4$ contg 2% $Na_2SO_4$.

(**c**) *Ethoxyquin std solns.*—(*1*) *Stock soln.*—10 μg/mL. Place 10 mg com. grade ethoxyquin in 1 L vol. flask; dissolve and dil. to vol. with isooctane. Store in refrigerator. (*2*) *Working solns.*—0.010, 0.020, 0.030, and 0.050 μg/mL. Transfer 1, 2, 3, and 5 mL aliquots stock soln to 1 L vol. flasks and dil. to vol. with isooctane. Prep. fresh on day of use.

#### *C. Preparation of Standard Curve*

Prep. std curve at time of analysis of final ethoxyquin exts. Read ethoxyquin stds with photofluorometer set at 0 with shutters closed and at 100 with most concd std. Plot instrument reading against μg ethoxyquin on linear graph paper.

#### *D. Preparation of Sample and Extraction*

(All glassware must be free of stopcock grease.)

(**a**) *Egg yolk.*—Carefully break egg to avoid rupturing yolk and sep. as much of egg white from yolk as possible. Wash yolk in running $H_2O$ to remove most of remaining egg white. Dry yolk on absorbent paper, break yolk sac, pour yolk into bottle, and stopper.

Weigh bottle contg yolk and pour ca 5 g yolk into mortar contg 25 g anhyd. granular $Na_2SO_4$ and 3 g anhyd. powd $Na_2CO_3$. Reweigh bottle and record wt yolk added. (Several samples may be prepd from same yolk.) Grind mixt. in mortar until uniform and dry 1 hr in desiccator contg Drierite.

Transfer dried mixt. to 4 oz (125 mL) screw-cap bottle and shake 30 min with 50 mL isooctane. Centrf. and filter supernate thru Whatman No. 1 paper into 250 mL separator. Repeat extn with second 50 mL isooctane and add ext to separator.

Gently shake isooctane ext 1 min each with two 50 mL portions 0.3*N* $H_2SO_4$-$Na_2SO_4$ soln. Combine acid exts and add 10 mL 6*N* NaOH. Ext alk. soln with two 50 mL portions isooctane. Combine isooctane exts and dry 15 min over anhyd. $Na_2SO_4$; decant, and dil. to 100 mL with isooctane.

**(b)** *Tissue* (*muscle and liver*).—Accurately weigh ca 5 g muscle or 1 g liver and add to 15 g anhyd. $Na_2SO_4$ and 2 g anhyd. $Na_2CO_3$ in mortar. Grind until uniform and place in desiccator 1 hr.

Shake dried mixt. 30 min in 4 oz (125 mL) screw-cap bottle with 100 mL isooctane. Centrf., and filter into 250 mL separator. Continue as in **(a)**, 4th par., beginning "Gently shake isooctane ext . . ."

**(c)** *Fat*.—Accurately weigh ca 1 g frozen fat and add to 10 g granular, anhyd. $Na_2SO_4$ and 1 g anhyd. $Na_2CO_3$ in *glass* mortar. Grind mixt. thoroly. Add 20 mL isooctane and continue grinding several min. Decant isooctane into 4 oz (125 mL) screw-cap bottle. Repeat grinding with isooctane 3 times. Transfer isooctane ext to bottle, shake, and centrf.

Decant supernate isooctane layer into 250 mL separator and continue as in **(a)**, 4th par., beginning "Gently shake isooctane ext . . ."

### *E. Determination*

Det. fluorescence of isooctane soln and calc. ethoxyquin content from std curve.

Ethoxyquin, ppm
= (μg ethoxyquin/mL) × (mL ext/g sample)

Refs.: JAOAC **50,** 844(1967); **51,** 453, 537(1968).

CAS-91-53-2 (ethoxyquin)

## 976.36 Melengestrol Acetate Residues in Animal Tissues

### Gas Chromatographic Method

**First Action 1976**
**Final Action 1978**

### *A. Principle*

Melengestrol acetate (MGA) is extd from lean tissue with $CH_3CN$ and ext is partitioned with hexane. MGA in fatty tissues is extd with hexane and then transferred into $CH_3CN$. Residue from either ext, after evapn of solv., is chromatgd on Florisil to remove interfering lipid materials with hexane and hexane-acetone (95 + 5). MGA is eluted with hexane-acetone (80 + 20). Residue is dissolved in hexane-acetone, and detd by GC.

For those liver samples where MGA is poorly resolved on chromatogram, hexane-acetone is evapd, partitioned with aq. 70% MeOH-hexane, transferred into $CHCl_3$, and evapd. Dry residue is dissolved in hexane-acetone and reinjected onto GC column.

### *B. Apparatus*

**(a)** *Adapters*.—₮24/40, Nos. 5225-10 and 5205 (Ace Glass, Inc., or equiv.).

**(b)** *High-speed blender*.—Waring Blendor Model 702-B with 1 L glass bowl having polyethylene gaskets (*see* **(i)**), or equiv.

**(c)** *Chromatographic tubes*.—Glass, 400 × 19 mm id, fitted with medium porosity fritted glass disks, Teflon stopcocks, and ₮ 24/40 tops.

**(d)** *Containers*.—Plastic, with lid. For storage of frozen tissues.

**(e)** *Flasks*.—R-b, 50, 500, and 1000 mL.

**(f)** *Funnels*.—Medium porosity fritted glass funnels, 350 mL.

**(g)** *Gas chromatograph*.—F&M Model 402, replaced by HP 5890A series, (available from Hewlett-Packard Co., Avondale Div.), or equiv., with all-glass on-column injection system, $^{63}Ni$ electron capture detector, and 1 mv strip chart recorder. Operating conditions: temps (°)—column 240–250, injection port 240–250, detector 270–275; flow rates—He carrier gas 60–80 mL/min (40 psi, 3.0–3.5 rotameter setting), Ar-$CH_4$ purge gas (95 + 5) 135–150 mL/min (40 psi); attenuation 16× or 32×; pulse interval 150; electrometer sensitivity $1 \times 10^{-12}$ amp full scale deflection with 1 mv recorder. Approx. retention time of MGA under these conditions is 5–6 min.

**(h)** *Gas chromatographic column*.—Use borosilicate glass tubing, 0.2362 ± 0.013″ (6.00 ± 0.33 mm) od and 0.118 ± 0.01″ (3.00 ± 0.25 mm) id. Bend 0.9 m (3′) piece of tubing into proper design for instrument. Pack column with 1% OV-17 on 100–120 mesh Gas Chrom Q (max. operating temp., 350°, Applied Science Laboratories, Inc., or equiv.), and plug both ends with 0.5 cm loosely packed silanized glass wool. Pack far enough from ends so that no part of column packing or glass wool is inside injection port or detector inlet fittings. Connect column to injection port and cap detector inlet. Condition column 1 hr at 240° with He carrier gas at 40 mL/min, and then 16 hr at 275° with He carrier gas at 80 mL/min. Remove cap and connect column to detector.

**(i)** *Gaskets*.—Polyethylene, cut from 1 qt (1 L) freezer containers.

**(j)** *Nitrogen pressure manifold for columns*.—(Optional). Adapters No. 5205 (Ace Glass, Inc., or equiv.) connected thru manifold regulated at 3 psi (20.7 kPa), with individual control valve.

**(k)** *Pipets*.—Transfer pipets, 9″ Dispo-pipettes (Scientific Products, Inc., or equiv.).

**(l)** *Reservoirs*.—250 mL ₮24/40 r-b flasks with 24/40 male joint in bottom, or equiv.

**(m)** *Rotary evaporator*.—4–6 small size Rinco evaporators (Valley Electromagnetics, One Wolfer Park, Spring Valley, IL 61362), or equiv., controlled with 4 mm bore stopcocks connected to manifold that leads to 2 condensation traps connected in series to vac. pump with free air capacity of 140 L/min. Cool traps with solid $CO_2$-alcohol mixt. Connect each sample in r-b flask with 2 adapters in series to evaporator, and heat in thermostatically controlled $H_2O$ bath at 45°.

**(n)** *Separators*.—With Teflon stopcocks, 500 and 1000 mL.

**(o)** *Silanized glass wool*.—Applied Science, or equiv.

**(p)** *Syringe*.—10 μL, Hamilton No. 701N, or equiv.

### *C. Reagents*

(All solvs must show no impurities when processed thru entire detn in absence of tissues.)

**(a)** *Argon-methane, 95 + 5*.—Purge gas (Matheson Gas Products, 30 Seaview Dr, PO Box 1587, Seacaucus, NJ 07096, or equiv.).

**(b)** *Diatomaceous earth*.—Celite 545 (Manville Filtration and Minerals, or equiv.).

**(c)** *Florisil*.—60–100 mesh (available from Floridin Co.). Activated by manufacturer at 650° (1225–1250°F). Heat in oven at 130° ≥48 hr before use.

(**d**) *Glassware cleaner*.—Haemo-Sol (Scientific Products, Inc., or equiv.).

(**e**) *Helium*.—99.5% min. purity (Matheson Gas Products, or equiv.).

(**f**) *Solid carbon dioxide*.

(**g**) *Solvents*.—Acetone, $CH_3CN$, benzene, $CHCl_3$, hexane, and MeOH. Distd-in-glass grade (Burdick & Jackson Laboratories, Inc., or equiv.).

(**h**) *Solvent mixtures*.—(v/v). (*1*) *Hexane-acetone*.—(8 + 2). (*2*) *Hexane-acetone*.—(95 + 5). (*3*) *70% Methanol*.

(**i**) *Anhydrous sodium sulfate*.—Mallinckrodt Chemical Works, or equiv. Wash with $CHCl_3$, dry in 110° oven, and store in g-s bottle until used.

### D. MGA Standard Solutions

(**a**) *Stock solns*.—(*1*) *A*.—1 mg/mL; 1000 ppm. Dissolve 100.0 mg melengestrol acetate (99.5% purity, Upjohn Co.) in 100 mL acetone. Soln is stable 2–3 months. (*2*) *B*.—100 ppm. Dil. 10.0 mL soln *A* to 100 mL with MeOH. Prep. soln fresh daily. (*3*) *C*.—10 ppm. Dil. 10.0 mL soln *B* to 100 mL with MeOH. Prep. soln fresh daily.

(**b**) *Intermediate solns*.—(*1*) *D*.—0.5 ppm. Dil. 5.0 mL soln *C* to 100 mL with MeOH. (*2*) *E*.—1.0 ppm. Dil. 10.0 mL soln *C* to 100 mL with MeOH. (*3*) *F*.—1.5 ppm. Dil. 15.0 mL soln *C* to 100 mL with MeOH.

(**c**) *Working solns*.—0.25, 0.50, 0.75 ppm. Transfer 5.0 mL solns *D*, *E*, and *F* into sep. 50 mL r-b flasks and evap. on rotary evaporator. Dissolve residues in 10.0 mL portions hexane-acetone (8 + 2).

### E. Extraction

(Wash all glassware in detergent and rinse in $H_2O$ to remove traces of cleaning agent. Then rinse with MeOH, acetone, or $CHCl_3$. *Caution:* See safety notes on blenders, acetonitrile, acetone, and cyclohexane. Store samples in freezer.)

(**a**) *From fat*.—Transfer 25.0 g sample to 250 mL beaker. Add 150 mL hexane and warm on steam bath in fume hood without boiling. Stir with spatula until fat dissolves. Place 20 g diat. earth (2 heaping tablespoons) in fritted funnel and wash with 100 mL $CH_3CN$. Discard wash. Filter warmed fat soln thru cake on funnel with vac. into 1 L filter flask. Rinse beaker with <50 mL hexane to remove solids, and transfer to funnel. Remove top 3 mm diat. earth cake and transfer to blender bowl. (Some diat. earth is left in funnel for next filtration.)

Add 150 mL hexane and homogenize 3 min at low speed. Filter soln thru diat. earth cake into filter flask. Rinse blender bowl with enough hexane to remove solids, and transfer to funnel. Adjust combined filtrates to ca 400 mL with hexane in filter flask. Rinse beaker and blender bowl with two 50 mL portions $CH_3CN$, and transfer to funnel. (Rinse cake thoroly, since MGA may adsorb onto diat. earth from hexane.) Warm filter flask on steam bath in hood and transfer filtrate to 1 L separator. Rinse flask with 5–15 mL $CH_3CN$, and transfer to separator. Shake vigorously 1 min. Let layers sep. 30 min. Drain lower layer into 1 L r-b flask. Add 100 mL $CH_3CN$ to separator. Repeat extn and sepn twice. Add 50 mL benzene to r-b flask and evap. on rotary evaporator.

(**b**) *From muscle, liver, and kidney*.—Transfer 25.0 g frozen tissue to blender bowl. Let thaw 5–10 min at room temp. Add 150 mL $CH_3CN$, 20 g diat. earth (2 heaping tablespoons), and 50 g anhyd. $Na_2SO_4$ (2 tablespoons). Homogenize at low speed 3 min. Place 20 g diat. earth into fritted funnel and wash with 100 mL $CH_3CN$. Discard wash. Filter soln thru cake with vac. into 1 L filter flask. Rinse blender bowl with <50 mL $CH_3CN$ to remove remaining solids. Sep. tissue cake from filter pad and transfer to blender. (Do not disturb diat. earth below tissue cake. Household fork is good transfer tool.)

Add 10 g diat. earth, 25 g anhyd. $Na_2SO_4$, and 150 mL $CH_3CN$ to blender bowl. Homogenize 3 min at low speed, filter, and rinse. Transfer combined filtrate to 1 L r-b flask and add 50 mL benzene. Evap. to dryness in rotary evaporator. (*Caution:* Bumping may occur. See safety note on benzene.) To dry residue, add 200 mL hexane and 100 mL $CH_3CN$ thru adapter. Remove adapter, and transfer solv. mixt. to 1 L separator. Add another 200 mL portion hexane to r-b flask and transfer to separator. Shake vigorously 1 min. Let layers sep. 30 min. Drain lower layer into 1 L r-b flask. Add 100 mL $CH_3CN$ to separator. Repeat extn and sepn twice. Add 50 mL benzene and evap. on rotary evaporator.

### F. Column Chromatography

Before analysis of samples, confirm, using MGA std soln, that hexane-acetone (8 + 2) elutes MGA completely, as follows: Pipet 1 mL 1 ppm MGA std soln into 50 mL r-b flask and evap. solv. on rotary evaporator. Chromatograph on Florisil column as indicated below. Det. recovery of MGA. If recovery is <95%, det. new elution vol. or obtain new batch of Florisil.

To 19 mm id glass tube, add cooled Florisil to ht of 10 cm with tapping. Push small wad of glass wool into tube until it touches Florisil. Place reservoir on top of column. Consecutively prewash column with 100 mL hexane, 100 mL acetone, and 100 mL hexane. (N pressure may be used to speed up this washing.) Remove reservoir.

Dissolve sample residue in 20 mL hexane and transfer to top of column. Replace reservoir and consecutively wash flask with 20 mL hexane, 200 mL hexane, and 300 mL hexane-acetone (95 + 5), and add each washing to column; if N pressure is used, add adapter. When last of solv. has reached top of column, place 500 mL r-b flask under column, wash sample residue flask with 150–170 mL hexane-acetone (8 + 2), and transfer to column for MGA elution. Elute sample until column goes dry, using N pressure to blow out last of solv. Evap. to dryness on rotary evaporator. Quant. transfer dried residue with five 2 mL portions acetone to 50 mL r-b flask and evap. on rotary evaporator. Dil. sample to 1.0 mL with hexane-acetone (8 + 2).

MGA gives poorly resolved chromatogram with some liver samples. Following addnl cleanup is necessary to remove interferences: Evap. remainder of 1 mL hexane-acetone soln on rotary evaporator. To dried residue, add three 20 mL portions hexane and transfer to 500 mL separator. Add 50 mL 70% MeOH, shake vigorously 1 min, let sep. 15 min, and drain lower layer into second 500 mL separator. Add 50 mL 70% MeOH to first separator. Repeat extn and sepn twice. To MeOH layer in second separator, add 1.0 mL satd $Na_2SO_4$ soln, 100 mL deionized $H_2O$, and 50 mL $CHCl_3$. Shake vigorously 1 min. (*Caution:* Vent frequently.) Let layers sep. 15 min and drain lower layer into 500 mL r-b flask. Add 50 mL $CHCl_3$ to separator and repeat extn and sepn twice. Evap. $CHCl_3$ on rotary evaporator. Quant. transfer dried residue with five 2 mL portions acetone to 50 mL r-b flask and evap. on rotary evaporator. Add 1.0 mL hexane-acetone (8 + 2) and reinject on column.

### G. Gas Chromatography

Alternately inject 2–4 μL aliquots sample blank and 0.25 ppm MGA std soln until reproducible peak hts are obtained for std. Inject 1–4 μL 0.25 ppm MGA std soln. Adjust gas flow and attenuation until 20–25 mm peak ht is obtained. Use this std soln for measurement and calcn of samples at ca 10 ppb (ng/g) level, 0.5 ppm std soln for samples at ca 20 ppb, and 0.75 ppm std soln for samples at 30 ppb.

Inject same sample vol. as used for std soln to obtain 20–

25 mm (or suitable) response. Measure peak ht of std, $H'$, and sample, $H$, at retention time of MGA by baseline technic.

$$\text{ppb MGA} = (H/H') \times C \times (V/I)/\text{g sample}$$

where $C$ = ng MGA std injected on column; $V$ = total mL soln (sample + solv.) in r-b flask (1.0 mL); and $I$ = mL sample soln injected onto column.

Ref.: JAOAC **59,** 507(1976).

CAS-2919-66-6 (melengestrol acetate)

## 970.84 Nalidixic Acid Residues in Animal Tissues

### Spectrofluorometric Method

**First Action 1970**
**Final Action 1974**

(Applicable to chicken liver and muscle contg ≥100 ppb nalidixic acid)

### A. Principle

Nalidixic acid is extd from aq. tissue homogenate with EtOAc. EtOAc is collected, concd, and passed thru alumina column which retains nalidixic acid. Nalidixic acid is removed from column with borate buffer, acidified, and re-extd with $CHCl_3$. After $CHCl_3$ removal, residual nalidixic acid is made to fluoresce with $H_2SO_4$ and resultant fluorescence is measured with spectrofluorometer.

### B. Apparatus

(**a**) *Spectrofluorometer*.—(*Caution:* See safety notes on photofluorometers.) Aminco-Bowman 4-8202, or equiv., with Xe lamp, IP 28 photomultiplier tube, and operated with manufacturer's slit arrangement No. 3. Precise wavelength settings for excitation and emission may vary slightly between instruments. Det. optimal wavelengths (ca 325 and 408 nm) after evapn of 2 mL working std soln (1 μg nalidixic acid) and soln of residue in 10 mL 21.5*N* $H_2SO_4$, **970.84C(c)**(*1*).

(**b**) *Chromatographic tubes*.—160 × 11.5 (id) mm (Kontes Glass Co., No. K-420000, or equiv.).

(**c**) *Shaker*.—Reciprocating (Sargent-Welch Scientific Co., No. S-74070, or equiv.).

### C. Reagents

(**a**) *Phosphate buffer soln*.—pH 6.0. Weigh 28 g $NaH_2PO_4.H_2O$ into 1 L beaker, add ca 600 mL $H_2O$, and adjust pH potentiometrically with aq. NaOH. Dil. to 1 L.

(**b**) *Borate buffer soln*.—pH 10.0. Dissolve 30 g $H_3BO_3$ in ca 600 mL $H_2O$ and adjust pH potentiometrically with aq. NaOH. Dil. to 1 L.

(**c**) *Dilute sulfuric acid*.—(*Caution:* See safety notes on sulfuric acid.) (*1*) *21.5N*.—Measure 200 mL $H_2O$ into 1 L flask and gradually add, with cooling, 300 mL $H_2SO_4$. Use soln at room temp. (*2*) *7N*.—Dil. 1 vol. (*1*) with 2 vols. $H_2O$.

(**d**) *Alumina*.—Neut. (Fisher Scientific Co., No. A-950, or equiv.).

(**e**) *Nalidixic acid std solns*.—(*1*) *Stock soln*.—500 μg/mL. Dissolve 50.0 mg nalidixic acid anal. std (available from Sterling Organics, 33 Riverside Ave, Rensselaer, NY 12144) in 100 mL MeOH. (*2*) *Intermediate soln*.—5.0 μg/mL. Dil. 2.0 mL stock soln to 200 mL with MeOH. (*3*) *Working soln*.—0.5 μg/mL. Dil. 10.0 mL intermediate soln to 100 mL with MeOH.

### D. Determination

(*Caution:* See safety notes on centrifuges, flammable solvents, chloroform, and ethyl acetate.)

Transfer 10 g chicken liver or muscle to high-speed blender. Add 100 mL phosphate buffer and blend 2–3 min. Transfer homogenate to 500 mL g-s extn bottle and add 300 mL EtOAc.

Add 100 mL phosphate buffer to each of five 500 mL g-s extn bottles. Transfer 0, 1.0, 2.0, 3.0, and 4.0 mL working soln contg 0.0, 0.50, 1.0, 1.5, and 2.0 μg nalidixic acid, resp. Add 300 mL EtOAc to each. Mech. shake all bottles contg sample and std 10–15 min and centrf. ca 5 min at 2500 rpm. Withdraw 250 mL EtOAc supernate from each and transfer to sep. 600 mL beakers. Evap. each under air current on steam bath to ca 60 mL.

Prep. adsorption column for sample and each std as follows: Place glass wool plug at bottom of chromatgc tube and add alumina to depth of 3 cm (ca 3 g). Place another glass wool pad at top of column. Wash each column with 25 mL EtOAc. Transfer tissue and std exts from beakers to respective columns. Rinse each beaker with 25 mL EtOAc followed by two 25 mL portions ether and two 25 mL portions MeOH. Transfer each solv. rinse to corresponding column and discard all eluates.

Add two 25 mL portions borate buffer and collect eluate in 50 mL graduate. Transfer eluate from graduate to 125 mL separator with Teflon stopcock. Ext with 25 mL ether and discard ether. Acidify aq. soln with 10 mL 7*N* $H_2SO_4$. Thoroly ext with 25 mL and 10 mL $CHCl_3$. Withdraw each $CHCl_3$ ext and combine in 100 mL beaker. (Do not introduce any aq. phase.) Evap. solv. just to dryness on steam bath.

Add 10.0 mL 21.5 *N* $H_2SO_4$ to each beaker. Mix thoroly ≥10 min. Det. relative fluorescence (product of linear scale meter reading and meter multiplier setting) of processed blank, stds, and tissue sample in 1 cm cell at excitation 325 nm and emission, 408 nm. Subtract relative fluorescence of reagent blank from relative fluorescence of all std and sample prepns.

Prep. std curve with reagent blank-corrected relative fluorescence values of processed stds as ordinate and corresponding μg nalidixic acid as abscissa. From std curve, det. amt nalidixic acid ($x$) which corresponds to reagent blank-corrected relative fluorescence of processed tissue sample.

$$\text{ppb (ng/g) Nalidixic acid} = (x \times 1000)/10 \text{ g (tissue wt)}$$

Ref.: JAOAC **53,** 464(1970).

CAS-389-08-2 (nalidixic acid)

## 982.40 Sulfamethazine Residues in Swine Tissues

### Gas Chromatographic–Mass Spectrometric Method

**First Action 1982**
**Final Action 1984**

(Applicable to residues at 0.05–0.20 ppm)

### A. Principle

Sulfamethazine is extd from tissue with $CHCl_3$-acetone. Ext is filtered and solv. is removed by evapn. Residue is redissolved in hexane and partitioned against 1N HCl. Acid phase is neutzd and sulfamethazine is extd with $CH_2Cl_2$. Solv. is removed and residue is methylated using diazomethane. Sulfamethazine is identified and quantitated using electron impact gas chromatgy/mass spectrometry (EIGC/MS) in selected ion mode. Six ions, m/z 92, 98, 227, 228, 233, and 234 are monitored. Ion current from each is accumulated thruout GC run, stored on mag. tape, and plotted as ion current vs time. Peaks appearing in ion current profiles are identified and retention times and areas for each peak are calcd. Sulfamethazine is quantitated from std curve prepd by least squares linear regression using data from analysis of known std solns. Identity of sulfonamide residues is confirmed by presence of significant ions appearing at proper retention time in proper relative abundances.

Procedure gives quant. results as well as data for confirmation of residues detected. Procedure is accurate at 0.1 ppm level with expected coefficient of variation of 4.6%. Min. detectable level is 0.002 ppm. To accommodate residues >0.2 ppm, reconstruct std curve as follows:

| Expected Concn Range | Use Stds (*in ppm*) A | B | C |
|---|---|---|---|
| 0 –0.2 ppm | 0.05 | 0.10 | 0.20 |
| 0.2–2.0 ppm | 0.50 | 1.0 | 2.0 |
| 2.0–20.0 ppm | 5.0 | 10.0 | 20.0 |

### B. Apparatus

(**a**) *Blender*.—Virtis Model 45, or equiv., with 500 mL flasks.

(**b**) *Evaporator*.—N-Evap Model III (Organomation Associates, PO Box 159, South Berlin, MA 01549).

(**c**) *Gas chromatograph–mass spectrometer*.—Hewlett-Packard Model 5992 quadrupole operated in multiple ion monitoring under following conditions: electron energy, 70 eV; electron multipler, 2000–2800 eV; source temp., 140°; integration time, 200 ms/mass monitored. Column: 2 mm id × 3 ft glass, packed with 3% OV-17 on 80–100 mesh Gas-Chrom Q (or equiv. packing). GC conditions: injection port 230°; column 220°, He flow 30 mL/min; GC/MS interface, silicone membrane separator. Total analysis time is ca 17 min. Sulfamethazine retention time is 9–12 min.

### C. Reagents

(**a**) *Solvents*.—Distd in glass, or equiv.: acetone; $CH_2Cl_2$; ether; MeOH (shake with and store over anhyd. $Na_2SO_4$); $CH_3Cl$ (no preservatives).

(**b**) *Trisodium citrate soln*.—Add 720 g trisodium citrate dihydrate to 1 L $H_2O$.

(**c**) *Diazomethane kits*.—Aldrich Chemical Co. No. Z10,159-1 and Z10,025-O. Prep. according to manufacturer's instructions. *Caution:* Prep. diazomethane in hood behind protective screen or shield. Wear gloves to prevent skin contact with reagents. Diazomethane is toxic and under some conditions explosive. Freshly made diazomethane soln is golden yellow. Store in freezer at ≤0°. Storage time may vary with method of prepn. *See* JAOAC **65,** 273–274(1982).

(**d**) *Sulfamethazine stock std soln*.—100 μg/mL. Accurately weigh 10.0 mg sulfamethazine into 100 mL vol. flask. Dissolve in and dil. to vol. with anhyd. MeOH.

(**e**) *Fortification std soln*.—5 μg/mL. Pipet 5.0 mL stock std soln into 100 mL vol. flask and dil. to vol. with $H_2O$. Prep. std fresh weekly.

(**f**) *GC/MS std soln*.—50 μg/mL. Accurately weigh 10.0 mg unlabeled sulfamethazine into 200 mL vol. flask. Dissolve in and dil. to vol. with anhyd. MeOH.

(**g**) *$^{13}$C-Labeled sulfamethazine std soln*.—50 μg/mL. Accurately weigh 10.0 mg $^{13}$C-labeled sulfamethazine into 200 mL vol. flask. Dissolve in and dil. to vol. with anhyd. MeOH.

### D. Extraction and Cleanup

Weigh 25 g (± 0.1 g) ground, frozen tissue into 250 mL centrf. bottle. Select blank sample as control. Fortify second blank sample at 0.2 ppm with 1 mL fortification std soln (5 μg/25 g = 0.2 ppm). Analyze both blanks in parallel with each set.

Add 100 mL acetone, washing any adhering tissue off walls of centrf. bottle; then blend 1 min with tissue grinder. Centrf. 10 min at 2000 rpm. Filter acetone ext thru 24 cm Whatman 2V fluted paper into 500 mL r-b flask. Add 10 mL 5N HCl to acetone ext and place on rotary evaporator in 55 ± 5° bath. Evap. until acetone odor is absent.

Transfer remaining acid phase to 125 mL separator with two 25 mL portions of ether. Gently rotate separator 1 min and let phases sep. 30 min. Transfer acid phase to 100 mL beaker and add 50 mL satd aq. trisodium citrate soln. Adjust pH to 6.0–6.5, using 10N NaOH. Transfer contents of beaker to 250 mL separator, add 30 mL $CH_2Cl_2$, and shake 2 min. Let phases sep. and transfer 10 mL $CH_2Cl_2$ phase to 15 mL centrf. tube. Evap. to dryness under stream of N at 45°.

### E. Thin Layer Chromatographic Screen

Dissolve residue in 0.1 mL MeOH. Aspirate entire sample into 100 μL syringe and place on automatic TLC spotter. Spot sample on TLC plate, along with 10 μL TLC spotting std soln (100 ng). Develop 10 cm at room temp. with $CHCl_3$–tert-butanol (80 + 20). Remove plate from tank and let air-dry 10 min at room temp. Spray plate with 1.0% sodium nitrite in 1.0N HCl. Dry with blow dryer or in 100° oven. Spray with 0.4% NEDA in MeOH. Dry plate as above to produce pink spots. Compare $R_f$ values with std for identification. If TLC results are pos., continue thru GC/MS below.

### F. GC/MS Quantitation/Confirmation

Weigh 50.0 g (± 0.1 g) ground, frozen tissue into 500 mL blender flask. Select blank tissue as control. Fortify second blank tissue sample at 0.1 ppm level with unlabeled sulfamethazine (100 μL GC/MS std). Spike all samples at 0.10 ppm level with $^{13}$C-labeled sulfamethazine std soln (100 μL).

Add 100 mL $CHCl_3$–acetone (1 + 1) to flask. Blend 1 min at low speed. Decant and filter liq. (no vac.) thru 24 cm Whatman 2V fluted paper into 1 L r-b flask.

Repeat extn and filtering twice more. Transfer all tissue to filter paper after third extn.

Rinse flask with ca 25 mL $CHCl_3$–acetone (1 + 1), transfer rinsing to filter paper, and let drain. Rinse filter paper and contents with three 20 mL aliquots of $CHCl_3$–acetone (1 + 1). If combined filtrates are not clear, refilter and wash filter paper with ca 20 mL $CHCl_3$–acetone (1 + 1).

Evap. on rotary evaporator at 55° (± 5°) to oily residue (ca 1–2 mL). Remove from rotary evaporator promptly. Quant. transfer residue to 250 mL separator using, in order, four 25 mL portions of hexane, two 3 mL portions of acetone, and two 25 mL portions of hexane. Add 10 mL 1N HCl to separator. Shake gently 2 min and let phases sep. Emulsions may be eliminated by placing separator in 60° $H_2O$ bath. Repeat extn 3 times with 5 mL portions of 1N HCl, drawing off acid phase and combining filtrates in the 125 mL separator. Add 3.0 mL 10N NaOH to 125 mL separator and mix. Det. pH, using pH paper. If pH is not 12–13, add addnl 10N NaOH with mixing to attain this pH.

Add 25 mL $CHCl_3$ to basic soln and shake 1 min. Let phases sep. completely, and discard $CHCl_3$. Repeat $CHCl_3$ extn a second time, discarding $CHCl_3$. Quant. transfer aq. phase to small beaker (ca 100 mL). Buffer by adding 25 mL satd aq. trisodium citrate. Adjust pH with pH meter to 5.55–5.65 by adding NaOH or HCl as required.

Quant. transfer contents of beaker to 125 mL separator, add 15 mL $CH_2Cl_2$, and shake 1 min. Let phases sep. and transfer $CH_2Cl_2$ to 50 mL conical centrf. tube. Check pH of aq. phase and re-adjust to 5.55–5.65 if necessary. Repeat $CH_2Cl_2$ extn twice.

Evap. contents of centrf. tube to dryness at 45° under stream of N on N-Evap. Dissolve residue in 1 mL anhyd. MeOH. Add 1 mL diazomethane soln, mix with vortex mixer, and let stand at room temp. 5 min. Transfer soln from tube to 15 mL or smaller concentrator tube and evap. to dryness at 45° under stream of N on N-Evap. Dissolve methylated residue in 200 μL anhyd. MeOH.

### *G. Preparation of Standard Curve*

Add 100 μL $^{13}$C-sulfamethazine std soln (50 μg/mL) to each of three 15 mL concentrator tubes. Label tubes A, B, and C. Add 50 μL GC/MS std soln (50 μg/mL) to tube A (equiv. to 0.05 ppm $^{12}$C). Add 100 μL GC/MS std soln (50 μg/mL) to B (equiv. to 0.10 ppm $^{12}$C). Add 200 μL GC/MS std soln (50 μg/mL) to tube C (equiv. to 0.20 ppm $^{12}$C). Add 1 mL freshly prepd diazomethane soln to each concentrator tube and mix with vortex mixer, let stand at room temp. 5 min. Evap. to dryness at 45° under stream of N on N-Evap. Dissolve each methylated residue in 200 μL anhyd. MeOH.

Set selected ion monitor (SIM) data acquisition program area as follows:

a Mass 1 = 227, dwell time = 200 ms
b Mass 2 = 228, dwell time = 200 ms
c Mass 3 = 233, dwell time = 200 ms
d Mass 4 = 234, dwell time = 200 ms
e Mass 5 = 92, dwell time = 200 ms
f Mass 6 = 98, dwell time = 200 ms
g Solvent elution time = 1.1 min

Make injections in order given below.

a Inject 2.0 μL std A
b Inject 2.0 μL std B
c Inject 2.0 μL std C

For each std injection, det. ratio of areas of m/z 227 peak to area of m/z 233 peak. Using method of least squares, calc. std curve for 227/233 mass ratio vs amt of unlabeled sulfamethazine added to each std (amt may be expressed as ppm based on 50 g sample). Similarly for each std, calc. following confirmation ratios: 228/227 and 234/233. Mean 228/227 and 234/233 ratios computed from 3 std injections will be used for confirmation of identity of sulfamethazine detected in processed samples.

### *H. Determination*

With SIM program set as for *Preparation of Standard Curve*, inject 2.0 μL from each sample to be analyzed. Plot reconstructed ion current profiles at end of each run and calc. 227/233 ion mass ratio. Read sulfamethazine content of sample from std curve.

For identification purposes: (a) Sample unlabeled sulfamethazine must co-elute with added $^{13}$C-labeled sulfamethazine. (b) The m/z 92, 277, and 228 from unlabeled sulfamethazine and m/z 98, 233, and 234 from $^{13}$C-labeled sulfamethazine must all be present. (c) Ratios of 228/227 and 234/233 ions in sample should be within 10% of mean ratio detd for stds.

Refs.: JAOAC **64,** 1386(1981).

CAS-57-68-1 (sulfamethazine)

## 982.41 Sulfamethazine Residues in Swine Tissues
### Gas Chromatographic Method
**First Action 1982**
**Final Action 1984**

(Applicable to residues ≥0.1 ppm)

### *A. Principle*

Tissue is extd with acetone–$CHCl_3$, 1N HCl is added, and solv. is evapd. Aq. soln is washed with hexane, pH is adjusted to 5.55–5.65, and sulfamethazine is extd with $CH_2Cl_2$, methylated with diazomethane, and detd by electron capture GC.

### *B. Reagents and Apparatus*

Rinse all clean glassware thoroly with MeOH and let dry. Use distd in glass solvs suitable for pesticide analyses (Burdick & Jackson Laboratories, Inc., or equiv.).

(**a**) *Sulfamethazine std solns.*—Prep. std solns contg 1, 2, and 10 μg sulfamethazine USP/mL acetone.

(**b**) *Diazomethane derivatizing reagent.*—*Caution*: Diazomethane is toxic, can cause specific sensitivity, and is potentially explosive. Prep. diazomethane reagent, methylate, and evap. in hood. Avoid ground glass joints, etched or scratched glassware, and sharp edges. Store diazomethane solns in freezer; do not expose to direct sunlight or strong artificial light. Prep. diazomethane by etheral basic distn of 21.5 g *N*-methyl-*N*-nitro-*p*-toluenesulfonamide (Diazald, Aldrich Chemical Co.), with 200 mL ether as described in Diazald kit, Cat. No. Z10, 025-0. (*Note:* Read Diazald kit instructions carefully for safe handling of diazomethane.) After distn of second portion (40 mL) of ether, transfer ether soln of diazomethane thru funnel to narrow-mouth bottle and cap tightly with a polyseal cap; store in freezer (*see* JAOAC **65**, 273–274(1982)). When stored in freezer, soln retains its efficiency as methylating agent ≥1 month.

(**c**) *Gas chromatograph.*—Tracor Model 222, or equiv., with $^{63}$Ni linearized electron capture detector and 6 ft × 2 mm id glass column packed with 5% OV-7 on 100–120 mesh Gas-Chrom Q (or equiv. packing). Operating conditions: injector 290°, column oven 260°; detector 290°; argon–methane (90 + 10) carrier gas at 30 mL/min; detector purge flow 20 mL/min. Retention time for methylated sulfamethazine is 4–4.5 min. Alternative column: 6 ft × 4 mm id, packed with 5% OV-25.

(**d**) *Food chopper.*—Model 84142D (Hobart Manufacturing Co., 711 Pennsylvania Ave, Troy, OH 45374), or equiv.

(**e**) *Flasks.*—100 mL pear-shaped, 24/40 joint (Kontes Glass Co. No. K-608700).

### *C. Procedure*

Cut tissue into 1.5 cu. in. pieces and freeze in plastic bags. Pulverize enough dry ice in bowl of Hobart food chopper to chill bowl and grater thoroly. Slowly add small portions (ca 50 g) of sample and continue chopping until complete sample is chopped. If necessary, add more dry ice to maintain sample in frozen state during chopping procedure. Store sample in a freezer (−20°F) until dry ice has dissipated.

Transfer 15 g sample to blender and blend 5 min at medium speed with 150 mL acetone–$CHCl_3$ (1 + 1). Filter thru glass-fiber paper, collecting first 100 mL filtrate in 100 mL graduate. Transfer 100 mL aliquot (equiv. to 10 g sample) to 250 mL r-b flask with 24/40 joint and add 10 mL 1N aq. HCl. Evap. org. solvs on rotary vac. evaporator with flask submerged in 32 ± 4° $H_2O$ bath. (For muscle and fat, some extd fat will prevent complete removal of solvs.) Add 50 mL *n*-hexane to 1N HCl phase and quant. transfer both phases to 125 mL separator. Rinse evapn flask with addnl 5 mL 1N HCl and transfer rinse to 125 mL separator. Shake contents of separator gently by inverting funnel and returning to upright position once a second for 50 s. Let funnel sit until phases sep. (ca 10 min). Draw off lower phase (1N HCl) into second 125 mL separator. (Centrifugation may be required to avoid transfer of emulsified solv. which tends to bump during hydrolysis step.) Rinse r-b flask with 5 mL 1N HCl, transfer to first separator, contg hexane, mix, and sep. as above. Drain HCl phase into second separator and discard hexane. For high fat samples only, add 15 mL $CH_2Cl_2$ to HCl phase in second separator, shake 30 s, and let phases sep. Draw off and discard lower $CH_2Cl_2$ phase.

Buffer aq. HCl ext in separator by adding 25 mL satd aq. trisodium citrate; then adjust pH with pH meter to 5.55–5.65 by adding 3N NaOH (ca 2.5 mL). Add 15 mL $CH_2Cl_2$ to separator and shake vigorously for 90 s. Let phases sep. and transfer lower $CH_2Cl_2$ layer to 100 mL pear-shaped flask. After first extn, check pH of aq. phase and re-adjust to 5.55–5.65 if necessary. In similar manner, ext with 3 addnl portions of $CH_2Cl_2$, combining exts in 100 mL pear-shaped flask. Evap. solv. in rotary vac. evaporator with $H_2O$ bath at 25–30°. Do not exceed 30°. Sample may be kept overnight at this stage.

Dissolve residue in 1.0 mL acetone. Swirl flask to dissolve any residue on walls of flask. In fume hood, add 1 mL diazomethane derivatizing reagent and let stand 15 min with intermittent gentle swirling. Evap. solv. under gentle stream of N. Dissolve residue in 1.0 mL acetone.

Prep. methylated stds by pipetting 1.0 mL aliquots of each sulfamethazine std soln into sep. 100 mL pear-shaped flasks. Add 1 mL diazomethane derivatization reagent and treat in same manner as sample.

### D. Gas Chromatography

Inject 2–8 µL methylated 1 µg/mL sulfamethazine std into gas chromatograph. (Resulting peak ht should be 30–40% FSD.) Inject up to 3 samples followed by std which approx. matches sample. If sample peak goes off scale, quant. dil. methylated sample soln with acetone to give response that is 30–60% FSD. Correct results for diln.

$$\text{Sulfamethazine, ppm} = 1.5\ (A \times C \times V')/(A' \times W \times V)$$

where $A$, $A'$ = peak area of sample and std, resp.; $V$, $V'$ = GC injection vol. (µL) of sample and std, resp.; $C$ = concn of std (µg sulfamethazine/mL); $W$ = wt sample (g); 1.5 = 150/100 to correct for 100 mL aliquot of 150 mL sample ext taken for analysis. Method reliably quantitates sulfamethazine at 0.1 ppm and above.

Ref.: JAOAC **64,** 1386(1981).

CAS-57-68-1 (sulfamethazine)

## 983.31 Sulfonamide Residues in Animal Tissues

### Thin Layer Chromatographic Screening Method

**First Action 1983**
**Final Action 1984**

(Applicable to swine, turkey, and duck tissues)

### A. Principle

Sulfonamides are extd with ethyl acetate after addn of sulfapyridine as internal std. Exts are cleaned up by partitioning between org. and aq. solvs, and chromatographed on silica gel TLC plates. Developed plates are treated with fluorescamine and scanned by fluorescence densitometer.

### B. Reagents

(**a**) *Ethyl acetate, hexane, methylene chloride, and methanol.*—Distd in glass (Burdick & Jackson Laboratories, Inc.).

(**b**) *Glycine buffer soln.*—Prep. glycine (Fisher Scientific Co.) as 0.2M aq. soln and adjust pH to 12.25 with NaOH.

(**c**) *Fluorescamine derivatizing soln.*—Dissolve 25 mg fluorescamine (Pierce Chemical Co.) in 250 mL acetone. Replace soln after treating 8–9 plates.

(**d**) *Sulfonamide stds.*—Com. sulfamethazine (SMZ), sulfadimethoxine (SDM), sulfaquinoxaline (SQX), sulfathiazole (STZ), sulfabromomethazine (SBR), and sulfapyridine (SPY).

(**e**) *Stock std solns.*—Dissolve 100 mg sulfonamide in 100 mL acetone. Store in refrigerator.

(**f**) *Fortification std solns.*—Combine sulfonamides of interest and dil. to 5.00, 2.50, and 1.25 µg/mL (equiv. to 0.2, 0.1, and 0.05 ppm in tissue), using 0.05M pH 7.5 phosphate buffer. All solns should contain 2.50 µg SPY/mL. Store fortification stds in refrigerator and prep. weekly.

(**g**) *Internal std soln.*—Using stock std soln (**e**), prep. 2.50 µg SPY/mL 0.05M pH 7.5 phosphate buffer.

### C. Apparatus

(**a**) *Densitometer.*—CAMAG TLC/HPTLC scanner (Applied Analytical Industries, Rt 6, PO Box 55, Wilmington, NC 28405) equipped with 400 nm interference filter on excitation source. Replace std 400 nm cutoff filter on photomultiplier by 500 nm interference filter. Slit dimensions 7.8 × 0.3 mm. Scan plates at either 1 or 2 mm/s.

(**b**) *TLC plates.*—20 × 20 cm channeled plates, channels 8 mm wide with 0.25 mm silica gel layer and pre-adsorbent spotting zones (No. 4865-821, Whatman, Inc.).

(**c**) *TLC spotting capillaries.*—20 µL glass capillary tubes (Corning Glass Works).

(**d**) *Heat strip.*—Automatic spotter heat strip set at 85° (Analytical Instrument Specialties, Inc., PO Box 596, Libertyville, IL 60048). Any equiv. heating device with temp. control can be substituted.

(**e**) *TLC tank.*—Std 2-trough tank (Alltech Associates, 2015 Waukegan Rd, Deerfield, IL 60015), atm. fully satd by lining with satn pads.

(**f**) *Derivatization tank.*—Stainless steel (Thomas Scientific).

(**g**) *Evaporator.*—N-Evap (Organomation Associates, Inc., PO Box 159, South Berlin, MA 01549).

(**h**) *Homogenizer.*—Tekmar SDT Tissumizer (Tekmar Co., PO Box 371856, Cincinnati, OH 45222).

(**i**) *Shaker.*—Horizontal reciprocating shaker set at ca 240 cycles/min (Eberbach Corp., PO Box 1024, Ann Arbor, MI 48106-1024).

(**j**) *Centrifuge.*—Set at 2500 rpm for 5 min (Model PR-7000, International Equipment Co., or equiv.).

(**k**) *Polypropylene centrifuge tubes.*—50 mL capacity (Corning Glass Works).

### D. Sample Extraction

Accurately weigh ca 2.5 g homogenized liver or muscle into 50 mL centrf. tube. Add 100 µL internal std soln. Prep. 3 control samples (using tissue known to be free of sulfonamides) and fortify with internal std (0.1 ppm) and each sulfonamide of interest, one control each at 0.05, 0.10, and 0.20 ppm. Wait 15 min and then add 25 mL ethyl acetate. Blend muscle samples 1 min with Tissumizer, and centrf. For liver samples, cap tube tightly, shake 20 min on horizontal shaker, and centrf. Transfer ethyl acetate to clean tube and discard tissue. Add 10 mL glycine buffer to ext, mech. shake 5 min, and centrf. Vac.-aspirate and discard org. phase. Adjust pH of aq. phase to 5.2–5.3 by adding 2 mL (1 + 1) mixt. of 2M pH 5.25 phosphate buffer and 1.7M HCl. Check pH and make final adjustments with either addnl buffer or 0.1N NaOH. Add 5 mL hexane, mech. shake 5 min, and centrf. Aspirate and discard hexane phase. Remove any solid or emulsified material remaining at interface. Add 10 mL $CH_2Cl_2$, shake 5 min, centrf., and aspirate and discard aq. phase. Add 10 µL diethylamine to $CH_2Cl_2$ ext and conc. just to dryness under stream of N at 40°. During evapn, occasionally rinse tube walls with $CH_2Cl_2$. Redissolve residue in 100 µL MeOH and mix 30 s on vortex mixer. Let stand 5 min before chromatgy to let insoluble oils settle.

### E. Chromatography

Apply 20 μL portion of sample to pre-adsorbent spotting zone of TLC plate. Avoid using lane at each side of plate, and spot the 3 fortified control samples at intervals across plate to minimize effects of across-plate variation. Develop plate 1 cm in MeOH followed by 2 developments, 6 cm and 12 cm, in $CHCl_3$–*tert*-BuOH (80 + 20). Between each development, dry plate 1 min at 110°. If STZ is suspected or present, prewash $CHCl_3$–*tert*-BuOH with $H_2O$. Maintain development tank temp. at 30–33° for optimum resolution of multiple sulfonamides. Derivatize compds by quickly dipping plate in fluorescamine soln. Bands are visible after 15–30 min. Scan each lane and obtain its response scanning curve. For each sample and std (each lane), det. ratio of response for sulfonamide of interest to response for internal std (SPY).

### F. Calculations

For each sulfonamide, calc. slope and intercept of std curve, concn vs response ratio, using linear regression and results for the 3 fortified samples. Plotting sulfonamide concn on $y$-axis results in std error of est. ($S_{y.x}$) having dimension of ppm, thus simplifying estn of confidence intervals. For quality assurance purposes, $S_{y.x}$ should be $\leq 0.02$ ppm, and correlation coefficient, $r$, should be $\geq 0.995$. Use linear regression slope and intercept to calc. concn of samples from their respective sulfonamide/internal std peak ht ratios.

Ref.: JAOAC **66,** 884(1983).

CAS-116-45-0 (sulfabromomethazine)
CAS-122-11-2 (sulfadimethoxine)
CAS-57-68-1 (sulfamethazine)
CAS-144-83-2 (sulfapyridine)
CAS-59-40-5 (sulfaquinoxaline)
CAS-72-14-0 (sulfathiazole)

## 966.26 Zoalene Residues in Animal Tissues

**Spectrophotometric Method**
**First Action 1966**
**Final Action 1967**

### A. Principle

Ground tissue is extd with acetone, and benzene is added to sep. soln into 2 layers. Org. layer is concd, and passed thru alumina column. Absorbed zoalene is washed with $CHCl_3$ and eluted with 80% alcohol. Alc. soln is evapd just to dryness and residue dissolved in alc. DMF soln. Colored complex formed by addn of 1,3-diaminopropane is measured at 560 nm.

### B. Apparatus

*See* **961.23B(a)**, **(b)**, and **(d)**.

### C. Reagents

**(a)** *Acetone-benzene soln.*—Mix 35 parts acetone with 65 parts benzene.
**(b)** *Alumina.*—*See* **961.23C(a)**.
**(c)** *1,3-Diaminopropane.*—Aldrich Chemical Co.
**(d)** *Dimethylformamide-alcohol soln.*—Mix 4 parts DMF with 1 part absolute ethanol.
**(e)** *Zoalene.*—Anal. std. (Available from Agricultural Dept, Dow Chemical Co.)

### D. Preparation of Alumina Column

*See* **961.23D**.

### E. Preparation of Standard Curve

Accurately weigh 100 mg zoalene into 1 L vol. flask, dissolve in 50 mL acetone, and dil. to vol. with $H_2O$. Dil. 10 mL of this stock soln to 100 mL with $H_2O$ to give working soln of 10 μg/mL. Pipet 0, 2, 4, 6, 8, and 10 mL aliquots working soln into sep. 100 mL beakers and evap. just to dryness under heat lamp. Add 5 mL alc. DMF soln to each beaker and stir 1–2 min. Add 5 mL 1,3-diaminopropane. After 10 min, measure $A$ of soln at 560 nm, using 1 cm cells against $H_2O$ as ref. Prep. std curve by plotting $A$ against μg zoalene.

### F. Determination

(*Caution:* See safety notes on blenders, distillation, flammable solvents, toxic solvents, benzene, and acetone.)

Collect tissue, freeze with solid $CO_2$, and keep frozen until analyzed. Grind tissue while at least partially frozen and weigh 50 g into 1 qt (1 L) Mason jar. Add 250 mL acetone and mix with high-speed mixer ca 5 min. Filter on buchner into 1 L filter flask, using 11 cm paper and ca 5 g Super-Cel as filter pad. Wash residue with 100 mL acetone, collecting washings in same flask. Transfer filtrate to 1 L separator and add 500 mL benzene. (*Caution*: See safety note on benzene.)

Vigorously shake ext in separator and let stand until layers sep. Swirl funnel and let stand again until layers sep. Drain aq. layer into 250 mL centrf. bottle. Transfer org. layer to 1 L beaker. Rinse separator with 100 mL acetone-benzene soln and add to centrf. bottle. Stopper, shake vigorously, and centrf. 20 min at ca 1700 rpm. Remove lower layer with suction tube and transfer org. layer to 1 L beaker.

Evap. to 10 mL under heat lamp with air current. Add 100 mL $CHCl_3$ and evap. to 50 mL. If soln is not clear, repeat addn and evapn of $CHCl_3$ to remove $H_2O$.

Add clear $CHCl_3$ soln to alumina column and drain to level of alumina. Wash with four 50 mL portions $CHCl_3$. Discard washings. Add 90 mL 80% alcohol to column to elute zoalene. Discard first 30 mL effluent and collect 60 mL in 100 mL beaker. Evap. soln under heat lamp with air current until residue no longer flows. Do not heat residue after beaker is dry.

Add 5 mL alc. DMF soln to beaker and warm with stirring to ca 45° to dissolve residue. When completely in soln, add 5 mL 1,3-diaminopropane to develop color. Filter thru small fluted paper. After 10 min, measure $A$ of soln at 560 nm, using 1 cm cells against $H_2O$ as ref.

### G. Calculations

Obtain μg zoalene corresponding to $A$ from std curve.

$$\text{ppm Zoalene in sample} = \mu\text{g zoalene/g sample}$$

Refs.: J. Agric. Food Chem. **9,** 201(1961). JAOAC **49,** 708 (1966).

CAS-148-01-6 (zoalene)

**Common and Chemical Names of Drugs in this Chapter**

| Common Name | Chemical Name |
|---|---|
| Clopidol | 3,5-Dichloro-2,6-dimethyl-4-pyridinol |
| Decoquinate | 6-(Decyloxy)-7-ethoxy-4-hydroxy-3-quinolinecarboxylic acid ethyl ester |
| Ethoxyquin | 6-Ethoxy-1,2-dihydro-2,2,4-trimethyl-quinoline |
| Melengestrol (acetate) | 17-Hydroxy-6-methyl-16-methylene-pregna-4,6-diene-3,20-dione |
| Nalidixic acid | 1-Ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid |
| Sulfabromomethazine | 4-Amino-*N*-(5-bromo-4,6-dimethyl-2-pyrimidinyl)-benzenesulfonamide |
| Sulfadimethoxine | 4-Amino-*N*-(2,6-dimethoxy-4-pyrimidinyl)-benzenesulfonamide |
| Sulfamethazine | 4-Amino-*N*-(4,6-dimethyl-2-pyrimidinyl)-benzenesulfonamide |
| Sulfapyridine | 4-Amino-*N*-2-pyridinyl-benzenesulfonamide |
| Sulfaquinoxaline | 4-Amino-*N*-2-quinoxalinyl-benzenesulfonamide |
| Sulfathiazole | 4-Amino-*N*-2-thiazolyl-benzenesulfonamide |
| Zoalene | 2-Methyl-3,5-dinitrobenzamide |

Sources: *USAN and the USP Dictionary of Drug Names* (1983) U.S. Pharmacopeial Convention, Rockville, MD; *The Merck Index* (1983) 10th ed., Merck & Co., Inc., Rahway, NJ.

# 24. Forensic Sciences

**Stanley M. Cichowicz, *Associate Chapter Editor***
*Bureau of Engraving & Printing*

### 974.35 Detection of Fingerprints (Latent) on Objects

**Powder Brushing Method**
**First Action 1974**
**Final Action 1975**

(Applicable to development of latent images deposited on nonporous surfaces within 120 hr and not subjected to extreme temp. or humidity changes)

Pour small amt fingerprint powder (Hi-Fi Volcano, Sirchie Fingerprint Laboratories, Inc., Gravelly Hollow Rd, Medford, NJ 08055, or equiv.) into shallow dish or onto piece of paper. Pick up small amt with end of camel hair brush. Hold brush over surface to be dusted, and tap handle lightly to permit powder to drift onto surface. Brush surface lightly until image begins to appear. Continue with light strokes, following ridge direction in pattern as it forms. Apply addnl powder, if necessary, to obtain good contrast, but retain ridge detail. When sufficient detail and contrast are obtained, remove excess powder with ostrich feather duster. Preserve image by photographing and then taping over, leaving tape in place if practical. If not, pull off tape slowly and evenly, and place on 3 × 5″ card of contrasting color.

Refs.: JAOAC **57,** 662(1974); **58,** 126(1975).

### 976.28 Detection of Fingerprints (Latent) on Papers

**Chemical Development Method**
**First Action 1976**

(Applicable to forced development of latent images deposited on bond and newsprint papers)

#### A. Apparatus and Reagent

(**a**) *Steam iron.*—With heat indicator.
(**b**) *Ninhydrin soln.*—0.5%. Dissolve 0.5 g 1,2,3-indantrione.$H_2O$ in 100 mL acetone, mix, and let stand 15 min.

#### B. Pretreatment

(If evidence contains handwriting, printing, or typewriting to be examined by document examiner, protect area by brushing pretreatment, allowing for migration of ninhydrin soln. In general, spray large objects.)

Perform one of following pretreatments on papers:
(**a**) *Dipping.*—Pour small vol. ninhydrin soln into flat dish, pick up paper with tweezers, submerge paper in soln until satd, hold paper above dish, letting excess liq. drip into dish, and place paper on clean blotter to air dry.
(**b**) *Brushing.*—With paint brush or cotton swab on wooden stick, pick up small vol. ninhydrin soln from flat dish, paint paper until surface is coated, transfer to clean blotter, and let air dry.
(**c**) *Spraying.*—Pour ca 30 mL ninhydrin soln into spraying cannister. Use com. compressed air or inert gas, or compressed air from laboratory line as propellant. Place papers in exhaust hood or similar cabinet. Hold spraying cannister upright ca 8–10″ (20–25 cm) from paper and spray until paper is coated. Transfer paper to blotter and let air dry.
Discard ninhydrin soln in flat dish. Return ninhydrin spraying soln to supply bottle for future use.

#### C. Development

Fill steam iron with $H_2O$ and turn heat indicator to "Steam". Place papers on clean blotter. When steam is being ejected from sole plate holes, hold iron ca 1″ (2.5 cm) above papers and move iron around to distribute heat and steam evenly. Vary ht above paper with rate steam is being projected beneath iron. Steam should just reach papers before rising to sole plate. Wipe sole plate frequently to prevent condensate from dripping on paper. Images will begin to develop in ca 45 sec and continue to intensify until plum or purple color is obtained. Move paper to clean blotter and let air dry; as alternative, after steam development of prints, shut off steam, wipe bottom of iron free of moisture, and then lightly pass iron over developed prints to enhance images. (Use caution not to burn or scorch papers.) Continue with each paper until all have been treated. When papers are dry, place each in appropriately labeled envelope or plastic protector, handling with tweezers.

Ref.: JAOAC **59,** 1003(1976).

### 973.65 Characterization and Matching of Glass Fragments

**Dispersion Microscopy (Double Variation Method)**
**First Action 1973**
**Final Action 1974**

#### A. Principle

Refractive indices ($n$) of glass and stdzd liqs are matched at different wavelengths by varying temp. of mixt. The $n$ of glass remains relatively const with temp. change; those of liqs decrease with increasing temp. Plot of wavelength, where $n$ of glass and liq. match, against temp. for specific stdzd liq., is characteristic of particular glass.

#### B. Apparatus

(**a**) *Microscope.*—Compd, transmitted light type, with illumination system capable of restricted substage aperture or, preferably, phase contrast optics which permits easier matching, with provision for long working distance.
(**b**) *Hot stage.*—Capable of attaining, holding, and indicating temp. accurately (±0.1°) and permitting use of required illumination (Model FP-5 (replacement Model FP-82), Mettler Instrument Corp., or equiv.).
(**c**) *Monochromatic light source.*—Accurately calibrated (±1 nm at 486) with sufficient intensity in small beam of low angular aperture over range at least 460–680 nm (continuous interference filter, 400–700 nm, band width ca 15 nm, No. 50 09 00, Carl Zeiss West Germany, PO Box 1369/1380, D-7082, Oberkochen, West Germany, is satisfactory).
(**d**) *Graph paper.*—Hartmann net (linear temp., nonlinear wavelength resulting in straight line) preferable; available from Walter C. McCrone Associates, Inc., 2820 S Michigan Ave, Chicago, IL 60616. If unavailable, use ordinary graph paper.

### C. Reagents

(**a**) *Refractive index liquids*.—Calibrated immersion liqs, range 1.50–1.65 (R. P. Cargille Laboratories, Inc., 55 Commerce Rd, Cedar Grove, NJ 07009), or NIST SRM 1823 silicone liqs. Use high dispersion set with most glasses.

(**b**) *Collodion soln*.—Dil. 1 mL 3% collodion, flexible (Fisher Scientific Co., No. C-409), to 100 mL with amyl acetate.

### D. Preparation of Samples

(All particles examined must be ≤100 μm in major dimension.)

(**a**) *Single small flake*.—Arrange 3 small (2–3 mm) cover slip flakes at corners of ca 1 cm triangle near end of 5/8 × 3″ microscope slide and cement in place by placing drop of dild collodion soln outside of triangle and drawing soln to each fragment in turn with fine tungsten needle. With very small drop of dild collodion soln, cement test fragment on slide within triangle, near 1 of fragments as locator. Avoid touching collodion cementing cover slip fragments. Second fragment can be cemented close by for simultaneous and direct comparison. Excessive collodion interferes with index readings; if necessary, thin collodion around test fragment with amyl acetate. Place cover slip on the 3 supports, and place small drop of liq. of known $n$ at edge to be drawn around fragments by capillary attraction. After $n$ measurement, clean slide, if necessary to change liq., by removing cover slip and rinsing tilted slide held over waste containers with drops of benzene added from dropper.

(**b**) *Many small flakes*.—Obtain many small flakes by crushing in anvil-striker type hammer mill. Place few flakes on slide without cementing, cover with cover slip, and add small drop of liq. at edge of cover slip. New prepn may be used for each liq. or slide may be cleaned by removing cover, pushing particles to side with razor cut edge of filter paper, adding drop of new liq., pushing again to side, and adding drop of fresh liq.

### E. Determination

Choose immersion liq. which matches $n$ of glass at far red end of spectrum at temp. slightly above room temp., ca 30°. With such match, all subsequent temp.-wavelength matches will be <60°, requiring small temp. corrections. Use narrow beam of light parallel to optic axis passing thru closed down substage aperture, but open enough to see Becke lines. (There will be best iris setting for each wavelength.) Place mounted sample on hot stage set at lowest even degree temp. at which wavelength match is observed (ca 660 nm). Darkened room and intense illumination are advantageous. Record av. of several matching wavelengths for that temp. Set temp. at successively higher even degree intervals, let equilibrate 30 sec, and read wavelength. Repeat match several times and det. av. Continue increasing temp. until no match can be obtained (ca 450 nm). Let hot stage cool, and recheck 1–2 of lower temp. matches. Difference indicates change in liq. at high temp. and requires repeat of second half of data at higher temp. with fresh liq. Rate should be 1 data point/min. Plot data on Hartmann net or ordinary graph paper.

### F. Calculations

From graph, read temp. corresponding to 486.1 (F), 589.3 (D), and 656.3 (C) nm. From Table **973.65**, obtain $n$ of liq. used at these wavelengths and temp. coefficient to be used in correcting table values at 25° to actual matching temp. Report matching temp. and corrected refractive index ($n^1$) for each of the 3 wavelengths specified.

**Table 973.65 Refractive Indices and Temperature Coefficients of Cargille Liquids**

| $n_F^{25}$ | $n_D^{25}$ | $n_C^{25}$ | d$n$/dt (25–35°) |
|---|---|---|---|
| | A Series | | |
| 1.46594 | 1.460 | 1.45762 | 0.00037 |
| 1.47666 | 1.470 | 1.46735 | 0.00037 |
| 1.48739 | 1.480 | 1.47709 | 0.00037 |
| 1.49812 | 1.490 | 1.48682 | 0.00038 |
| 1.50884 | 1.500 | 1.49656 | 0.00038 |
| 1.51957 | 1.510 | 1.50629 | 0.00038 |
| 1.53030 | 1.520 | 1.51603 | 0.00038 |
| 1.54103 | 1.530 | 1.52576 | 0.00038 |
| 1.55175 | 1.540 | 1.53550 | 0.00039 |
| 1.56248 | 1.550 | 1.54523 | 0.00039 |
| 1.57321 | 1.560 | 1.55497 | 0.00039 |
| 1.58393 | 1.570 | 1.56470 | 0.00039 |
| 1.59466 | 1.580 | 1.57444 | 0.00040 |
| 1.60539 | 1.590 | 1.58417 | 0.00040 |
| 1.61611 | 1.600 | 1.59391 | 0.00040 |
| | High Dispersion Series | | |
| 1.5134 | 1.500 | 1.4943 | 0.00045 |
| 1.5247 | 1.510 | 1.5039 | 0.00046 |
| 1.5360 | 1.520 | 1.5134 | 0.00046 |
| 1.5451 | 1.530 | 1.5242 | 0.00047 |
| 1.5557 | 1.540 | 1.5339 | 0.00047 |
| 1.5664 | 1.550 | 1.5437 | 0.00047 |
| 1.5770 | 1.560 | 1.5534 | 0.00047 |
| 1.5877 | 1.570 | 1.5632 | 0.00047 |
| 1.5983 | 1.580 | 1.5729 | 0.00047 |
| 1.6090 | 1.590 | 1.5827 | 0.00047 |
| 1.6196 | 1.600 | 1.5924 | 0.00047 |
| 1.6303 | 1.610 | 1.6021 | 0.00047 |
| 1.6410 | 1.620 | 1.6119 | 0.00047 |
| 1.6516 | 1.630 | 1.6216 | 0.00047 |
| 1.6623 | 1.640 | 1.6314 | 0.00047 |

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*Example:* Using Cargille liq. 1.520:

| Wavelength | Matching temp. from graph | n at this wavelength (Table ***973:65***) at 25° | Calcd n at matching temp. |
|---|---|---|---|
| 486 | 42.8 | 1.53030 | 1.52354 |
| 589 | 33.6 | 1.52000 | 1.51673 |
| 656 | 29.6 | 1.51603 | 1.51428 |

Calcd $n$ is obtained by using d$n$/dt = 0.00038/° in equation:

$$n^1 = n_{25} - (\text{matching temp.} - 25)(\mathrm{d}n/\mathrm{dt})$$
$$n_{486} = 1.53030 - (42.8 - 25)(0.00038)$$
$$= 1.53030 - 0.00676 = 1.52354$$

Repeat for 589 and 656 nm.

Refs.: JAOAC **56,** 1223(1973); **57,** 668(1974). McCrone, W. C., and Delly, J. G., "The Particle Atlas," 2nd ed., Vol. 4, Ann Arbor Science Publishers, PO Box 1425, Ann Arbor, MI 48106 (1973), Table: Optical Constants for Cargille Refractive Index Liquids.

## 981.23 Mineral Wool Insulation

**Comparison of Properties**

**First Action 1981**

### A. Apparatus and Reagents

(**a**) *Microscopes*.—(*1*) Phase contrast, with provision for long working distances; (*2*) Low power inspection type.

(**b**) *Illuminators.*—White and UV (253.7 nm) incident light sources.

(**c**) *Monochromator.*—*See* **973.65B(c)**; or for transmitted light illumination.

(**d**) *Hot stage.*—*See* **973.65B(b)**.

(**e**) *Graph paper.*—*See* **973.65B(d)**

(**f**) *Annealing oven.*—650°. Controlled temp. muffle furnace.

(**g**) *Refractive index liquids.*—*See* **973.65C(a)**.

### *B. Microscopic Examination*

Compare known and unknown source materials under low power microscope in incident light to det. color of resin, if any, and disposition of resin on fibers (e.g. evenly coated, in globs, etc). Compare diams of fibers, and relative abundance of slugs and shot.

### *C. Annealing*

Place known source and unknown source fibers and slugs in porcelain crucibles with covers and heat to 650° (1200° F) in controlled temp. muffle furnace. Hold at 650° $\geq$10 min; then lower temp. at rate of ca 28° (50° F)/30 min to ca 365° (690° F) when rate of cooling becomes immaterial.

### *D. Comparison of Properties*

(**a**) *Fluorescence.*—If enough material is available, compare fluorescence of annealed fibers under UV light, (**b**). Note color and brightness of fluorescence.

(**b**) *Solubility in acid.*—Place annealed fibers on glass slide under low power microscope, add drop HCl (1 + 3), and note solubility. Repeat, if indicated, with concd HCl.

(**c**) *Optical properties.*—Place representative fibers and slugs from annealed known and unknown source materials on microscope slides under cover slips. Introduce refractive index liq., (**g**), under cover slip. Insert on hot stage and adjust temp. until fibers essentially disappear when viewed thru phase microscope and monochromator set near red end of visible spectrum (near 656 nm). Note temp. and monochromator setting. Increase temp. in 5° (9° F) increments to find $\geq$3 match points of glass and oil within 656–486 nm range. Plot results on graph paper, (**e**), on which oil dispersion curves have been plotted and calibrated. If variations along fibers or among fibers and slugs have been observed, curve for material is best expressed by band covering all match points observed.

Because of wide variation of *n* among mineral wool insulations, it may be necessary to make several trials before appropriate oil is found. If fibers are clearly visible in all wavelengths of light at temps between 35 and 90°, choose different oil. Repeat trial-and-error procedure until oil is found in which fibers match oil at 3 different wavelengths beween 656 and 486 nm.

Compare properties of known and unknown source materials.

Refs.: JAOAC **60,** 772(1977); **62,** 792(1979).

## 975.52 Voice Print Identification

**Sound Spectrographic Method**

**First Action 1975**
**Final Action 1976**

### *A. Principle*

Voice print method of speaker identification consists of aural and visual comparison of one or more known voices to unknown or questioned voice. Aural examination dets if acoustic properties of known and unknown voices exhibit preponderance of similarities or differences. Visual analysis with speech spectrograms compares spectrographic features of similar sounds in both known and unknown voices.

### *B. Apparatus*

(**a**) *Sound spectrograph.*—Model 4691 or 700 from Voice Identification, Inc., PO Box 714, Somerville, NJ 08876, or equiv.

(**b**) *Tape recorders.*—*(1)* Model 110 Sony cassette (ac capability, 115–120 v, 60 Hz; frequency response, 50–10,000 Hz), or equiv. *(2)* Multitrack, for aural evaluation (optional).

(**c**) *Patch cord.*

(**d**) *Spectrograph paper.*

### *C. Determination*

Record unknown and known voices onto spectrograph. Use patch cord to record speech samples from cassette recorder to spectrograph. Adjust signal peaks to zero volume units (VU) for proper record level, and monitor recording.

Listen to known and questioned voices until familiar with context of each call and each speaker's voice. Set spectrograph modes for normal (bar), wide-band, expanded linear frequency scale with high shaping. Adjust scan playback level to zero VU for production of each spectrogram. Label unknown spectrogram by call number. Label known spectrogram by name given for each speaker. Properly label all speech sounds produced on each spectrogram.

Aurally compare questioned and known voices, using multitrack recorder, or 2 recorders. Visually examine similar sounds between unknown and known voices as displayed in speech spectrograms. Conduct aural and visual examinations simultaneously in arriving at conclusion, without limitations on time or restrictions on number of speech samples necessary.

Make 1 of 5 alternative judgments: (1) positive identification; (2) positive elimination; (3) probable identification; (4) probable elimination; (5) unable to arrive at conclusion. Base all positive judgments on $\geq$10 pairs of like sounds in known and unknown voices.

Refs.: JAOAC **58,** 453(1975); **59,** 927(1976).

# Appendix: Standard Solutions and Certified Reference Materials


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## 942.25 Standard Solutions and Materials

Use accurately calibrated equipment, which meets NIST specifications. Because alk. and other corrosive solns dissolve glass, to avoid vol. errors do not store such solns in calibrated app. Burets used continuously with such solns should be recalibrated periodically.

Working temp. of std soln should approximate that of its temp. during stdzn. If temp. corrections are necessary, sufficient accuracy may be obtained by use of Table **942.25**.

Ref.: JAOAC **25,** 650(1942).

## 942.26 Standard Solutions of Ammonium and Potassium Thiocyanates

**Final Action**

### *A. Reagents*

(**a**) *Purified silver nitrate.*—Dissolve 50 g $AgNO_3$ in 20 mL boiling $H_2O$ contg ca 5 drops $HNO_3$. Heat to dissolve, filter while still hot thru fritted glass filter, using suction, and collect filtrate in clean Pyrex beaker. Wash beaker and filter with ca 5 mL hot $H_2O$, adding washings to filtrate. Cool in ice bath, stirring to induce crystn, and place in refrigerator at ca 10° until equilibrium is reached. Decant liq. thru fritted glass filter and transfer crystals to filter. Cover filter with watch glass and draw air thru filter to remove adhering liq. Transfer crystals to small, clean Pyrex beaker. Cover beaker with watch glass and place inside larger covered Pyrex beaker. Dry at 105° and fuse at 220–250° (mp 208°), holding at this temp. ca 15 min after crystals are melted. Protect from dust during prepn. Cool in desiccator, remove product from beaker, powder in mortar, dry 0.5 hr at 105°, and store in brown g-s bottle in dark over good desiccant.

(**b**) *Reference soln.*—To mixt. of 5 mL $HNO_3$ (1 + 1), 2 mL Fe alum soln, **941.18D (a)**, and 115 mL $H_2O$, add ca 0.02 mL 0.1*N* thiocyanate, **942.26B**, noting exact vol. used.

### *B. Preparation of Standard Solution*

Prep. ca 0.1*N* soln from reagent that shows no Cl, using 7.612 g $NH_4SCN$ or 9.718 g KSCN/L.

### *C. Standardization*

Accurately weigh, on tared watch glass, enough purified $AgNO_3$ to give titrn of ca 40 mL (ca 0.7 g for 0.1*N* soln) and transfer with $H_2O$ thru glass funnel to 250 mL g-s erlenmeyer. Dissolve in ca 75 mL $H_2O$ (halogen-free), and add 5 mL $HNO_3$ (1 + 1) and 2 mL Fe alum soln, **941.18D(a)**. Titr. with thiocyanate soln until titrd soln is reddish brown, which remains after shaking vigorously 1 min. Record buret reading and set flask aside 5 min, shaking occasionally and maintaining end point color by addn of thiocyanate soln as required. Then add addnl thiocyanate soln, if necessary, to produce permanent end point color, matching with color of ref. soln, **942.26A(b)**. From total vol. thiocyanate soln used in titrn, subtract vol. contained in ref. soln.

$$\text{Normality} = \text{g } AgNO_3 \times 1000/\text{mL titer} \times 169.87$$

Refs.: JAOAC **25,** 661(1942); **30,** 105, 496(1947).

## 939.12 Standard Solution of Arsenious Oxide

**Final Action**

### *A. Reagent*

*Arsenious oxide.*—Use NIST SRM 83. Dry 1 hr at 105° immediately before using.

### *B. Preparation of Standard Solution*

Accurately weigh $As_2O_3$ by difference from small g-s weighing bottle (use ca 4.95 g/L for 0.1*N*). Dissolve in 1*N* NaOH (50 mL/5 g $As_2O_3$) in flask or beaker by heating on steam bath. Add ca same vol. 1*N* $H_2SO_4$. Cool, quant. transfer mixt. to vol. flask, and dil. to vol. (Soln must be neut. to litmus, not alk.)

$$\text{Normality} = \text{g } As_2O_3 \times 4000/\text{mL final vol.} \times 197.84$$

Refs.: JAOAC **22,** 568(1939); **24,** 100, 639(1941).

## 964.24 Buffer Solutions for Calibration of pH Equipment

**First Action 1964**
**Final Action 1965**

Use $H_2O$ with pH of ≥6.5 but ≤7.5, obtained by boiling $H_2O$ 15 min and cooling under $CO_2$-free conditions. Store std buffer solns except $Ca(OH)_2$ in bottles of chem. resistant glass. Protect phosphate, borax, and $Ca(OH)_2$ buffers from $CO_2$. pH values as function of temp. are given in Table **964.24**.

(**a**) *Potassium tetroxalate buffer soln.*—0.0496*M*; 0.05*m*. Transfer 12.61 g $KHC_2O_4.H_2C_2O_4.2H_2O$ (air wt) (NIST SRM 189) to 1 L vol. flask, dil. to vol. with $H_2O$, and mix thoroly. (It is not necessary to remove dissolved $CO_2$ from the $H_2O$ or to dry salt before weighing.) Prep. fresh every 2 months.

(**b**) *Potassium hydrogen tartrate buffer soln.*—Satd soln at 25°, 0.034*M*. Add excess (ca 100%) of $KHC_4H_4O_6$ (NIST SRM 188) to $H_2O$ in g-s bottle or flask, and shake vigorously; few min shaking is enough for satn (100 mL $H_2O$ at 25° dissolves ca 0.7 g $KHC_4H_4O_6$). Adjust to 25°, let solid settle, and decant clear soln, or filter if necessary. Discard when mold appears. Few crystals of thymol added during prepn will retard mold growth, and will alter pH by <0.01 unit. For accuracy of ±0.01 pH unit, temp. of soln at satn must be between 20 and 30°.

(**c**) *Acid potassium phthalate buffer soln.*—0.0496*M*; 0.05*m*. Dissolve 10.12 g dried (2 hr at 110°) $KHC_8H_4O_4$ (NIST SRM 185) in $H_2O$ and dil. to 1 L. (Elaborate precautions for exclusion of atm. $CO_2$ are unnecessary, altho soln should be pro-

**Table 942.25 Temperature Corrections for Volume of Aqueous Solutions**

| | Correction in Milliliters at— | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vol. Std Soln | 6° | 8° | 10° | 12° | 14° | 16° | 18° | 20° | 22° | 24° | 26° | 28° | 30° |
| *mL* | | | | | | | | | | | | | |
| 10 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | −0.01 | −0.02 | −0.02 |
| 20 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.01 | 0.01 | 0.00 | −0.01 | −0.02 | −0.03 | −0.03 | −0.03 |
| 25 | 0.04 | 0.03 | 0.03 | 0.03 | 0.02 | 0.02 | 0.01 | 0.00 | −0.01 | −0.02 | −0.03 | −0.04 | −0.05 |
| 30 | 0.04 | 0.04 | 0.04 | 0.03 | 0.03 | 0.02 | 0.01 | 0.00 | −0.01 | −0.02 | −0.04 | −0.05 | −0.07 |
| 40 | 0.06 | 0.06 | 0.05 | 0.04 | 0.04 | 0.03 | 0.01 | 0.00 | −0.02 | −0.03 | −0.05 | −0.07 | −0.09 |
| 50 | 0.07 | 0.07 | 0.06 | 0.06 | 0.05 | 0.03 | 0.02 | 0.00 | −0.02 | −0.04 | −0.06 | −0.09 | −0.12 |

tected against evapn and contamination with molds. Replace soln if mold appears.)

(**d**) *Phosphate buffer soln.*—0.0249*M*; 0.025*m*. Dissolve 3.387 g $KH_2PO_4$ and 3.533 g $Na_2HPO_4$ (NIST SRM 186-I and II) in $H_2O$ and dil. to 1 L. (Dry salts 2 hr at 110–130° before use.)

(**e**) *Phosphate buffer soln.*—0.008663*M*, 0.008695*m* $KH_2PO_4$ and 0.03030*M*, 0.03043*m* $Na_2HPO_4$. Dissolve 1.179 g $KH_2PO_4$ and 4.303 g $Na_2HPO_4$ (NIST SRM 186-I and II) in $H_2O$ and dil. to 1 L. (Dry salts 2 hr at 110–130° before use.)

(**f**) *Borax buffer soln.*—0.00996*M*; 0.01*m*. Dissolve 3.80 g $Na_2B_4O_7.10H_2O$ (NIST SRM 187) in $H_2O$ and dil. to 1 L. (Salt must not be dried in oven before use.) To avoid contamination with $CO_2$, stopper bottle except when in use or protect with soda-lime tube. Use buffer soln within 10 min after removal from bottle.

(**g**) *Sodium bicarbonate-carbonate buffer soln.*— 0.0249*M*; 0.025*m* (each). Transfer 2.092 g $NaHCO_3$ (NIST SRM 191; do not heat) and 2.640 g $Na_2CO_3$ (NIST SRM 192; dry 2 hr at 275°) to 1 L vol. flask. Dissolve and dil. to vol. with $CO_2$-free $H_2O$.

(**h**) *Calcium hydroxide buffer soln.*—Satd soln at 25°, 0.02025*M*. Slowly heat finely granular $CaCO_3$, low in alkalies, to 1000° in Pt dish and maintain at this temp. 45–60 min. Cool in desiccator, and add to $H_2O$ with stirring. Heat to bp with continuous stirring. Cool, and filter on medium fritted glass filter. Dry at 110°, cool, and crush to fine, granular powder.

Place crushed CaO in polyethylene bottle, add $H_2O$, shake vigorously, let settle, and record temp. (Keep large excess of $Ca(OH)_2$ in bottle.) For use, filter soln thru medium fritted glass filter. Use at same temp. at which satn took place, and discard filtered soln if it becomes turbid. When more buffer soln is needed, add addnl $H_2O$ to suspension, re-sat., and filter as above.

Refs.: NIST Certificates for Standard Reference Materials 185e, 186c, 187b, 188, 189, 191, and 192. JAOAC **33,** 223(1950); **41,** 302(1958); **47,** 43(1964).

## 941.17 Standard Buffers and Indicators for Colorimetric pH Comparisons

**Final Action**

### *A. Preparation of Sulfonphthalein Indicators*

| | X | pH |
|---|---|---|
| Bromocresol green | 14.3 | 3.8–5.4 |
| Chlorophenol red | 23.6 | 4.8–6.4 |
| Bromothymol blue | 16.0 | 6.0–7.6 |
| Phenol red | 28.2 | 6.8–8.4 |

X = mL 0.01*N* NaOH/0.1 g indicator required to form mono-Na salt. Dil. to 250 mL for 0.04% reagent.

### *B. Preparation of Stock Solutions*

Use recently boiled and cooled $H_2O$.

(**a**) *Acid potassium phthalate soln.*—0.2*M*. Dry to const wt at 110–115°. Dissolve 40.836 g in $H_2O$ and dil. to 1 L.

(**b**) *Monopotassium phosphate soln.*—0.2*M*. Dry $KH_2PO_4$ to const wt at 110–115°. Dissolve 27.232 g in $H_2O$ and dil. to 1 L. Soln should be distinctly red with Me red, and distinctly blue with bromophenol blue.

(**c**) *Boric acid-potassium chloride soln.*—0.2*M*. Dry $H_3BO_3$ to const wt in desiccator over $CaCl_2$. Dry KCl 2 days in oven at 115–120°. Dissolve 12.405 g $H_3BO_3$ and 14.912 g KCl in $H_2O$, and dil. to 1 L.

(**d**) *Sodium hydroxide std soln.*—0.2*M*. Prep. and stdze as in **936.16**; 0.04084 g $KHC_8H_4O_4$ = 1 mL 0.2*M* NaOH. It is preferable to use factor with soln rather than try to adjust to exactly 0.2*M*.

**Table 964.24 pH Values for Standard Buffer Solutions as Function of Temperature**

| Temperature | 0.05*m* Potassium Tetroxalate | Satd Potassium Hydrogen Tartrate | 0.05*m* Acid Potassium Phthalate | 0.025*m* Phosphate | 0.008695*m* and 0.03043*m* Phosphate | 0.01*m* Borax | 0.025*m* $NaHCO_3$ and 0.025*m* $Na_2CO_3$ | Satd Calcium Hydroxide |
|---|---|---|---|---|---|---|---|---|
| °C | pH | pH | pH | pH | pH | pH | pH | pH |
| 0 | 1.666 | — | 4.003 | 6.982 | 7.534 | 9.460 | 10.321 | 13.423 |
| 5 | 1.668 | — | 3.998 | 6.949 | 7.501 | 9.392 | 10.248 | 13.207 |
| 10 | 1.670 | — | 3.996 | 6.921 | 7.472 | 9.331 | 10.181 | 13.003 |
| 15 | 1.672 | — | 3.996 | 6.898 | 7.449 | 9.276 | 10.120 | 12.810 |
| 20 | 1.675 | — | 3.999 | 6.878 | 7.430 | 9.227 | 10.064 | 12.627 |
| 25 | 1.679 | 3.557 | 4.004 | 6.863 | 7.415 | 9.183 | 10.014 | 12.454 |
| 30 | 1.683 | 3.552 | 4.011 | 6.851 | 7.403 | 9.143 | 9.968 | 12.289 |
| 35 | 1.688 | 3.549 | 4.020 | 6.842 | 7.394 | 9.107 | 9.928 | 12.133 |
| 37 | 1.691 | 3.548 | 4.024 | 6.839 | 7.392 | 9.093 | – | 12.043 |
| 40 | 1.694 | 3.547 | 4.030 | 6.836 | 7.388 | 9.074 | 9.891 | 11.984 |
| 45 | 1.700 | 3.547 | 4.042 | 6.832 | 7.385 | 9.044 | 9.859 | 11.841 |
| 50 | 1.707 | 3.549 | 4.055 | 6.831 | 7.384 | 9.017 | 9.831 | 11.705 |
| 55 | 1.715 | 3.554 | 4.070 | – | – | – | – | 11.574 |
| 60 | 1.723 | 3.560 | 4.085 | – | – | – | – | 11.449 |

### C. Preparation of Buffer Solutions

Prep. std buffer solns from designated amts stock solns, **941.17**, and dil. to 200 mL. For use as colorimetric std, mix 20 mL buffer soln with 0.5 mL indicator soln, **941.17A**.

*Phthalate-NaOH Mixtures*

| pH | *0.2M* KH Phthalate (mL) | *0.2M* NaOH (mL) |
|---|---|---|
| 5.0 | 50 | 23.65 |
| 5.2 | 50 | 29.75 |
| 5.4 | 50 | 35.25 |
| 5.6 | 50 | 39.70 |
| 5.8 | 50 | 43.10 |
| 6.0 | 50 | 45.40 |
| 6.2 | 50 | 47.00 |

*$KH_2PO_4$-NaOH Mixtures*

| pH | *0.2M* $KH_2PO_4$ (mL) | *0.2M* NaOH (mL) |
|---|---|---|
| 5.8 | 50 | 3.66 |
| 6.0 | 50 | 5.64 |
| 6.2 | 50 | 8.55 |
| 6.4 | 50 | 12.60 |
| 6.6 | 50 | 17.74 |
| 6.8 | 50 | 23.60 |
| 7.0 | 50 | 29.54 |
| 7.2 | 50 | 34.90 |
| 7.4 | 50 | 39.34 |
| 7.6 | 50 | 42.74 |
| 7.8 | 50 | 45.17 |
| 8.0 | 50 | 46.85 |

*$H_3BO_3$-KCl-NaOH Mixtures*

| pH | *0.2M* $H_3BO_3$, KCl (mL) | *0.2M* NaOH (mL) |
|---|---|---|
| 7.8 | 50 | 2.65 |
| 8.0 | 50 | 4.00 |
| 8.2 | 50 | 5.90 |
| 8.4 | 50 | 8.55 |
| 8.6 | 50 | 12.00 |

Refs.: JAOAC **24**, 583(1941). Clark, "Determination of Hydrogen-ions," 3rd Ed., pp. 91, 94, 192–202.

## 936.15 Standard Solution of Hydrochloric Acid

**Final Action**

### A. Preparation of Standard Solutions

Following table gives approx. vols of 36.5–38% HCl required to make 10 L std solns:

| *Approx. normality* | *mL HCl to be dild to 10 L* |
|---|---|
| 0.01 | 8.6 |
| 0.02 | 17.2 |
| 0.10 | 86.0 |
| 0.50 | 430.1 |
| 1.0 | 860.1 |

### B. Standard Sodium Hydroxide Method

Titr. 40 mL against std alkali soln, **936.16C–E**, of ca same concn as acid being stdzd in 300 mL flask that has been swept free from $CO_2$, using $CO_2$-free $H_2O$ and 3 drops phthln.

Normality = (mL std alkali × normality of alkali)/mL HCl

If more concd than desired, dil. soln to required normality value by following formula:

$$V_1 = V_2 \times N_2/N_1$$

where $N_2$ and $V_2$ represent normality and vol. of stock soln, resp., and $V_1$ = vol. to which stock soln should be dild to obtain desired normality, $N_1$.

Check exact concn of final soln by titrn as above. Normality will be exact only if same indicator is used in detn as in stdzn. Restdze if indicators other than phthln are used.

Refs.: JAOAC **19**, 107, 194(1936). **49**, 250(1966). Kolthoff & Stenger, "Volumetric Analysis," **II**, 52(1947).

### C. Constant Boiling Method

Dil. 822 mL HCl (36.5–38% HCl) with 750 mL $H_2O$. Check sp gr with spindle and adjust to 1.10. Place 1.5 L in 2 L flat-bottom distg flask, add ca 10 SiC grains (ca "20 mesh"), and connect to long, straight inner-tube condenser. Heat on elec. hot plate and distil at 5–10 mL/min, keeping end of condenser open to air. When 1125 mL has distd, change receivers and catch next 225 mL, which is const boiling HCl, in erlenmeyer with end of condenser inserted into flask, but above surface of liq. Read barometer to nearest mm at beginning and end of collection of 225 mL portion and note barometer temp. Average readings.

Calc. air wt in g ($G$) of this const boiling HCl required to give one equiv. wt of HCl from one of following equations:

For $P_0$ = 540–669 mm Hg:

$$G = 162.255 + 0.02415\ P_0$$

For $P_0$ = 670–780 mm Hg:

$$G = 164.673 + 0.02039\ P_0$$

where $P_0$ = barometric pressure in mm Hg corrected to 0°C for expansion of Hg and of barometer scale. For brass scale barometer, following correction is accurate enough:

$$P_0 = P_t(1 - 0.000162t),$$

where $t$ = barometer temp. in °C

Weigh required amt of const boiling HCl in tared, stoppered flask to at least 1 part in 10,000. Dil. immediately, and finally dil. to vol. with $CO_2$-free $H_2O$ at desired temp.

Refs.: JAOAC **25**, 653(1942); **36**, 96, 354(1953); **37**, 122, 462(1954).

**Standard Borax Method**

### D. Reagents

(**a**) *Methyl red indicator*.—Dissolve 100 mg Me red in 60 mL alcohol and dil. with $H_2O$ to 100 mL.

(**b**) *Reference soln*.—Prep. ref. soln of $H_3BO_3$, NaCl, and indicator corresponding to composition and vol. of soln at equivalence point. For use in detn of end point of titrn with 0.1$N$ HCl, ref. soln should be 0.1$M$ in $H_3BO_3$ and 0.05$M$ in NaCl.

(**c**) *Std borax*.—Sat. 300 mL $H_2O$ at 55° (not higher) with $Na_2B_4O_7.10H_2O$ (ACS) (ca 45 g). Filter at this temp. thru folded paper into 500 mL erlenmeyer. Cool filtrate to ca 10°, with continuous agitation during crystn. Decant supernate, rinse ppt once with 25 mL cold $H_2O$, and dissolve crystals in just enough $H_2O$ at 55° to ensure complete soln (ca 200 mL). Recrystallize by cooling to ca 10°, agitating flask during crystn.

Filter crystals onto small buchner with suction, wash ppt once with 25 mL ice-cold $H_2O$, and dry crystals by washing with two 20 mL portions alcohol, drying after each washing with suction. Follow with two 20 mL portions ether. (Just before use, free alcohol and ether from any possible reacting acids by vigorously shaking each with 2–3 g of the pure, dry $Na_2B_4O_7.10H_2O$ and then filtering.) Spread crystals on watch glass, immediately place dried $Na_2B_4O_7.10H_2O$ in closed container over soln satd with respect to both sucrose and NaCl, and let it remain ≥24 hr before using. Then transfer the pure $Na_2B_4O_7.10H_2O$ to g-s container and store in closed container over soln satd with respect to both sucrose and NaCl (stable under these conditions 1 year).

### E. Standardization

Accurately weigh enough std $Na_2B_4O_7.10H_2O$ to titr. ca 40 mL and transfer to 300 mL flask. Add 40 mL $CO_2$-free $H_2O$, **936.16B(a)**, and stopper flask. Swirl gently until sample dissolves. Add 4 drops Me red and titr. with soln that is being stdzd to equivalence point as indicated by ref. soln.

$$\text{Normality} = \text{g } Na_2B_4O_7.10H_2O \times 1000/\text{mL acid} \times 190.69$$

Ref.: JAOAC **22,** 102, 563(1939).

### Standard Sodium Carbonate Method

### F. Reagents

(**a**) *Methyl orange indicator*.—0.1% in $H_2O$.

(**b**) *Reference soln*.—80 mL $CO_2$-free $H_2O$ contg 3 or 4 drops Me orange.

(**c**) *Anhydrous sodium carbonate*.—Heat 250 mL $H_2O$ to 80° and add $NaHCO_3$ (ACS), stirring until no more dissolves. Filter soln thru folded paper (use of hot $H_2O$ funnel is desirable) into erlenmeyer. Cool filtrate to ca 10°, swirling constantly during crystn. Fine crystals of trona that sep. out have approx. composition: $Na_2CO_3.NaHCO_3.2H_2O$. Decant supernate, drain crystals by suction, and wash once with cold $H_2O$.

Transfer ppt, being careful not to include any paper fibers, to large flat-bottom Pt dish. Heat 1 hr at 290° in elec. oven or furnace with pyrometer control. Stir contents occasionally with Pt wire. Cool in desiccator. Place the anhyd. $Na_2CO_3$ in g-s container and store in desiccator contg efficient desiccant. Dry at 120° and cool just before weighing.

Refs.: Kolthoff & Stenger, "Volumetric Analysis," **II,** 80(1947). Ind. Eng. Chem., Anal. Ed. **9,** 141(1937). JAOAC **22,** 563(1939).

### G. Standardization

Accurately weigh enough anhyd. $Na_2CO_3$, (**c**), to titr. ca 40 mL, transfer to 300 mL erlenmeyer, and dissolve in 40 mL $H_2O$. Add 3 drops Me orange and titr. until color begins to deviate from $H_2O$ tint (ref. soln). (Equivalence point has not been reached.) Boil soln gently 2 min, and cool. Titr. until color is barely different from $H_2O$ tint of indicator.

$$\text{Normality} = \text{g } Na_2CO_3 \times 1000/\text{mL acid} \times 52.994$$

Ref.: JAOAC **22,** 102, 563(1939).

## 939.13 Standard Solution of Iodine

**Final Action**

### A. Preparation of Standard Solution

Dissolve weighed amts of I (12.7 g/L for 0.1*N* soln) and KI, in proportion of 20 g KI to 13 g I, in 50 mL $H_2O$. When I dissolves, transfer soln to g-s vol. flask. Dil. to vol. with $H_2O$ and mix thoroly. Store in dark brown, g-s bottle away from light and restdze as frequently as necessary.

### B. Standardization

Transfer accurately measured portion of std $As_2O_3$ soln, **939.12B** (40–50 mL ca 0.1*N* soln for 0.1*N* soln), to erlenmeyer. Acidify slightly with $H_2SO_4$ (1 + 10), neutze with solid $NaHCO_3$, and add ca 2 g excess. Titr. with I soln, using ca 0.2% starch soln (5 mL/100 mL) as indicator. Sat. soln with $CO_2$ at end of titrn by adding 1 mL $H_2SO_4$ (1+10) just before end point is reached.

$$\text{Normality} = \text{mL } As_2O_3 \times \text{normality } As_2O_3/\text{mL I}$$

Refs.: JAOAC **22,** 568(1939); **24,** 100, 639(1941).

## 947.13 Standard Solution of Potassium Bromide-Bromate

**Final Action**

### A. Preparation of Standard Solution

Dissolve ca 2.8 g $KBrO_3$ and 12 g KBr in boiled $H_2O$ and dil. to 1 L with boiled $H_2O$ for ca 0.1*N* soln.

### B. Standardization

Measure 40 mL std $As_2O_3$ soln, **939.12B**, from buret into 300 mL erlenmeyer. Add 10 mL HCl and 3 drops Me orange, **936.15F(a)**. Titr. with KBr-$KBrO_3$ soln until ≤1 drop causes color of Me orange to fade completely. Swirl soln constantly and add last mL dropwise, swirling between drops.

$$\text{Normality} = \text{mL } As_2O_3 \times \text{normality } As_2O_3/\text{mL KBr-}KBrO_3$$

Refs.: JAOAC **30,** 502(1947); **31,** 119, 572(1948).

## 949.13 Standard Solution of Potassium Dichromate

**Final Action**

### A. Reagent

*Starch soln*.—Mix ca 1 g arrowroot starch with 10 mL $H_2O$ and pour slowly, with const stirring, into 200 mL boiling $H_2O$. Boil until thin, translucent fluid is obtained. Let settle and use clear supernate. Preserve with Hg.

### B. Assay of Stock Potassium Dichromate

If $K_2Cr_2O_7$ is in small crystals, mix by shaking thoroly in large, clean jar; if it is in lumps, grind representative sample to pass No. 60 sieve, and then mix by shaking. Dry portion for weighings 2 hr at 100°.

Weigh, into each of 3 g-s erlenmeyers, enough $K_2Cr_2O_7$ (NIST SRM 136) to give titer of 100.5–102.0 mL 0.1*N* $Na_2S_2O_3$, **942.27A** (0.4928–0.5001 g for 0.1*N* soln). Completely dissolve in 100 mL $H_2O$, add 4.0 g KI, and swirl mixt. until dissolved. With buret, add 4.0 mL HCl, stopper flask, mix by swirling, and let stand in dark 10 min. Cool flask ca 1 min in ice-$H_2O$.

While swirling flask, pipet in 100 mL $Na_2S_2O_3$ soln. Add 5 mL starch soln and complete titrn with $Na_2S_2O_3$ soln added from 10 mL microburet (graduated in 0.05 mL). End point is from bluish green to clear green; change takes place within 0.01 mL. Record vol. to nearest 0.01 mL. Calc. apparent normality of $Na_2S_2O_3$ soln for each of the 3 titrns, and average. Designate this av. as $N_{NIST}$.

Similarly titr. 3 portions of stock $K_2Cr_2O_7$ and calc. the 3 apparent normalities. Designate each of these results as $N_{stock}$. Calc. % purity of stock $K_2Cr_2O_7$ = ($N_{NIST}$ × 100)/$N_{stock}$.

Take av. of the 3 results as % purity of stock $K_2Cr_2O_7$.

### C. Preparation of Standard Solution

Dissolve theoretical wt $K_2Cr_2O_7$ (NIST SRM 136) (4.9032 g for 0.1*N* soln), or wt stock $K_2Cr_2O_7$, **949.13B**, found to have oxidimetric value 99.95–100.05% of NIST SRM, in enough $H_2O$ to make 1 L. (Dry $K_2Cr_2O_7$ 2 hr at 100° before using.)

Refs.: JAOAC **32,** 587(1949); **33,** 225(1950).

## 940.35 Standard Solution of Potassium Permanganate

**Final Action**

### *A. Preparation of Standard Solution*

Dissolve slightly more than desired equiv. wt (3.2 g for 0.1*N*) of $KMnO_4$ in 1 L $H_2O$. Boil soln 1 hr. Protect from dust and let stand overnight. Thoroly clean 15 cm glass funnel, perforated porcelain plate from Caldwell crucible, and g-s bottle (preferably of brown glass) with warm chromic acid cleaning soln. Digest asbestos for use in gooches on steam bath 1 hr with ca 0.1*N* $KMnO_4$ that has been acidified with few drops $H_2SO_4$ (1 + 3). Let settle, decant, and replace with $H_2O$. To prep. glass funnel, place porcelain plate in apex, make pad of asbestos ca 3 mm thick on plate, and wash acid-free. (Pad should not be too tightly packed and only moderate suction should be applied.) Insert stem of funnel into neck of bottle and filter $KMnO_4$ soln directly into bottle without aid of suction.

### *B. Standardization*

For 0.1*N* soln, transfer 0.3 g dried (1 hr at 105°) Na oxalate (NIST SRM 40) to 600 mL beaker. Add 250 mL $H_2SO_4$ (5 + 95), previously boiled 10–15 min and then cooled to 27 ± 3°.

Stir until $Na_2C_2O_4$ dissolves. Add 39–40 mL $KMnO_4$ soln at rate of 25–35 mL/min, stirring slowly. Let stand until pink disappears (ca 45 sec). If pink persists because $KMnO_4$ soln is too concd, discard and begin again, adding few mL less of $KMnO_4$ soln. Heat to 55–60°, and complete titrn by adding $KMnO_4$ soln until faint pink persists 30 sec. Add last 0.5–1 mL dropwise, letting each drop decolorize before adding next.

Det. excess of $KMnO_4$ soln required to turn soln pink by matching with color obtained by adding $KMnO_4$ soln to same vol. of boiled and cooled dil. $H_2SO_4$ at 55–60°. This correction is usually 0.03–0.05 mL. From net vol. $KMnO_4$, calc. normality:

$$\text{Normality} = \text{g } Na_2C_2O_4 \times 1000/\text{mL } KMnO_4 \times 66.999$$

Refs.: JAOAC **23,** 543(1940); **31,** 568(1948). J. Research NBS **15,** 493(1935), Research Paper No. 843.

## 941.18 Standard Solution of Silver Nitrate

**Final Action**

### *A. Preparation of Standard Solution*

Dissolve slightly more than theoretical wt of $AgNO_3$ (equiv. wt, 169.87) in halogen-free $H_2O$ and dil. to vol. Thoroly clean glassware, avoid contact with dust, and keep prepd soln in amber g-s bottles away from light.

***Mohr Method***

### *B. Reagents*

(**a**) *Potassium chloride.*—Recrystallize KCl 3 times from $H_2O$, dry at 110°, and then heat at ca 500° to const wt. Equiv. wt KCl = 74.555. Or, preferably, use NIST SRM 999.

(**b**) *Potassium chromate soln.*—5% soln of $K_2CrO_4$ in $H_2O$.

### *C. Standardization*

Accurately weigh enough KCl to yield titrn of ca 40 mL (ca 0.3 g for 0.1*N* soln), and transfer to 250 mL g-s erlenmeyer with 40 mL $H_2O$. Add 1 mL $K_2CrO_4$ soln and titr. with $AgNO_3$ soln until first perceptible pale red-brown appears. From titrn vol., subtract mL of the $AgNO_3$ soln required to produce end point color in 75 mL $H_2O$ contg 1 mL $K_2CrO_4$ soln. From net vol. $AgNO_3$, calc. normality:

$$\text{Normality} = \text{g KCl} \times 1000/\text{mL } AgNO_3 \times 74.555$$

***Volhard Method***

### *D. Reagents*

(**a**) *Ferric alum indicator soln.*—Satd soln of $FeNH_4(SO_4)_2.12H_2O$ in $H_2O$.

(**b**) *Potassium or ammonium thiocyanate std soln.*—Prep. ca 0.1*N* soln, **942.26B**. Det. working titer by accurately measuring 40–50 mL std $AgNO_3$ soln, adding 2 mL Fe alum soln and 5 mL $HNO_3$ (1 + 1), and titrg with the thiocyanate soln until soln appears pale rose after vigorous shaking.

### *E. Standardization*

Accurately weigh enough KCl, **941.18B(a)**, to yield titrn of ca 40 mL (ca 0.3 g for 0.1*N* soln) and transfer to 250 mL g-s erlenmeyer with 40 mL $H_2O$. Add 5 mL $HNO_3$ (1 + 1) and excess $AgNO_3$ soln. Mix, and let stand few min protected from light. Filter thru gooch prepd with medium pad of asbestos previously rinsed with 2% $HNO_3$. Wash flask and ppt with several small portions of 2% $HNO_3$, passing washings thru crucible until filtrate and washings measure ca 150 mL. Add 2 mL Fe alum soln and titr. residual $AgNO_3$ with thiocyanate soln. From titrn, together with ratio of the 2 solns, calc. net vol. $AgNO_3$ soln. (Errors of blank are compensating and may be disregarded.) From net vol. $AgNO_3$, calc. normality as in **941.18C**.

Ref.: JAOAC **24,** 100, 631(1941).

## 936.16 Standard Solution of Sodium Hydroxide

**Final Action**

***Standard Potassium Hydrogen Phthalate Method***

### *A. Apparatus*

Use buret and pipet calibrated by NIST or by analyst. Protect exits to air of automatic burets from $CO_2$ contamination by suitable guard tubes contg soda-lime. Use containers of alkali-resistant glass.

### *B. Reagents*

(**a**) *Carbon dioxide-free water.*—Prep. by one of following methods: (*1*) Boil $H_2O$ 20 min and cool with soda-lime protection; (*2*) bubble air, freed from $CO_2$ by passing thru tower of soda-lime, thru $H_2O$ 12 hr.

(**b**) *Sodium hydroxide soln.*—(1 + 1). To 1 part NaOH (reagent quality contg <5% $Na_2CO_3$) in flask add 1 part $H_2O$ and swirl until soln is complete. Close with rubber stopper. Set aside until $Na_2CO_3$ has settled, leaving perfectly clear liq. (ca 10 days).

(**c**) *Acid potassium phthalate.*—NIST SRM for Acidimetry 84. Crush to pass No. 100 sieve. Dry 2 hr at 120°. Cool in desiccator contg $H_2SO_4$.

### *C. Preparation of Standard Solution*

Following table gives approx. vols of NaOH soln (1 + 1) necessary to make 10 L of std solns:

| *Approx. normality* | *mL NaOH to be dild to 10 L* |
|---|---|
| 0.01 | 5.4 |
| 0.02 | 10.8 |
| 0.10 | 54.0 |
| 0.50 | 270.0 |
| 1.0 | 540.0 |

Add required vol. of NaOH soln (1 + 1) to 10 L $CO_2$-free $H_2O$. Check normality, which should be slightly high, as in **936.16D**, and adjust to desired concn by following formula:

$$V_1 = V_2 \times N_2/N_1$$

where $N_2$ and $V_2$ represent normality and vol. stock soln, resp., and $V_1$, vol. to which stock soln should be dild to obtain desired normality, $N_1$. Stdze final soln as in **936.16D** or **E**.

### *D. Standardization*

Accurately weigh enough dried $KHC_8H_4O_4$ to titr. ca 40 mL and transfer to 300 mL flask that has been swept free from $CO_2$. Add 50 mL cool $CO_2$-free $H_2O$. Stopper flask and swirl gently until sample dissolves. Titr. to pH 8.6 with soln being stdzd, taking precautions to exclude $CO_2$ and using as indicator either glass-electrode pH meter or 3 drops phthln. In latter case, det. end point by comparison with pH 8.6 buffer soln, **941.17C**, contg 3 drops phthln. Det. vol. NaOH required to produce end point of blank by matching color in another flask contg 3 drops phthln and same vol. $CO_2$-free $H_2O$. Subtract vol. required from that used in first titrn and calc. normality.

$$\text{Normality} = \text{g } KHC_8H_4O_4 \times 1000/\text{mL NaOH} \times 204.229$$

Refs.: JAOAC **19**, 107, 194(1936). NIST Certificate for Standard Reference Material 84.

### *Constant Boiling Hydrochloric Acid Method*

### *E. Standardization*

Accurately weigh from weighing buret enough const boiling HCl, **936.15C**, to titr. ca 40 mL, into erlenmeyer previously swept free from $CO_2$. Add ca 40 mL $CO_2$-free $H_2O$, then 3–5 drops desired indicator, and titr. with soln being stdzd.

$$\text{Normality} = \text{g HCl} \times 1000/\text{mL titer} \times G$$

where $G$ has value given in **936.15C**.

Refs.: JAOAC **25**, 653(1942); **36**, 96, 354(1953); **37**, 122, 462 (1954).

## 942.27 Standard Solutions of Sodium Thiosulfate

**Final Action**

### *A. Preparation of Standard Solution*

Dissolve ca 25 g $Na_2S_2O_3.5H_2O$ in 1 L $H_2O$. Boil gently 5 min and transfer while hot to storage bottle previously cleaned with hot chromic acid cleaning soln and rinsed with warm boiled $H_2O$. (Temper bottle, if not heat-resistant, before adding hot soln.) Store soln in dark, cool place; do not return unused portions to stock bottle. If solns less concd than 0.1$N$ are desired, prep. by diln with boiled $H_2O$. (More dil. solns are less stable and should be prepd just before use.)

### *B. Standardization*

Accurately weigh 0.20–0.23 g $K_2Cr_2O_7$ (NIST SRM 136 dried 2 hr at 100°) and place in g-s I flask (or g-s flask). Dissolve in 80 mL Cl-free $H_2O$ contg 2 g KI. Add, with swirling, 20 mL ca 1$N$ HCl and immediately place in dark 10 min. Titr. with $Na_2S_2O_3$ soln, **942.27A**, adding starch soln after most of I has been consumed.

$$\text{Normality} = \text{g } K_2Cr_2O_7 \times 1000/\text{mL } Na_2S_2O_3 \times 49.032$$

Refs.: JAOAC **25**, 659(1942); **27**, 557(1944); **28**, 594(1945); **38**, 382(1955); **47**, 43, 46(1964); **48**, 103(1965).

## 890.01 Standard Solutions of Sulfuric Acid

**Final Action**

### *A. Preparation of Standard Solution*

Following table gives approx. vols of 95–98% $H_2SO_4$ necessary to make 10 L std solns:

| *Approx. normality* | *mL $H_2SO_4$ to be dild to 10 L* |
|---|---|
| 0.01 | 2.8 |
| 0.02 | 5.6 |
| 0.10 | 27.7 |
| 0.50 | 138.1 |
| 1.0 | 276.1 |

### *B. Standard Borax Method Standardization*

*See* **936.15E**.

### *C. Specific Gravity Method*

Dil. $H_2SO_4$ with enough $H_2O$ to make convenient vol. of ca 70% $H_2SO_4$ by wt. Det. sp gr in air at convenient temp. (0–40°) as in **945.06C** (or sp gr may be detd with Sprengel pycnometer), protecting soln from contact with air. Calc. exact % $H_2SO_4$ by wt,

$$P = S(85.87 + 0.05T - 0.0004t^2) - 69.82$$

where $S$ = sp gr (in air) at $T$°, compared with $H_2O$ at $t$°.

Weigh exactly $W$ g prepd acid contg $P$% $H_2SO_4$ and dil. to $n$ L to make required soln contg $G$ g $H_2SO_4$/L. Calc. $W$ from equation:

$$W = nG \times 100/P$$

Refs.: J. Chem. Soc. Trans. **57**, 64(1890). J. Soc. Chem. Ind. (1899), 1091. JAOAC **24**, 636(1941).

## 948.28 Standard Solutions of Titanium Trichloride

**Final Action**

### *A. Preparation of Standard Solution*

To 200 mL com. 15% $TiCl_3$ soln add 150 mL HCl and dil. to 2 L. Make soln ca 0.1$N$, place in container with H atm. provision (e.g., JAOAC **5**, 207(1921)), and let stand 2 days for absorption of residual O.

### *B. Standardization*

Weigh 3 g $FeSO_4(NH_4)_2SO_4.6H_2O$ and transfer to 500 mL flask. Introduce stream of $CO_2$, and add 50 mL recently boiled $H_2O$ and 25 mL 40% (by wt) $H_2SO_4$. Then, without interrupting current of $CO_2$, rapidly add 40 mL 0.1$N$ $K_2Cr_2O_7$, **949.13C**. Add $TiCl_3$ soln until near calcd end point. Then quickly add 5 g $NH_4SCN$, and complete titrn. Det. blank on 3 g $FeSO_4(NH_4)_2SO_4.6H_2O$, using same vols of $H_2O$, $H_2SO_4$, and $NH_4SCN$, and current of $CO_2$. From net vol. $TiCl_3$, calc. normality:

$$\text{Normality} = \text{mL } K_2Cr_2O_7 \times \text{normality } K_2Cr_2O_7/\text{ mL } TiCl_3$$

Refs.: JAOAC **31**, 573(1948); **32**, 589(1949).

## 982.35 Certified Reference Materials

### *A. Definitions*

*Reference Material (RM)*.—Material or substance one or more properties of which are sufficiently well established to be used for calibrating app., or assessing measurement method, or assigning values to materials.

*Certified Reference Material (CRM)*.—Ref. material one or more of whose property values are certified by valid procedure, or accompanied by or traceable to certificate or other documentation which is issued by certifying body.

### *B. General Information*

Following tables list selected CRMs, certification, and code of issuing organization. (Tables **982.35A–F**). Table **982.35A** shows source codes together with names and addresses of these organizations.

Particular CRMs were selected on basis of present and potential applicability to AOAC activities. Listing of CRMs from organization does not imply either AOAC endorsement of CRMs or AOAC recommendation of organization supplying them.

Organizations listed are only representative of those supplying CRMs. No claim is made as to completeness of information supplied. More complete listing of organizations supplying CRMs is available from the International Organization for Standardization. Catalogs or literature describing CRMs are available from individual organizations. However, because inventories of CRMs are continually changing, inquiries should be made of the organizations concerning current availability of specific CRMs.

**Table 982.35A Sources of Certified Reference Materials—Organizations and Addresses**

| Source Code | Name and Address |
|---|---|
| BCR | Community Bureau of Reference—BCR<br>Directorate General XII<br>Commission of the European Communities<br>200, rue de la Loi<br>B-1049 Brussels, Belgium |
| IAEA | International Atomic Energy Agency<br>Analytical Quality Control Services<br>Laboratory Seibersdorf<br>PO Box 100<br>A-1400 Vienna, Austria |
| NIST | National Institute of Standards and Technology<br>Office of Standard Reference Materials<br>Room B311, Chemistry Building<br>Washington, DC 20234, USA |
| NIES | National Institute for Environmental Studies<br>Yatabe-Machi, Tsukuba, Ibaraki 305, Japan |
| LGC | National Physical Laboratory<br>Laboratory of the Government Chemist<br>Office of Reference Materials<br>Teddington, Middlesex TW11 OLY, UK |
| NRCC | National Research Council<br>Marine Analytical Chemistry Standards<br>Montreal Rd<br>Ottawa, Ontario, Canada K1A OR9 |
| USP | U.S. Pharmacopeial Convention, Inc.<br>USP-NF Reference Standards<br>12601 Twinbrook Parkway<br>Rockville, MD 20852, USA |
| WHO | World Health Organization<br>Collaborating Center for Chemical Reference Substances<br>Apotekens Centrallaboratorium<br>Box 3045<br>T-17103 Solna, Sweden |

Refs.: ISO Directory of Certified Reference Materials (CRM), International Organization for Standardization, 1982. Available from (*1*) ISO Central Secretariat, Case postale 56, CH-1211, Geneva, Switzerland; (*2*) American National Standards Institute, 1430 Broadway, New York, NY 10018.

**Table 982.35B Certified Reference Materials for Animal Tissues, Plant Tissues, Foods, Alcoholic Beverages, Animal Feedstuffs**

| | Designation | Certification | Source Code |
|---|---|---|---|
| 1566a | Oyster Tissue | Note a | NIST |
| 1577a | Bovine Liver | Note a | NIST |
| H-4 | Muscle | Note a | IAEA |
| 1572 | Citrus Leaves | Note a | NIST |
| 1573 | Tomato Leaves | Note a | NIST |
| 1575 | Pine Needles | Note a | NIST |
| No. 1 | Pepperbush | Note a | NIES |
| 60 | Aquatic Plant (*Lagarosiphon m Major*) | Note a | BCR |
| 61 | Aquatic Plant (*Platihypnidium riparioides*) | Note a | BCR |
| 62 | Olive Leaves (*Olea europaea*) | Note a | BCR |
| 1567a | Wheat Flour | Note a | NIST |
| 1568 | Rice Flour | Note a | NIST |
| 1549 | Nonfat Milk Powder | Note a | NIST |
| 1569 | Brewers Yeast | Cr | NIST |
| 17c | Sucrose | Note b | NIST |
| 41c | Dextrose | Note c | NIST |
| 1590 | Wine | EtOH | NIST |
| A15-01 | DL-5-Vinyloxazolidin-2-thione (VOT) | Purity | LGC |
| 063 | Skim Milk Powder, natural | Note a | BCR |
| 150 | Skim Milk Powder, spiked | Note a | BCR |
| 151 | Skim Milk Powder, spiked | Note a | BCR |
| 162 | Soya-maize Oil Blend | Note d | BCR |
| 163 | Beef-pig Fat Blend | Note d | BCR |
| 184 | Bovine Muscle | Note a | BCR |
| 185 | Bovine Liver | Note a | BCR |
| 186 | Pig Kidney | Note a | BCR |
| 189 | Wholemeal Flour | Note a | BCR |
| 191 | Brown Bread | Note a | BCR |
| 273 | Single Cell Protein | Matrix | BCR |
| 274 | Single Cell Protein | Tr. Elem. | BCR |
| 279 | Sea Lettuce | Note a | BCR |
| 282 | Aflatoxin $M_1$ in Whole Milk Powder | Very low level | BCR |
| 283 | Aflatoxin $M_1$ in Whole Milk Powder | Low level | BCR |
| 284 | Aflatoxin $M_1$ in Whole Milk Powder | Medium level | BCR |
| 285 | Aflatoxin $M_1$ in Whole Milk Powder | High level | BCR |
| 1563 | Cholesterol & Fat-Sol. Vitamins in Coconut Oil | Fortified levels | NIST |
| 1588 | Organics in Cod Liver Oil | Note e | NIST |
| TORT-1 | Lobster Hepatopancreas | Note a | NRCC |
| DORM-1 | Dogfish Muscle | Note a | NRCC |
| DOLT-1 | Dogfish Liver | Note a | NRCC |

[a] Elemental composition.
[b] Optical rotation, index of refraction, and density.
[c] Purity and specific rotation.

**Table 982.35C Certified Reference Materials for Biochemicals, Clinicals, Drugs, Industrial Hygiene, Pharmaceuticals**

| | Designation | Certification | Source Code |
|---|---|---|---|
| 900 | Antiepilepsy Drug Level | Note a | NIST |
| 1599 | Anticonvulsant Drug Level | Note b | NIST |
| 909 | Human Serum | Selected electrolytes, orgs | NIST |
| 2671a | Freeze-dried Urine | F− (0.55 and 5.7 mg/L) | NIST |
| 2672a | Freeze-dried Urine | Hg (2 concs) | NIST |
| 2670 | Freeze-dried Urine | Selected elements | NIST |
| | | Purity, other properties (Note c) | NIST |
| — | Clinical CRMs | Purity, other properties (Note d) | NIST |
| — | Drugs, clinicals . . . | | USP |
| — | Pharmaceuticals | Purity, other properties | WHO |
| 147 | Lyophilized thromboplastin Human plain | Note e | BCR |
| 148 | Lyophilized thromboplastin Bovine | Note e | BCR |
| 149 | Lyophilized thromboplastin Rabbit | Note e | BCR |
| 194 | Lyophilized bovine blood (low conc'n) | Pb, Cd | BCR |
| 195 | Lyophilized bovine blood (medium conc'n) | Pb, Cd | BCR |
| 196 | Lyophilized bovine blood (high conc'n) | Pb, Cd | BCR |
| 303 | Calcium in Human Blood I | Ca | BCR |
| 304 | Calcium in Human Blood II | Ca | BCR |
| 192 | Lyophilized Human Serum (low conc'n) | Cortisol | BCR |
| 193 | Lyophilized Human Serum (high conc'n) | Cortisol | BCR |
| 319 | Lyophilized CGT from pig kidney | Note f | BCR |
| 8430 | AST (E.C.2.6.1.1)—Human Erythrocyte | Note f | NIST |
| 1951 | Cholesterol in Human Serum (Frozen) | Chol. | NIST |
| 1952 | Cholesterol in Human Serum (Freeze-Dried) | Chol. | NIST |
| 1507 | Tetrahydrocannabinol (THC) in Urine | THC | NIST |
| 1598 | Inorganic Constituents in Bovine Serum | Tr. Elem. | NIST |
| 5 | Human Hair | Tr. Elem. | NIES |
| A-13 | Freeze dried animal blood | Tr. Elem. | IAEA |

[a] Phenytoin, ethosuximide, phenobarbital, and primidone at 3 concn levels plus serum blank.
[b] Carbamazepine and valproic acid at 3 concn levels plus serum blank.
[c] Approximately 20 clinical calibration materials.
[d] Drugs of abuse, veterinary drugs, enzymes, food chemicals clinicals, vitamins . . .
[e] Calibrated against Inter. Ref. Preparation of WHO (IRP 67/40).
[f] Based on IFCC method.

**Table 982.35D Certified Reference Materials for Fertilizers and Related Materials**

| | Designation | Certification | Source Code |
|---|---|---|---|
| 120c | Phosphate Rock (Florida) | Note a | NIST |
| 694 | Phosphate Rock (Western) | Note a | NIST |
| 32 | Phosphate Rock (Moroccan) | Note a | BCR |
| 33 | Superphosphate | Note a | BCR |
| 193 | Potassium Nitrate | N and K concn | NIST |
| 194 | Ammonium Dihydrogen Phosphate | N and P concn | NIST |
| 200 | Potassium Dihydrogen Phosphate | P and K concn | NIST |

[a] Chem. composition.

**Table 982.35E Certified Reference Materials for Pesticides**

| Designation | Certification | Source Code |
|---|---|---|
| P11-01 to P11-31 Chlorinated compds | High purity | LGC |
| P12-04 to P12-07 Organophosphorus compds | High purity | LGC |
| P13-01 to P13-12 Phenoxy-acids and related compds | High purity | LGC |
| P14-01 to P14-05 Substituted urea compds | High purity | LGC |
| P16-01 to P16-34 Heterocyclic and miscellaneous compds | High purity | LGC |
| P17-01 to P17-02 Pyrethroids | Note a | LGC |
| 1583 Chlorinated compds in isooctane | Note b | NIST |
| 1492 Chlorinated Pesticides in Hexane | Note c | NIST |
| 1491 Aromatic Hydrocarbons in Hexane/Toluene | 23 org. compds | NIST |
| 1579 Powdered Pb-base Paint | Pb | NIST |
| 1582 Petroleum Crude Oil | 6 org. compds. | NIST |
| 1584 Priority Pollutant Phenols in Methanol | 10 org. compds. | NIST |
| 1585 Chlorinated Biphenyls in Isooctane | 8 org. compds. | NIST |
| 1586 Isotopically Labeled & Unlabeled Priority Pollutants in Methanol | 10 org. compds. | NIST |
| 1587 Nitrated Polycyclic Aromatic Hydrocarbons in Methanol | 6 org. compds. | NIST |
| 1589 Polychlorinated Biphenyls (As Aroclor 1260) in Human Serum | Aroclor 1260 | |
| 1596 Dinitropyrene Isomers & 1-Nitropyrene in Methylene Chloride | 4 org. compds. | NIST |
| 1597 Complex Mixture of Polycyclic Aromatic Hydrocarbons from Coal Tar | 12 PAHs | NIST |
| 1614 Dioxin (2,3,7,8 TCDD) in Isooctane Dioxin | | NIST |
| 1618 V & Ni in Residual Fuel Oil | V, Ni | NIST |
| 1636 Pb in Reference Fuel | Pb | NIST |
| 1639 Halocarbons (in $CH_3OH$) for $H_2O$ Anal. | 7 org. compds. | NIST |
| 1650 Diesel Particulate Matter | 6 org. compds. | NIST |
| 2694 Simulated Rainwater | Note c | NIST |

[a] No purity figure is given but full anal. data supplied.
[b] Concns at μg/mL levels.
[c] Concns approx. 200 ng/g

**Table 982.35F Certified Reference Materials for Water, Sediments, Gases, Particulates, Fuels**

| | Designation | Certification | Source Code |
|---|---|---|---|
| 1641b | Mercury in Water | µg/mL level | NIST |
| 1643b | Trace Elements in Water | ng/mL level | NIST |
| 1644 | Generator Columns | Note a | NIST |
| 1647a | Priority Pollutants (in $CH_3CN$) | Note b | NIST |
| NASS-2 | Seawater | Trace elements | NRCC |
| CRM 046-097 | Polynuclear Aromatic Hydrocarbons | Purity | BCR |
| 2 | Pond Sediment | Note c | NIES |
| SL-1 | Lake Sediment | Note c | IAEA |
| 2704 | Buffalo River Sediment | Note c | NIST |
| 1646 | Estuarine Sediment | Note c | NIST |
| BCSS-1 | Marine Sediment I | Note c | NRCC |
| MESS-1 | Marine Sediment II | Note c | NRCC |
| Soil-5 | Soil | Note c | IAEA |
| 1658a–1659a | Methane in Air | 1–10 µmol/mol | NIST |
| 1661/1696 | Sulfur Dioxide in $N_2$ | 50–3500 µmol/mol | NIST |
| 1665b–1669b | Propane in Air | 3–500 µmol/mol | NIST |
| 2643–2648 | Propane in $N_2$ | 100–5000 µmol/mol | NIST |
| 2649–2650 | Propane in $N_2$ | 1.0–2.0 mol % | NIST |
| 2651–2652 | Propane/Oxygen in $N_2$ | $O_2$ (5.0–10.0 mol %) $C_3H_8$ (0.01 mol %) | NIST |
| 1683/2631 | Nitric Oxide in $N_2$ | 5–3000 µmol/mol | NIST |
| 1677c/2638 | Carbon Monoxide in $N_2$ | 25–5000 µmol/mol) | NIST |
| 2639–2642 | Carbon Monoxide in $N_2$ | 1.0–8.0 mol % | NIST |
| 2612a–2614a | Carbon Monoxide in Air | 9.9–43 mol % | NIST |
| 2632–2634 | Carbon Dioxide in $N_2$ | 300–800 µmol/mol | NIST |
| 2619a–1675b | Carbon Dioxide in $N_2$ | 0.5–15 mol % | NIST |
| 2657–2659 | Oxygen in $N_2$ | 2.0–21 mol % | NIST |
| 1805–1806 | Benzene in $N_2$ | 0.25–10 µmol/mol | NIST |
| 1625–1627 | Sulfur Dioxide Permeation Tube | 2.8–0.56 µg/min (Note d) | NIST |
| 1629a | Nitrogen Dioxide Permeation Tube | 0.5–1.5 µg/min | NIST |
| 1911 | Benzene Permeation Tube | 0.3–0.5 µg/min (Note d) | NIST |
| 1648 | Urban Particulate, Inorganic | As, Cd, Pb + 11 others | NIST |
| 1649 | Urban Dust, Organics | Polynuclear aromatic hydrocarbons | NIST |
| 2676b | Toxic Metals on Filters | Cd, Mn, Pb, Zn at µg levels | NIST |
| Air-3/1 | Trace Elements on Filters | 13 elements at µg levels | IAEA |
| 1619–1624a | Sulfur in Fuel Oil | 0.15–5 wt % | NIST |
| 1634a | Trace Elements in Fuel Oil | Pb, V, + 6 others | NIST |
| 1581 | Polychlorinated Biphenyls in Oil | 100 µg/g | NIST |
| 1580 | Shale Oil | 9 org. compds | NIST |
| 1636a–1638a | Lead in Reference Fuel | 11.2–764 µg/g | NIST |
| 2682–2685 | Sulfur in Coal | S(0.5–4.6 wt %); plus ash and calorific values | NIST |
| 1632a | Trace Elements in Coal (Bituminous) | As, Cd, Pb + 15 others | NIST |
| 1635 | Trace Elements in Coal (Subbituminous) | As, Cd, Pb + 11 others | NIST |
| 1630 | Trace Mercury in Coal | 0.13 µg/g | NIST |
| 1633a | Trace Elements in Coal Fly Ash | As, Cd, Pb + 17 others | NIST |

[a] Generates certified concns of anthracene, benz(a)anthracene, and benzo(a)pyrene in $H_2O$.
[b] Certified concns of 16 polynuclear aromatic hydrocarbons.
[c] Certified for chem. composition.
[d] Permeation rate is certified.

# Appendix: Laboratory Safety

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## Introduction

This chapter is not intended to be an exhaustive treatise on laboratory safety. These precautionary notes serve only as a reminder of possible hazards involved in the use of particular operations or substances. Refer to recommended texts at end of chapter for fuller treatment of subject. Follow safety requirements of your organization and state, provincial, or federal government. Consult guidelines issued by professional associations and government agencies.

## Cautionary Statements

Nature and amt of each chemical and its prescribed use were criteria used in detg if cautionary statement for method was indicated.

Safety hazard was considered to exist when nature, amt, and use of chemical or equipment specified in method appeared likely to produce any of following:

(**a**) Concn of vapors from flammable liq. exceeding 25% of lower flammability limit of that liq. described by National Fire Protection Association, Boston, MA.

(**b**) Contact between analyst and amts of material highly active physiologically or toxic to humans in excess of Threshold Limit Values published by American Conference of Governmental Industrial Hygienists, P.O. Box 1937, Cincinnati, OH 45201.

(**c**) Contact between analyst and amts of highly corrosive material sufficient to produce serious injury.

(**d**) Contact between analyst and radiations which could be harmful.

(**e**) Explosion or violent reaction.

(**f**) Injury to analyst by hazards in equipment or processes which are not readily detectable by analyst.

When in doubt about possible hazards not covered in this chapter, consult refs at end of chapter and other sources of information such as hazard warnings on labels and manufacturers' material safety data sheets.

## Potential Hazards of Equipment

### *Refrigerators*

Should be explosion proof or explosion resistant when used for storage of ether and other highly volatile, flammable liqs. Ordinary refrigerator can be made explosion resistant by removal of light switch, receptacle, and associated wiring and placing thermoregulation controls on outside of refrigerator.

### *Glass*

Dispose of chipped or broken glassware in special containers; minor chips may be fire-polished and glassware retained. If glassware is to be repaired, mark defective area plainly and store in special location until repairs are completed.

Use heat-resistant glassware for prepn of solns that generate heat (e.g., not bottles or graduates).

### *Fire Extinguishers*

Class B and C dry chemical fire extinguishers (for flammable liq. and elec. fires) should be conveniently available to each laboratory room. Carbon dioxide fire extinguishers should be used on fires in electronic equipment.

Become familiar with their location and methods for effective use.

### *Blenders*

Motor on high-speed blenders used to mix flammable solv. with other materials should be explosion proof. Blend toxic or flammable liqs in effective fume removal device.

### *Centrifuges*

Adjust all tubes to equal wt before loading them into centrf. Make certain that stoppers of tubes placed in pivot-type head will clear center when tubes swing to horizontal. Do not open centrf. cover until machine stops completely. Before removing tubes, turn elec. switch to "off." Do not rely on zero-set rheostat. Use only tubes specially designed for centrfg. Do not exceed safe speed for various tube materials (glass, cellulose nitrate, polyethylene, etc.) recommended by tube manufacturer. Cellulose nitrate tubes may explode if autoclaved. Heating cellulose nitrate tubes >60° may cause them to produce harmful nitrogen oxide fumes.

### *Atomic Absorption Spectrophotometer*

Follow all manufacturer's instructions for installation, operation, safety, and maintenance. Use only hose/tubing to conduct gases approved by manufacturer and supplier. Use effective fume removal device to remove gaseous effluents from burner. Use only $C_2H_2$ which is dissolved in solvent recommended by manufacturer. Open $C_2H_2$ tank stem valve only $^1/_4$ turn. Change tank when $C_2H_2$ pressure shows 75–100 lb. If instrument has a drain trap, ensure that it is filled with $H_2O$ before igniting burner. Following repair to $C_2H_2$ supply line, check for gas tightness at all connections with soap solution or combustible gas detection system. Whenever solutions are aspirated which contain high concentrations of Cu, Ag, or Hg, spray chamber should be rinsed with 50–100 mL $H_2O$ before shutting down to clean these metals from chamber. See safety notes on compressed gas cylinders.

### *Flame Photometer*

Use effective fume removal device to remove gaseous burner effluents.

### *Photofluorometer*

Considerable amts of $O_3$ are formed by UV light radiated by quartz lamp. Ozone is toxic even in low concns; remove thru effective fume removal device placed near quartz lamp.

### *Monitoring Equipment*

Monitor unattended operations with equipment that will automatically shut down process if unsafe condition develops.

Ref.: N. V. Steere, "Handbook of Laboratory Safety" (1971); CRC Press, Inc., 2255 Palm Beach Lakes Blvd, West Palm Beach, FL 33409.

### Compressed Gas Cylinders

Identify by name(s) of gas(es) contents of compressed gas cylinders on attached decal, stencil, or tag, instead of by color codes. Move cylinders (with protective cap) upright secured to cart. Secure cylinders in upright position by means of strap, chain, or non-tip base. Let contents of $C_2H_2$ cylinders settle and let all cylinders come to room temp. prior to opening. Use only correct pressure gages, pressure regulator, flow regulator, and hose/tubing, for each size of gas cylinder and type of gas as specified by supplier. Use soap solution or combustible gas detection system to check all connections, especially when system is pressurized and gas is not flowing, to check for slow leak. Use special heater on $N_2O$ gas line. Close gas tank valve and diaphragm on regulator (turn counter-clockwise) when gas not in use. Service regulator at least yearly. Use toxic gases only in effective fume removal device. When burning gas, use flashback prevention device in gas line on output side of regulator to prevent flame being sucked into cylinder.

Ref.: Handbook of Compressed Gases (1981) Compressed Gas Assoc., Van Nostrand Reinhold Co., New York, NY.

### Distillation, Extraction, and Evaporations

(**a**) *Flammable liquids*.—Perform operations behind safety barrier with hot $H_2O$, steam, or elec. mantle heating. Use effective fume removal device to remove flammable vapors as produced. Set up app. on firm supports and secure all connections. Leave ample headroom in flask and add boiling chips *before* heating is begun. All controls, unless vapor sealed, should be located outside vapor area. Dispose of waste flammable solvs by evapn as above unless other provisions for safe disposal are available.

(**b**) *Toxic liquids*.—Use effective fume removal device to remove toxic vapors as produced. Avoid contact with skin. Set up app. on firm supports and secure all connections. Dispose of waste toxic solvs by evapn, using effective fume removal device unless other provisions for safe disposal are available.

### Electrical Equipment

Accidents involving elec. equipment may result in *mech. injury*, e.g., fingers being caught in chopping mill knives; *elec. shock*, which may be due to lack of or improper grounding, defective equipment, exposed wiring, or inadequate maintenance; and *fire* thru ignition of flammable vapors by electrically produced spark. Ground all elec. equipment to avoid accidental shock. Installation, maintenance, and repair operations should be performed by qualified electricians.

### Parr Bomb

Follow manufacturer's directions closely to avoid explosion.

### Pressure

Do not conduct pressure operations with std glassware. In certain circumstances, glassware specifically designed to withstand pressure may be used. Observe manufacturer's recommended safeguards when using pressure app. such as calorimeter bomb, hydrogenator, etc.

### Vacuum

Tape or shield with safety barrier containers and app. to be used under vac. to minimize effects of possible implosion. Vac. pump drive belts must have effective guards.

### Hazardous Radiations

UV radiation is encountered in AA spectrophotometry, fluorometry, UV spectrophotometry, germicidal lamps, and both long- and shortwave UV lamps used to monitor chromatgc sepns. Never expose unprotected eyes to UV light from any source either direct or reflected (e.g., flames in flame photometer, lamps, elec. arcs, etc.). Always wear appropriate eye protection such as goggles having uranium oxide lenses, welder's goggles, etc., when such radiations are present and unshielded. Keep skin exposure to UV radiations to min.

## Safety Technics and Practices

### Spraying Chromatograms

When strong corrosive and toxic reagents are sprayed on chromatograms, use gloves, face shield, respiratory protection, and appropriate fume removal device to protect skin, eyes, and respiratory tract against mists or fumes generated by spraying device.

### Pipets

Do not pipet hazardous liqs by using mouth suction to fill pipet. Use pipet fillers or rubber tubing connected thru trap to vac. line for this purpose.

### Wet Oxidation

This technic is among most hazardous uses of acids but can be performed safely. Observe precautions in this chapter for particular acids used and rigorously follow directions given in specific method being used.

### Hazardous or After Hours Work

Anyone working alone after hours or on hazardous procedures should arrange to be contacted periodically as safety measure.

### Glass Tubing

Protect hands with heavy towel or gloves when inserting glass tubing into cork or rubber stopper. Fire polish all raw glass cuts.

Open ampules in fume removal device over tray large enough to hold contents if ampule should break. If contents are volatile, cool before opening.

## Safe Handling of Acids

Use effective *acid-resistant* fume removal device whenever heating acids or performing reactions which liberate acid fumes. In dilg, always add acid to $H_2O$ unless otherwise directed in method. Keep acids off skin and protect eyes from spattering. If acids are spilled on skin, wash immediately with large amts of $H_2O$.

### Acetic Acid and Acetic Anhydride

React vigorously or explosively with $CrO_3$ and other strong oxidizers. Wear face shield and heavy rubber gloves when using.

CAS-108-24-7 (acetic anhydride)

### Chromic and Perchromic Acids

Can react explosively with $Ac_2O$, HOAc, EtOAc, isoamyl alcohol, and benzaldehyde. Less hazardous with ethylene glycol, furfural, glycerol, and MeOH. Conduct reactions behind safety barrier. Wear face shield and heavy rubber gloves.

### Formic and Performic Acids

Strong reducing agents; react vigorously or explosively with oxidizing agents. Irritating to skin, forming blisters. Performic acid (formyl hydroperoxide) has detonated for no apparent reason while being poured. Wear face shield and heavy rubber gloves when using.

CAS-64-18-6 (formic acid)

### Hydrofluoric Acid

Very hazardous with $NH_3$. It can cause painful sores on skin and is extremely irritating to eyes. Use effective removal device. Wear goggles and acid-resistant gloves.

CAS-7664-39-3 (hydrofluoric acid)

### Nitric Acid

Reacts vigorously or explosively with aniline, $H_2S$, flammable solvs, hydrazine, and metal powders (especially Zn, Al, and Mg). Gaseous nitrogen oxides from $HNO_3$ can cause severe lung damage. Copious fumes are evolved when concd $HNO_3$ and concd HCl are mixed. Avoid premixing. Use effective fume removal device when fumes are generated. Handle with disposable polyvinyl chloride, not rubber, gloves.

### Oxalic Acid

Forms explosive compd with Ag and Hg. Oxalates are toxic. Avoid skin contact and ingestion.

CAS-144-62-7 (oxalic acid)

### Perchloric Acid

Contact with oxidizable or combustible materials or with dehydrating or reducing agents may result in fire or explosion. Persons using this acid should be thoroly familiar with its hazards. Safety practices should include following:

(**a**) Remove spilled $HClO_4$ by immediate and thoro washing with large amts of $H_2O$.

(**b**) Hoods, ducts, and other devices for removing $HClO_4$ vapor should be made of chem. inert materials and so designed that they can be thoroly washed with $H_2O$. Exhaust systems should discharge in safe location and fan should be accessible for cleaning.

(**c**) Avoid use of org. chems in hoods or other fume removal devices used for $HClO_4$ digestions.

(**d**) Use goggles, barrier shields, and other devices as necessary for personal protection; use polyvinyl chloride, not rubber, gloves.

(**e**) In wet combustions with $HClO_4$, treat sample first with $HNO_3$ to destroy easily oxidizable org. matter unless otherwise specified. *Do not evap. to dryness.*

(**f**) Contact of $HClO_4$ soln with strong dehydrating agents such as $P_2O_5$ or concd $H_2SO_4$ may result in formation of anhyd. $HClO_4$ which reacts explosively with org. matter and with reducing agents. Exercise special care in performing analyses requiring use of $HClO_4$ with such agents. Extremely sensitive to shock and heat when concn is $>72\%$.

(**g**) Also observe precautions outlined in (1) "Perchloric Acid Solution," Chemical Safety Data Sheet SD-11 (1965), Manufacturing Chemists Association of the US, 1825 Connecticut Ave, NW, Washington, DC 20009; (2) "Applied Inorganic Analysis," W. F. Hillebrand, G. E. F. Lundell, H. A. Bright, and J. I. Hoffman, 2nd ed. (1953), pp. 39–40, John Wiley and Sons, Inc., New York, NY; (3) "Notes on Perchloric Acid and Its Handling in Analytical Work," Analyst **84**, 214–216(1959); (4) "Perchlorates," ACS Monograph No. 146, J. C. Schumacher, ed., Reinhold (1960). *See also* refs at end of this chapter.

### Picric Acid

Highly sensitive to shock when in dry state. In contact with metals and $NH_3$, it produces picrates which are more sensitive to shock than picric acid. Readily absorbed thru skin and irritating to eyes. Wear heavy rubber gloves and eye protection.

CAS-88-89-1 (picric acid)

### Sulfuric Acid

Always add $H_2SO_4$ to $H_2O$. Wear face shield and heavy rubber gloves to protect against splashes.

CAS-7664-93-9 (sulfuric acid)

### Fuming Acids

Prep. and use with effective fume removal device. Wear acid-resistant gloves and eye protection.

## Safe Handling of Alkalies

Alkalies can burn skin, eyes, and respiratory tract severely. Wear heavy rubber gloves and face shield to protect against concd alkali liqs. Use effective fume removal device or gas mask to protect respiratory tract against alkali dusts or vapors.

### Ammonia

Extremely caustic liq. and gas. Wear skin, eye, and respiratory protection when handling in anhyd. liq. or gaseous state. $NH_3$ vapors are flammable. Reacts violently with strong oxidizing agents, halogens, and strong acids.

### Ammonium Hydroxide

Caustic liq. Forms explosive compds with many heavy metals such as Ag, Pb, Zn, and their salts, especially halide salts.

CAS-1336-21-6 (ammonium hydroxide)

### Sodium, Potassium, Lithium, and Calcium Metals

Violently reactive with $H_2O$ or moisture, $CO_2$, halogens, strong acids, and chlorinated hydrocarbons. Emit corrosive fumes when burned. Can cause severe burns. Wear skin and eye protection when handling. Use only dry alcohol when preparing Na alcoholate and add metal directly to alcohol, one small piece at a time. Avoid adding metallic Na to reaction thru condenser.

CAS-7440-23-5 (sodium)

### Sodium Peroxide

Less caustic than Na and K hydroxides but reacts violently with $H_2O$, org. matter, charcoal, glycerol, $Et_2O$, or P. Wear skin, eye, and respiratory protection when handling multigram amts.

CAS-1313-60-6 (sodium peroxide)

### Calcium Oxide (Burnt Lime)

Strongly caustic! Reacts violently with $H_2O$. Protect skin, eyes, and respiratory tract against contact with dust.

CAS-1305-78-8 (calcium oxide)

### Sodium and Potassium Hydroxides

Extremely caustic. Can cause severe burns. Protect skin and eyes when working with these alkalies as solids or concd solns. Add pellets to $H_2O$, not vice versa.

CAS-1310-58-3 (potassium hydroxide)
CAS-1310-73-2 (sodium hydroxide)

### Sodium Biphenyl, Sodium Methylate, and Sodium Ethylate

Less caustic than NaOH but can be injurious. React vigorously with $H_2O$. Protect skin and eyes when handling.

## Safe Handling of Organic Solvents

(Do not mix waste solvs.)

### *Flammable Solvents*

Do not let vapors conc. to flammable level in work area, since it is nearly impossible to eliminate all chance of sparks from static electricity even tho elec. equipment is grounded. Use effective fume removal device to remove these vapors when released.

### *Toxic Solvents*

Vapors from some volatile solvs are highly toxic. Several of these solvs are readily absorbed thru skin. Use effective fume removal device to remove vapors of these solvs as they are liberated.

Refs.: Gosselin, Smith, and Hodge, "Clinical Toxicology of Commercial Products (Home and Farm)," 5th ed. (1976); The Williams & Wilkins Co., 428 E Preston St, Baltimore, MD 21202.
American Conference of Governmental Industrial Hygienists, "Threshold Limit Values"; PO Box 1937, Cincinnati, OH 45201.
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Journal of the American Society of Safety Engineers **7**, Feb. 1964.
See also references at end of chapter.

## Safe Handling of Special Chemical Hazards

### *Pesticides*

Many pesticide chemicals are extremely toxic by various routes of exposure, especially in concd form. These chemicals include org. Cl, carbamate, and org. P insecticides, mercurials, arsenicals, nicotine, and other chemicals. As an example, org. P family of pesticides is consistently highly toxic, not only by oral ingestion, but dermally and by inhalation as well. Observe following min. precautions at all times. Consult safety data sheets or labels for addnl information.

(**a**) Do all laboratory sampling, mixing, weighing, etc., under effective fume removal device in area having good forced ventilation of nonrecirculated air, or wear gas mask of proper type. If mask is used, replace cartridges as recommended, since using contaminated mask may be worse than no mask.

(**b**) Keep off skin. Wear clean protective clothing and nonpermeable gloves (such as polyethylene gloves) as necessary. Wash thoroly with soap and water to avoid contaminating food and smoking materials.

(**c**) Label all sample containers with name and approx. content of all pesticides.

(**d**) Have readily available and study information on symptoms of poisoning and first aid treatment for each type of pesticide being handled.

(**e**) Consult physician about preventive measures and antidotes for use in emergencies when pesticide poisoning is suspected.

(**f**) Follow your organization's procedures when disposing of waste pesticides. The manufacturer can be contacted for advice on disposal problems.

(**g**) Do not enter pesticide *residue* or other laboratories after handling pesticide formulations until protective clothing and gloves have been removed and face and hands thoroly washed with soap and water.

U.S. Environmental Protection Agency operates "hotline" staffed to handle pesticide questions, called National Pesticide Telecommunications Network (NPTN). To reach this hotline, dial: 800-858-7378.

Refs.: Gosselin, Smith, and Hodge, "Clinical Toxicology of Commercial Products (Home and Farm)," 5th ed. (1984), The Williams and Wilkens Co., Baltimore, MD 21202.
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Morgan, D.P., Recognition and Management of Pesticide Poisonings, 4th ed. (1989), U.S. Environmental Protection Agency, Office of Pesticide Programs, Washington, DC.
Citizens Guide to Pesticides (1987), U.S. Environmental Protection Agency, Office of Pesticides and Toxic Substances, Washington, DC.

### *Aniline*

Toxic. Avoid contact with skin and eyes. Use effective fume removal device. Highly toxic when heated to decomposition. Flammable. May react vigorously with oxidizing agents. Ignites in presence of fuming $HNO_3$. May react violently with $O_3$.

CAS-62-53-3 (aniline)

### *Acetonitrile*

Toxic. Avoid contact with skin and eyes. Use effective fume removal device.

### *Ammoniacal Silver Nitrate*

Use soon after prepn and do not allow to stand for long periods of time.

### *Benzene*

Toxic. Highly flammable. Avoid contact with skin. Do not breathe vapors. Use effective fume removal device. Decomposes violently in presence of strong oxidizing agents. Reacts violently with Cl. Considered to be tumor producing agent.

CAS-71-43-0 (benzene)

### *Acetone*

Highly flammable. Forms explosive peroxides with oxidizing agents. Use effective fume removal device. Do not mix with $CHCl_3$.

CAS-67-64-1 (acetone)

### *Bromine and Chlorine*

Hazardous with $NH_3$, H, petroleum gases, turpentine, benzene, and metal powders. Extremely corrosive. Use effective fume removal device. Protect skin against exposure.

### *Carbon Disulfide*

Extremely flammable with low ignition temp. Toxic. Use effective fume removal device. Can react vigorously to violently with strong oxidizing agents, azides, and Zn. Avoid static electricity.

CAS-75-15-0 (carbon disulfide)

#### *Carbon Tetrachloride*

Reacts violently with alkali metals. Toxic. Fumes may decompose to phosgene when heated strongly. Use effective fume removal device.

CAS-56-23-5 (carbon tetrachloride)

#### *Cyanides*

React with acids to form highly toxic and rapid acting HCN gas. Use only in effective fume removal device. Destroy residues with alk. NaOCl soln.

#### *Cyclohexane*

Highly flammable. Use effective fume removal device. Can react vigorously with strong oxidizing agents.

CAS-110-82-7 (cyclohexane)

#### *Di- and Triethylamine*

Flammable. Toxic. Corrosive to skin and eyes. Use effective fume removal device. Can react vigorously with oxidizing materials.

#### *Dimethylformamide*

Toxic. Flammable. Avoid contact with skin and eyes. Use effective fume removal device. Can react vigorously with oxidizing agents, halogenated hydrocarbons, and inorg. nitrates.

#### *Diethyl Ether*

Store protected from light. Extremely flammable. Unstable peroxides can form upon long standing or exposure to sunlight in bottles. Can react explosively when in contact with Cl, $O_3$, $LiAlH_4$, or strong oxidizing agents. Use effective fume removal device. Avoid static electricity. See also safety notes on peroxides.

CAS-60-29-7 (ether)

#### *Ethanol*

Flammable. Use effective fume removal device when heating or evapg.

#### *Chloroform*

Can be harmful if inhaled. Forms phosgene when heated to decomposition. Use effective fume removal device. Can react explosively with Al, Li, Mg, Na, K, disilane, $N_2O_4$, and NaOH plus MeOH. Considered to be tumor producing agent.

CAS-67-66-3 (chloroform)

#### *Ethyl Acetate*

Flammable, especially when being evapd. Irritating to eyes and respiratory tract. Use effective fume removal device.

CAS-141-78-6 (ethyl acetate)

#### *Formaldehyde*

A suspect human carcinogen. Exposure to high concns may cause skin irritation and inflammation of mucous membranes, eyes, and respiratory tract. Use skin protection and effective fume removal device.

CAS-50-00-0 (formaldehyde)

#### *Hydrogen Sulfide*

Hazardous with oxidizing gases, fuming $HNO_3$, and $Na_2O_2$. Forms explosive mixts with air. Toxic. Use effective fume removal device.

CAS-7783-06-4 (hydrogen sulfide)

#### *Hypophosphorus Acid*

Reacts violently with oxidizing agents. On decomposition, emits highly toxic fumes (phosphine) and may explode. Use effective fume removal device.

#### *Hexane*

Highly flammable. Use effective fume removal device.

CAS-110-54-3 (hexane)

#### *Isooctane*

Highly flammable. Use effective fume removal device.

CAS-26635-64-3 (isooctane)

#### *Magnesium*

When finely divided, liberates H in contact with $H_2O$. Burns in air when exposed to flame. Can be explosive in contact with $CHCl_3$ or $CH_3Cl$.

#### *Magnesium Perchlorate*

Explodes on contact with acids and reducing materials. Use as drying agent on inorg. gases and materials only.

CAS-10034-81-8 (magnesium perchlorate)

#### *Mercury*

Hazardous in contact with $NH_3$, halogens, and alkali. Vapors are extremely toxic and cumulative. Regard spills on hot surfaces as extremely hazardous and clean up promptly. Powd S sprinkled over spilled Hg can assist in cleaning up spills. High degree of personal cleanliness is necessary for persons who use Hg. Handle only in locations where any spill can be readily and thoroly cleaned up. When Hg evapn is necessary, use effective fume removal device.

To avoid environmental contamination, dil. liq. remaining in Kjeldahl distn flask to ca 300 mL with $H_2O$, cool to room temp., and add 50 mL 30% $H_2O_2$. (If Raney powder method is used, 6 mL is enough.) Warm gently to initiate reaction, let reaction go to completion in warm flask, and sep. pptd HgS. Reserve ppt in closed labeled container for recovery of Hg or disposal appropriate for Hg.

*See* also safety notes on mercury salts.

#### *Methanol*

Flammable. Toxic. Avoid contact with eyes. Avoid breathing vapors. Use effective fume removal device. Can react vigorously with NaOH plus $CHCl_3$, and KOH plus $CHCl_3$ or $HClO_4$.

CAS-67-56-1 (methanol)

#### *Methyl Cellosolve*

Vapors can be harmful. Use effective fume removal device.

CAS-109-86-4 (methyl cellosolve)

#### *Nitrobenzene and Other Nitroaromatics*

Readily absorbed thru skin. Symptoms of intoxication are sense of well-being and bluish tint on tongue, lips, and fingernails. Wear resistant rubber gloves when handling. Heat or evap. in effective fume removal device.

CAS-98-95-3 (nitrobenzene)

#### *Oxidizers*

(Perchlorates, peroxides, permanganates, persulfates, perborates, nitrates, chlorates, chlorites, bromates, iodates, concd $H_2SO_4$, concd $HNO_3$, $CrO_3$)

Can react violently with most metal powders, $NH_3$, and $NH_4$ salts, P, many finely divided org. compds, flammable liqs, acids, and S. Use exactly as specified in method. Handle in effective fume removal device from behind explosion-resistant barrier. Use face shield.

### Peroxides

(**a**) *Hydrogen peroxide.*—30% strength is hazardous; can cause severe burns. Drying $H_2O_2$ on org. material such as paper or cloth can lead to spontaneous combustion. Cu, Fe, Cr, other metals, and their salts cause rapid catalytic decomposition of $H_2O_2$. Hazardous with flammable liqs, aniline, and nitrobenzene. Since it slowly decomposes with evolution of O, provide stored $H_2O_2$ with vent caps. Wear gloves and eye protection when handling.

(**b**) *Ether peroxides.*—These peroxides form in $Et_2O$, dioxane, and other ethers during storage. They are explosive and must be destroyed chem. before distn or evapn. Exposure to light influences peroxide formation in ethers. Filtration thru activated alumina is reported to be effective in removing peroxides. Store over Na ribbon to retard peroxide formation.

CAS-7722-84-1 (hydrogen peroxide)

### Phosphotungstic Acid

Emits highly toxic fumes when heated to decomposition or in strong alkali.

### Pyridine

Toxic. Flammable. Use effective fume removal device. Releases toxic cyanides when heated to decomposition.

CAS-110-86-1 (pyridine)

### Petroleum Ether

Extremely flammable. Use effective fume removal device. Avoid static electricity.

CAS-8030-30-6 (petroleum ether)

### Pentane

Extremely flammable. Use effective fume removal device. Avoid static electricity.

CAS-109-66-0 (pentane)

### Radioactive Chemicals

Consult NBS Handbook No. 92, "Safe Handling of Radioactive Materials" (available as NCRP Report No. 30 from National Council on Radiation Protection, Publications Dept., 4201 Conn. Ave. NW, Washington, DC 20008) and NCRP Report No. 39 "Basic Radiation Protection Criteria," before handling these materials.

### Silver Nitrate

Powerful oxidizing agent; strongly corrosive. Dust or solid form is hazardous to eyes. Handle as noted for oxidizers.

### Silver Iodate

Powerful oxidizing agent. Can initiate combustion in contact with org. material (e.g., paper or cloth). Can react vigorously with reducing agents. Handle as noted for oxidizers.

### Arsenic Trioxide

Toxic. Forms toxic volatile halides in contact with halide acids. Forms volatile, highly toxic arsine when reduced in acid soln. Protect skin and respiratory tract when handling. Use effective fume removal device when arsine or arsenic trihalide is formed.

CAS-1327-53-3 (arsenic trioxide)

### Mercury Salts

Mercuric salts are quite toxic and mostly $H_2O$-sol. Use skin and respiratory protection when dry mercuric salts are to be used. Use skin protection when concd aq. solns of mercuric salts are used. Mercurous salts are generally less toxic than mercuric salts. Use of personal protection is advisable when handling these salts and their concd solns.

*See also* safety notes on mercury.

### Permanganates

Moderately toxic. Readily sol. in $H_2O$. Strong oxidizing agent. May form explosive mixt. with $H_2SO_4$ or $HClO_4$. When using with strong acids to destroy org. matter, perform reaction behind safety barrier.

### Sulfur Dioxide

Toxic gas. Forms $H_2SO_3$ in contact with moisture. Use effective fume removal device to remove $SO_2$ vapors released by reaction or from gas cylinder. Avoid contact with skin, eyes, and respiratory tract.

CAS-7446-09-5 (sulfur dioxide)

### Di- and Trichloroacetic and Trifluoroacetic Acids

Protein precipitants. Can cause severe burns to skin and respiratory tract. Use rubber gloves, eye protection, and effective fume removal device to remove vapors generated.

### Uranyl Acetate

Highly toxic. Avoid skin contact and breathing dusts.

### Toxic Dusts

Use gloves and goggles to avoid contact with skin and eyes. Use effective fume removal device or other respiratory protection.

### Carcinogens

Regulations of U.S. Department of Labor require special precautions to avoid exposure of persons to carcinogenic chems. Consult 29CFR1910.93c (U.S. Government Printing Office, Washington, DC 20402) and Guidelines for the Laboratory Use of Chemical Substances Posing a Potential Occupational Carcinogenic Risk, USDHEW, 1978.

### Asbestos

Dry asbestos fibers are hazardous when inhaled. Wet fibers form a mat which does not constitute a hazard. Transfer dry fibers in hood to container of distd $H_2O$ and store under $H_2O$ until needed, e.g., for prepn of mats in Gooch crucibles. Do not dry asbestos in forced draft oven, only in convection oven. Open oven doors slowly to avoid developing convection currents that will make fibers airborne. Reuse of filtering mats is often possible by washing, drying, and ignition, as appropriate.

CAS-8012-01-9 (asbestos)

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# Appendix: Reference Tables



SURPLUS TABLES



w Element for which known variations in isotopic composition in normal terrestrial material prevent a more precise atomic weight being given; $A_r$(E) values should be applicable to any "normal" material.

x Element for which geologic specimens are known in which the element has an anomalous isotopic composition, such that the difference between the atomic weight of the element in such specimens and that given in the Table may exceed considerably the implied uncertainty.

y Element for which substantial variations in $A_r$ from the value given can occur in commercially available material because of inadvertent or undisclosed change of isotopic composition.

z Element for which the value of $A_r$ is that of the radioisotope of longest half-life.

**977.38 Table of atomic weights (1981) from Commission of Atomic Weights, International Union of Pure and Applied Chemistry**

Scaled to the relative atomic mass, $A_r(^{12}C) = 12$

The atomic weights of many elements are not invariant but depend on the origin and treatment of the material. The footnotes to this Table elaborate the types of variation to be expected for individual elements. The values of $A_r(E)$ given here apply to elements as they exist naturally on earth and to certain artificial elements. When used with due regard to the footnotes, they are considered reliable to ±1 in the last digit or ±3 when followed by an asterisk*. Values in parentheses are used for certain radioactive elements whose atomic weights cannot be quoted precisely without knowledge of the origin of the elements; the value given is the atomic mass number of the isotope of that element of longest known half life.

| Name | Symbol | Atomic Number | Atomic Weight | Footnotes |
|---|---|---|---|---|
| Actinium | Ac | 89 | 227.0278 | z |
| Aluminum | Al | 13 | 26.98154 | |
| Americium | Am | 95 | (243) | |
| Antimony | Sb | 51 | 121.75* | |
| Argon | Ar | 18 | 39.948* | w,x |
| Arsenic | As | 33 | 74.9216 | |
| Astatine | At | 85 | (210) | |
| Barium | Ba | 56 | 137.33 | x |
| Berkelium | Bk | 97 | (247) | |
| Beryllium | Be | 4 | 9.01218 | |
| Bismuth | Bi | 83 | 208.9804 | |
| Boron | B | 5 | 10.81 | w,y |
| Bromine | Br | 35 | 79.904 | |
| Cadmium | Cd | 48 | 112.41 | x |
| Calcium | Ca | 20 | 40.08 | x |
| Californium | Cf | 98 | (251) | |
| Carbon | C | 6 | 12.011 | w |
| Cerium | Ce | 58 | 140.12 | x |
| Cesium | Cs | 55 | 132.9054 | |
| Chlorine | Cl | 17 | 35.453 | |
| Chromium | Cr | 24 | 51.996 | |
| Cobalt | Co | 27 | 58.9332 | |
| Copper | Cu | 29 | 63.546* | w |
| Curium | Cm | 96 | (247) | |
| Dysprosium | Dy | 66 | 162.50* | |
| Einsteinium | Es | 99 | (252) | |
| Erbium | Er | 68 | 167.26* | |
| Europium | Eu | 63 | 151.96 | x |
| Fermium | Fm | 100 | (257) | |
| Fluorine | F | 9 | 18.998403 | |
| Francium | Fr | 87 | (223) | |
| Gadolinium | Gd | 64 | 157.25* | x |
| Gallium | Ga | 31 | 69.72 | |
| Germanium | Ge | 32 | 72.59* | |
| Gold | Au | 79 | 196.9665 | |
| Hafnium | Hf | 72 | 178.49* | |
| Helium | He | 2 | 4.00260 | x |
| Holmium | Ho | 67 | 164.9304 | |
| Hydrogen | H | 1 | 1.00794 | w,x,y |
| Indium | In | 49 | 114.82 | x |
| Iodine | I | 53 | 126.9045 | |
| Iridium | Ir | 77 | 192.22* | |
| Iron | Fe | 26 | 55.847* | |
| Krypton | Kr | 36 | 83.80 | x,y |
| Lanthanum | La | 57 | 138.9055* | x |
| Lawrencium | Lr | 103 | (260) | |
| Lead | Pb | 82 | 207.2 | w,x |
| Lithium | Li | 3 | 6.941* | w,x,y |
| Lutetium | Lu | 71 | 174.967* | |
| Magnesium | Mg | 12 | 24.305 | x |
| Manganese | Mn | 25 | 54.9380 | |
| Mendelevium | Md | 101 | (258) | |
| Mercury | Hg | 80 | 200.59* | |
| Molybdenum | Mo | 42 | 95.94 | x |
| Neodymium | Nd | 60 | 144.24* | x |
| Neon | Ne | 10 | 20.179* | x,y |
| Neptunium | Np | 93 | 237.0482 | z |
| Nickel | Ni | 28 | 58.69 | |
| Niobium | Nb | 41 | 92.9064 | |
| Nitrogen | N | 7 | 14.0067 | |
| Nobelium | No | 102 | (259) | |
| Osmium | Os | 76 | 190.2 | x |
| Oxygen | O | 8 | 15.9994* | w,x |
| Palladium | Pd | 46 | 106.42 | x |
| Phosphorus | P | 15 | 30.97376 | |
| Platinum | Pt | 78 | 195.08* | |
| Plutonium | Pu | 94 | (244) | |
| Polonium | Po | 84 | (209) | |
| Potassium | K | 19 | 39.0983* | |
| Praseodymium | Pr | 59 | 140.9077 | |
| Promethium | Pm | 61 | (145) | |
| Protactinium | Pa | 91 | 231.0359 | z |
| Radium | Ra | 88 | 226.0254 | x,z |
| Radon | Rn | 86 | (222) | |
| Rhenium | Re | 75 | 186.207 | |
| Rhodium | Rh | 45 | 102.9055 | |
| Rubidium | Rb | 37 | 85.4678* | x |
| Ruthenium | Ru | 44 | 101.07* | x |
| Samarium | Sm | 62 | 150.36* | x |
| Scandium | Sc | 21 | 44.9559 | |
| Selenium | Se | 34 | 78.96* | |
| Silicon | Si | 14 | 28.0855* | |
| Silver | Ag | 47 | 107.8682* | x |
| Sodium | Na | 11 | 22.98977 | |
| Strontium | Sr | 38 | 87.62 | x |
| Sulfur | S | 16 | 32.06 | w |
| Tantalum | Ta | 73 | 180.9479* | |
| Technetium | Tc | 43 | (98) | |
| Tellurium | Te | 52 | 127.60* | x |
| Terbium | Tb | 65 | 158.9254 | |
| Thallium | Tl | 81 | 204.383 | |
| Thorium | Th | 90 | 232.0381 | x,z |
| Thulium | Tm | 69 | 168.9342 | |
| Tin | Sn | 50 | 118.69* | |
| Titanium | Ti | 22 | 47.88* | |
| Tungsten (Wolfram) | W | 74 | 183.85* | |
| Uranium | U | 92 | 238.0289 | x,y |
| Vanadium | V | 23 | 50.9415* | |
| Xenon | Xe | 54 | 131.29* | x,y |
| Ytterbium | Yb | 70 | 173.04* | |
| Yttrium | Y | 39 | 88.9059 | |
| Zinc | Zn | 30 | 65.38 | |
| Zirconium | Zr | 40 | 91.22 | x |

(*See* preceeding page for footnotes.)

## 935.70 Various strength solutions of the common acids, alkalies, and alcohol[a]

**(a)** *Ammonia solns:* Specification requires ≥28–≤30% $NH_3$ by wt. Sp gr of 28.0% $NH_3$ soln = 0.9 at 15°. Mix and dil. to 1 L.

| $NH_3$ Strength Desired | Reagent $NH_3$ Required | |
|---|---|---|
| g/L | g | ml |
| 5 | 17.86 | 19.8 |
| 10 | 35.71 | 39.7 |
| 15 | 53.57 | 59.5 |
| 20 | 71.43 | 79.4 |
| 25 | 89.29 | 99.2 |
| 50 | 178.57 | 198.4 |
| 75 | 267.86 | 297.6 |
| 100 | 357.14 | 396.8 |
| 150 | 535.71 | 595.2 |
| 200 | 714.29 | 793.7 |

**(b)** *Sodium hydroxide solns:* Specification requires ≥97% NaOH in sticks or pellets of caustic soda. Dissolve and dil. to 1 L.

| NaOH Strength Desired | NaOH Required | |
|---|---|---|
| g/L | g | |
| 12.5 | 12.89 | For crude fiber |
| 30 | 30.93 | |
| 40 | 41.24 | 1*N* soln |
| 50 | 51.55 | |
| 75 | 77.32 | |
| 100 | 103.09 | |
| 150 | 154.64 | |
| 200 | 206.19 | |
| 250 | 257.73 | |
| 300 | 309.28 | |

**(c)** *Hydrochloric acid solns:* Specification requires ≥36.5–≤38.0% HCl by wt. Sp gr of 37.2% HCl soln = 1.19 at 15°. Mix with $H_2O$ and dil. to 1 L.

| HCl Strength Desired | HCl Required | | |
|---|---|---|---|
| g/L | g | ml | |
| 5 | 13.44 | 11.29 | |
| 10 | 26.88 | 22.59 | |
| 15 | 40.32 | 33.88 | |
| 20 | 53.77 | 45.18 | |
| 36.46 | 98.01 | 82.36 | 1*N* soln |
| 50 | 134.41 | 112.95 | |
| 100 | 268.82 | 225.90 | |
| 150 | 403.23 | 338.85 | |
| 200 | 537.63 | 451.79 | |
| 222.6 | 598.39 | 502.85 | Constant boiling |
| 278.4 | 748.39 | 628.90 | Sp gr 1.125 |
| 300 | 806.45 | 677.69 | |

**(d)** *Nitric acid solns:* Specification requires ≥69.0–≤71.0% $HNO_3$ by wt. Sp gr of 70.4% $HNO_3$ soln = 1.42 at 15°. 1 ml concd $HNO_3$ contains ca 1.00 g $HNO_3$. Mix with $H_2O$ and dil. to 1 L.

| $HNO_3$ Strength Desired | $HNO_3$ Required | | |
|---|---|---|---|
| g/L | g | ml | |
| 5 | 7.10 | 5.0 | |
| 10 | 14.20 | 10.0 | |
| 20 | 28.41 | 20.0 | |
| 30 | 42.61 | 30.0 | |
| 40 | 56.82 | 40.0 | |
| 50 | 71.02 | 50.0 | |
| 63 | 89.49 | 63.0 | 1*N* soln |
| 70 | 99.43 | 70.0 | |
| 100 | 142.05 | 100.0 | |
| 150 | 213.07 | 150.0 | |
| 200 | 284.09 | 200.1 | |
| 300 | 426.14 | 300.1 | |

**(e)** *Sulfuric acid solns:* Specification requires ≥95.0–≤98.0% $H_2SO_4$ by wt. Sp gr of 96.0% soln = 1.84 at 15°. Pour acid into excess of $H_2O$ and dil. to 1 L.

| $H_2SO_4$ Strength Desired | $H_2SO_4$ Required | | |
|---|---|---|---|
| g/L | g | ml | |
| 5 | 5.21 | 2.8 | |
| 12.5 | 13.02 | 7.1 | For crude fiber |
| 20 | 20.83 | 11.3 | |
| 30 | 31.25 | 17.0 | |
| 40 | 41.67 | 22.6 | |
| 49 | 51.04 | 27.7 | 1*N* soln |
| 100 | 104.17 | 56.6 | |
| 150 | 156.25 | 84.9 | |
| 250 | 260.42 | 141.5 | |
| 300 | 312.50 | 169.8 | |
| 400 | 416.67 | 226.5 | |

**(f)** *Alcoholic solns:*[b] Specification requires 95% $C_2H_5OH$ by vol. Sp gr = 0.810 at 25°. Mix and dil. to 1 L.

| Alcohol Strength Desired | Alcohol Required | |
|---|---|---|
| ml/L | g | ml |
| 50 | 42.63 | 52.6 |
| 100 | 85.26 | 105.3 |
| 150 | 127.89 | 157.9 |
| 200 | 170.52 | 210.5 |
| 250 | 213.16 | 263.2 |
| 300 | 255.78 | 315.9 |
| 400 | 341.04 | 421.1 |
| 500 | 426.32 (proof) | 526.3 |
| 700 | 596.84 | 736.8 |

[a] Prepd by G. C. Spencer and H. J. Fisher, 1935 and updated by W. D. Hubbard, 1970.
[b] Alcohol of any desired strength may be obtained by taking number of ml 95% alcohol equiv. to desired strength and dilg soln to 95 ml; e.g., to obtain soln of 70% alcohol, take 70 ml 95% alcohol and dil. to 95 ml.

**955.57 Optical crystallographic properties of some crystalline drugs[a]**

| Compound | α | β | γ | Optic Sign | Extinction | Elongation | 2V | Remarks |
|---|---|---|---|---|---|---|---|---|
| **Alkaloids and Related Amines** | | | | | | | | |
| Aconitine | 1.560 | — | 1.575 | + | | – | 36° | |
| Alphaprodine.HCl | 1.499 | 1.572 | 1.597 | – | p, i | | 63° | |
| Apomorphine.HCl | 1.638 | 1.658 | 1.701 | + | | | | |
| Arecoline.HBr | 1.555 | 1.590 | 1.655 | + | p | – | | |
| Atropine | 1.550 | 1.583 | 1.595 | – | | | | $n_\alpha$ very common |
| Atropine sulfate | 1.555 | — | 1.60 | | | | | |
| Benzethonium chloride | 1.560 | 1.565 | 1.589 | + | | | 48° | Most fragments do not extinguish completely |
| Berberine | 1.490 | 1.701 | >1.734 | – | p | – | | Yellow needles and rods |
| Berberine.HCl.2H₂O | 1.500 | 1.535 | >1.733 | + | p | | | |
| Brucine | 1.562 | — | >1.65 | | p | | | |
| Brucine sulfate | 1.512 | 1.595 | 1.688 | + | p | + | | 6-sided plates and rods |
| Cetylpyridinium chloride | 1.509 | 1.566 | 1.613 | – | i | | 88° | Op. ax. fig. common |
| Cinchonidine | 1.610 | 1.625 | 1.675 | + | p | + | 59° | r>v weak |
| Cinchonidine sulfate | 1.562 | 1.604 | 1.660 | + | p | + | | |
| Cinchonine | 1.570 | 1.685 | 1.690 | – | p | + | 21° | Plates and rods |
| Cinchonine.HCl.2H₂O | 1.545 | 1.617 | 1.661 | – | | + | large | |
| Cocaine.HCl | 1.570 | 1.596 | 1.618 | – | | | | |
| Codeine | 1.543 | 1.636 | 1.684 | – | | | 53° | |
| Codeine.HCl | 1.559 | 1.580 | 1.676 | + | | | | |
| Codeine sulfate | 1.561 | 1.642 | 1.661 | – | p | – | | Rods |
| Desipramine.HCl | 1.586 | 1.622 | 1.698 | + | p, s | – | large | Elongate prisms, many 6-sided in outline; op. ax. figs. rare, flash figs. common |
| Dextropropoxyphene napsylate | 1.568 | 1.636 | 1.638 | – | | | 23° | Marked dispersion, r<v |
| Diacetylmorphine (Heroin) | 1.556 | 1.607 | 1.627 | – | | | | Sl. sol. in R. I. oils |
| Diacetylmorphine.HCl.H₂O | $1.578_\omega$ | | $1.613_\epsilon$ | + | | | | Uniaxial |
| Diphenylhydantoin | 1.600 | — | 1.635 | | p | – | | |
| *l*-Ephedrine.HCl | 1.530 | 1.603 | 1.638 | – | p | – | 70° | Prisms and rods |
| Ethoheptazine citrate | 1.537 | — | 1.556 | | | | | |
| Ethylhydrocupreine.HCl | 1.513 | — | 1.619 | | | | | |
| Hydrastine | 1.550 | 1.734 | >1.734 | | | | | $n_\alpha$ common |
| Hyoscyamine | 1.562 | — | 1.581 | | p, i | + | | |
| Isobucaine.HCl | 1.522 | 1.574 | 1.612 | – | p, i | ± | 82° | |
| Levallorphan tartrate | 1.545 | 1.595 | 1.653 | + | | | 86° | |
| Meperidine.HCl | 1.545 | 1.581 | 1.618 | + | p | + | | |
| Methaqualone.HCl | 1.568 | 1.659 | sl >1.800 | + | p | – | | Long rods and rectangular plates; α lengthwise, β and γ crosswise |
| Methscopolamine bromide | 1.580 | 1.615 | 1.617 | – | | | 33° | |

[a] Abbreviations: p = parallel; s = symmetrical; i = inclined; n = index; $n_i$ = intermediate index; Bx.ac. = acute bisectrix; Bx.ob. = obtuse bisectrix; Op.ax. = optic axis; fig. = figure; sl = slightly; r = red; v = violet.

*(Continued)*

**955.57 Optical crystallographic properties of some crystalline drugs[a]—*Continued.***

| Compound | $\alpha$ | $\beta$ | $\gamma$ | Optic Sign | Extinction | Elongation | 2V | Remarks |
|---|---|---|---|---|---|---|---|---|
| **Alkaloids and Related Amines—*Continued*** | | | | | | | | |
| Methylphenidate.HCl | 1.558 | 1.581 | 1.585 | – | | | 43° | |
| $O^6$-Monoacetylmorphine.HCl | 1.526 | 1.597 | 1.639 | – | p | – | fairly large | Elongate prisms; $\alpha$ lengthwise, $\beta$ and $\gamma$ crosswise |
| Morphine.HCl, anhyd. | 1.612 | 1.637 | 1.644 | – | p | – | med. to large | Irregular plates, some rectangular or lath-shaped showing $\alpha$ and $\gamma$ |
| Morphine.$H_2O$ | 1.580 | 1.625 | 1.645 | – | p | ± | | |
| Morphine.HCl.$3H_2O$ | 1.540 | $n_i$1.590 | 1.635 | – | p | – | | |
| Morphine sulfate.$5H_2O$ | 1.545 | 1.620 | 1.632 | – | p | – | | |
| Papaverine | 1.625 | 1.690 | >1.690 | | p | – | large | Orthorhombic |
| Papaverine.HCl | 1.555 | $n_i$1.733 | >1.733 | | | | | $n_\alpha$ common |
| Pentazocine | 1.575 | 1.590 | 1.627 | + | p | – | | $\alpha$ lengthwise, $\gamma$ crosswise |
| Pentazocine.HCl | 1.577 | — | 1.594 | | p, i | + | | Mostly inclined extinction; flash figs. common; $\alpha$ crosswise, $\gamma$ lengthwise |
| Phenmetrazine.HCl | 1.508 | 1.516 | 1.628 | + | | | | |
| Phensuximide | 1.536 | 1.617 | >1.673 | | p, i | | very large | |
| Phenylbutazone | 1.600 | — | 1.620 | + | p | – | | |
| Quinidine | 1.580 | 1.665 | 1.690 | – | p | + | 55° | |
| Quinidine sulfate | 1.565 | 1.607 | 1.670 | + | p | + | | |
| Quinine | 1.620 | 1.625 | 1.630 | | p | – | very large | |
| Quinine.HCl | 1.590 | 1.610 | 1.669 | + | p | + | 61° | r>v |
| Racephedrine.HCl | 1.570 | 1.608 | 1.630 | – | | | | Irregular fragments; op. ax. figs. occasional |
| Scopolamine.HBr | 1.567 | 1.585 | 1.623 | + | p | | medium | Rods and prisms |
| Strychnine | 1.617 | 1.660 | >1.690 | – | p | + | large | |
| Strychnine.HCl.$2H_2O$ | 1.610 | 1.626 | 1.668 | + | | – | large | |
| Syrosingopine | 1.529 | 1.538 | 1.646 | + | p | – | 34° | |
| Yohimbine | 1.548 | 1.563 | 1.688 | + | | | 42° | |
| Yohimbine.HCl | 1.57 | 1.61 | 1.69 | + | | | | |
| **Antibiotics** | | | | | | | | |
| Carbomycin | 1.474 | 1.484 | 1.513 | + | p | ± | | |
| Chloramphenicol | 1.523 | 1.608 | 1.659 | – | p | – | 70–80° | |
| Chloramphenicol palmitate | 1.527 | — | 1.569 | | p | ± | | |
| Chlortetracycline.HCl | 1.635 | 1.706 | 1.730 | – | p, s | | 59° | |

*(Continued)*

**955.57 Optical crystallographic properties of some crystalline drugs[a]—*Continued.***

| Compound | α | β | γ | Optic Sign | Extinction | Elongation | 2V | Remarks |
|---|---|---|---|---|---|---|---|---|
| | | | Antibiotics—*Continued* | | | | | |
| Cycloserine | 1.583 | — | 1.630 | | | | | Rod-shaped aggregates |
| Dihydrostreptomycin.3HCl | 1.522 | 1.548 | 1.566 | − | p | + | 80° | |
| Dihydrostreptomycin sulfate | 1.552 | 1.558 | 1.566 | + | p, i | | 89° | Ext. angle = 18° |
| Erythromycin estolate | 1.483 | 1.488 | 1.515 | + | p | − | 52° | |
| Erythromycin ethylcarbonate | 1.496 | 1.506 | 1.510 | − | p | + | medium | Op. ax. figs. common |
| Erythromycin ethylsuccinate | 1.490 | 1.515 | 1.567 | + | p | − | | $n_\gamma$ rare |
| Erythromycin gluceptate | 1.506 | — | 1.528 | | p | + | | |
| Erythromycin.HI.$H_2O$ | 1.528 | 1.536 | 1.550 | + | p | + | 75° | |
| Erythromycin.$2H_2O$ | 1.512 | 1.523 | 1.532 | − | p | − | 84° | |
| Erythromycin oxalate.$2H_2O$ | 1.484 | 1.492 | 1.516 | + | p | + | 60° | |
| Erythromycin stearate | 1.498 | 1.507 | 1.563 | + | p | ± | small | $n_\gamma$ rare |
| Fumagillin | 1.518 | ca 1.572 | >1.780 | + | p, i | | small | |
| Gramicidin | 1.541 | ca 1.553 | 1.573 | + | | | | |
| Neomycin sulfate | n 1.541 | | | | | | | Isotropic |
| Novobiocin acid, form 2 | 1.608 | 1.638 | 1.654 | − | | | 71° | r>v |
| Novobiocin sodium | 1.565 | — | 1.629 | | p | − | | Tiny needles |
| Nystatin | 1.512 | 1.583 | 1.682 | + | p | − | | Small, pale yellow rods |
| Oxytetracycline.$2H_2O$ | 1.634 | 1.646 | >1.700 | + | p | | 28° | |
| Oxytetracycline.HCl | 1.546 | 1.635 | 1.730 | + | p, i | + | very large | Op. ax. figs. common |
| Penicillin G benzathine | 1.523 | 1.622 | 1.630 | − | p | + | very small | $n_\alpha$ and $n_\gamma$ common |
| Penicillin G dibenzylamine | 1.567 | — | 1.613 | | p | + | | |
| Penicillin G *l*-ephedrine | 1.575 | — | 1.610 | | p | − | | |
| Penicillin G *l*-ephenamine | 1.583 | 1.590 | 1.648 | + | i | | very small | Bx. ac. figs. |
| Penicillin G hydrabamine | 1.556 | ca 1.590 | 1.619 | | p, i | − | | |
| Penicillin G.HI diethylaminoethyl ester | 1.601 | 1.608 | 1.632 | + | p | − | medium | |
| Penicillin G potassium | 1.550 | — | 1.603 | − | p | + | | Elongated rectangular plates |
| Penicillin G procaine | 1.545 | 1.570 | 1.685 | + | p, i | ± | | $n_\beta$ common |
| Penicillin G sodium | 1.550 | 1.609 | 1.620 | − | p | + | large | $n_\beta$ common |
| Penicillin O chloroprocaine | 1.541 | 1.585 | 1.656 | + | p | ± | large | |
| Penicillin O potassium | 1.545 | — | 1.593 | | p | + | | |
| Tetracycline.HCl | 1.603 | 1.685 | 1.714 | − | p | | large | Bx. ac. and op. ax. figs. |
| Tetracycline.$3H_2O$ | 1.538 | 1.646 | sl >1.787 | + | p, i | | large | Occasional op. ax. figs. |
| Tyrocidine.HCl | 1.553 | — | 1.584 | | p | + | | |

(*Continued*)

**955.57 Optical crystallographic properties of some crystalline drugs[a]—*Continued.***

| Compound | α | β | γ | Optic Sign | Extinc-tion | Elonga-tion | 2V | Remarks |
|---|---|---|---|---|---|---|---|---|
| | | | | Antihistamines | | | | |
| Anthallan®.HCl | 1.505 | 1.585 | 1.617 | – | p | – | | Small rods & irregular fragments; no figs. |
| Bromothen.HCl | 1.617 | 1.654 | 1.734 | + | i | + | | Very small rods |
| Chlorcyclizine.HCl | 1.590 | 1.610 | 1.665 | + | | | | Thin platy fragments; op. ax. figs. common |
| Chlorcyclizine.2HCl | 1.610 | 1.660 | 1.665 | – | p | | very small | Short rods & thin 6-sided plates; op. ax. figs. common |
| Chlorothen citrate | 1.583 | 1.603 | 1.645 | | | | | Minute plates & shreds; op. ax. figs. rare |
| Chlorothen.HCl | 1.553 | 1.625 | >1.734 | | | | | Massive fragments, some rectangular; op. ax. figs. occasional |
| Chlorpheniramine maleate | 1.533 | $n_i$1.668 | I <1.734 | | | | | Box-like prisms & irregular fragments; figs. infrequent |
| Cyproheptadine.HCl | 1.620 | 1.647 | 1.738 | + | p | | 60° | |
| Dexchlorpheniramine maleate | 1.509 | 1.564 | 1.683 | + | p | | 70° | |
| Dimenhydrinate (unsatisfactory for optical crystallographic study) | | | | | | | | Platy material & rods |
| Diphenhydramine.HCl | 1.602 | 1.625 | 1.630 | – | p | – | | 6-sided plates |
| Doxylamine succinate | ca 1.525 | 1.563 | 1.598 | – | | | 86° | |
| *p*-Fluorotripelennamine.HCl | 1.585 | 1.600 | 1.668 | + | p | – | large | Rods & square plates; op. ax. figs. occasional |
| Methaphenilene.HCl | 1.604 | 1.675 | 1.733 | – | | | | Elongated 6-sided rods with obtuse ends; op. ax. figs. frequent |
| Methapyrilene.HCl | 1.588 | 1.654 | >1.695–<1.734 | – | | | | Thick hexagonal plates |
| 2-(4-Morpholinyl) ethyl benzhydryl ether.HCl (Linadryl.HCl) | 1.577 | 1.631 | 1.672 | – | p, i | ± | | Elongated 6-sided & irregular fragments; figs. rare |
| Phenbenzamine.HCl | 1.587 | 1.635 | 1.734 | + | | | | Short prisms |
| Pheniramine maleate | 1.548 | 1.574 | 1.665 | + | p | + | small | Rods & plates |
| Promethazine.HBr | 1.667 | 1.675 | >1.733 | + | | | small | Massive prisms; elongated or short & stubby |
| Promethazine.HCl | 1.617 | 1.691 | 1.733 | – | p | + | | Rods & irregular fragments |
| Pyrathiazine.HCl | 1.690 | — | 1.737 | | | | | Stout prismatic forms; no figs. |
| Pyrilamine maleate (unsatisfactory for optical crystallographic study) | | | | | | | | Rods & irregular fragments |
| Pyrrobutamine phosphate | 1.566 | 1.614 | 1.653 | | | | 82° | |
| Thenyldiamine.HCl | 1.590 | — | 1.680 | | | | large | Square plates & stubby prisms |
| Thonzylamine.HCl | 1.612 | 1.679 | 1.691 | – | p | + | | Rods & platy material |
| Tripelennamine.HCl | 1.580 | 1.655 | 1.705 | – | | | | Rectangular plates & prisms from water; op. ax. figs. common |

*(Continued)*

**955.57 Optical crystallographic properties of some crystalline drugs[a]—*Continued.***

| Compound | α | β | γ | Optic Sign | Extinc-tion | Elonga-tion | 2V | Remarks |
|---|---|---|---|---|---|---|---|---|
| | | | | Barbiturates | | | | |
| Allobarbital | 1.516 | 1.572 | 1.625 | − | s | | large | Op. ax. fig. common |
| Alphenal (5-allyl-5-phenylbarbituric acid) | 1.551 | 1.578 | 1.645 | + | p | − | 67° | Op. ax. fig. common |
| Amobarbital | 1.467 | 1.533 | 1.560 | − | p | + | | |
| Amobarbital sodium | n 1.505 | | | | | | | Isotropic |
| Aprobarbital | 1.520 | 1.581 | 1.600 | − | i | | medium | Rods |
| Barbital | 1.445 | 1.548 | 1.580 | | | | | All n's common |
| Barbital sodium | 1.512 | 1.532 | 1.615 | | | | 40° | |
| Butabarbital sodium | 1.465 | 1.529 | 1.532 | − | p | + | very small | |
| Butalbital | 1.508 | $n_i$1.521 | 1.577 | + | p | + | medium | Rods and plates |
| Butallylonal | 1.524 | 1.577 | 1.603 | − | p | + | medium | Rosettes of tiny rods and blades; bx. ac. figs. occasional |
| Butethal | 1.454 | 1.518 | 1.556 | − | i | | large | Rods & needles; op. ax. and bx. ac. figs. common |
| Cyclobarbital | 1.515 | 1.546 | 1.621 | + | | ± | 69° | Bx. ac. & bx. ob. figs. common |
| Cyclopal® (5-allyl-5-(2-cyclopenten-1-yl) barbituric acid) | 1.520 | 1.575 | 1.626 | − | | − | 85° | Bx. ac. fig. common |
| Hexethal (5-ethyl-5-*n*-hexylbarbituric acid) | 1.473 | 1.519 | 1.549 | − | | − | 76° | |
| Hexobarbital | 1.546 | 1.608 | 1.634 | − | p | + | 64° | Bx. ac. & op. ax. figs. common |
| Mephobarbital | 1.594 | 1.610 | 1.651 | + | p | − | 65° | Bx. ac. fig. common |
| Pentobarbital | 1.465 | — | 1.565 | − | i | | very large | |
| Pentobarbital sodium | 1.477 | — | 1.523 | | | | | |
| Phenobarbital | 1.557 | 1.620 | 1.667 | | p | − | | β very common |
| Phenobarbital sodium (unstable) | | | | | | | | |
| Probarbital | 1.477 | 1.573 | 1.624 | − | i | + | 73° | Rods |
| Probarbital sodium | 1.532 | — | 1.629 | | p | + | | Rods & needles |
| Secobarbital | 1.487 | 1.557 | 1.563 | − | p | + | 31° | |
| Secobarbital sodium | 1.490 | $n_i$1.500 | 1.525 | | | | | |
| Sigmodal® (5-(2-bromoallyl)-5-(1-methylbutyl) barbituric acid) | 1.519 | 1.583 | 1.634 | − | | + | 80° | |
| Thiopental | 1.534 | 1.634 | — | − | i | | 40–45° | Lamellar |
| Vinbarbital | 1.506 | 1.544 | 1.672 | + | p | − | 61° | |

*(Continued)*

**955.57 Optical crystallographic properties of some crystalline drugs[a]—*Continued.***

| Compound | α | β | γ | Optic Sign | Extinction | Elongation | 2V | Remarks |
|---|---|---|---|---|---|---|---|---|
| **Hallucinogens** | | | | | | | | |
| *d*-Lysergic acid diethylamide tartrate (LSD-25) | 1.540 | 1.596 | 1.676 | + | i | | 83° | |
| 4-Methyl-2,5-dimethoxyamphetamine.HCl ("STP".HCl) (DOM®) | 1.518 | 1.622 | 1.632 | – | | | 33° | Bx. ob. figs. common |
| 3,4-Methylenedioxyamphetamine carbonate (MDA carbonate) | 1.564 | 1.586 | 1.598 | – | p or sl i | – | | $\alpha$ lengthwise, $\gamma$ crosswise |
| 3,4-Methylenedioxyamphetamine.HCl (MDA.HCl) | 1.517 | 1.612 | 1.679 | – | i, s | | large | Broad plates; extinction sl i and almost symmetrical |
| 3,4-Methylenedioxyamphetamine sulfate (MDA sulfate) | 1.537 | — | 1.629 | | | | | Uniaxial figs. and biaxial figs. with small 2V |
| Phencyclidine.HBr | 1.572 | 1.620 | 1.654 | | | | | |
| Phencyclidine.HCl | 1.572 | 1.618 | 1.654 | – | | | 80° | |
| Psilocybin | 1.527 | 1.554 | 1.672 | + | | | 55° | $n_\beta$ and $n_\gamma$ common |
| **Steroids** | | | | | | | | |
| Betamethasone | 1.554 | — | 1.667 | | p | + | | Very small rods |
| Cholesterol | 1.520 | 1.532 | 1.566 | + | | | 60° | Elongated plates |
| Cortisone | 1.552 | 1.572 | 1.625 | + | p | + | medium | $n_\alpha$ and $n_\beta$ most common |
| Cortisone acetate | 1.512 | 1.552 | 1.621 | + | p | ± | medium | |
| Dehydrocholic acid | 1.510 | 1.542 | 1.572 | – | p, i | ± | 80° | |
| Desoxycorticosterone acetate | 1.529 | 1.550 | 1.630 | + | | | 54° | r > v |
| Dexamethasone | 1.553 | 1.572 | 1.648 | + | | | 52° | Bx. ac. common |
| Diethylstilbestrol, trans | 1.594 | 1.611 | 1.73 | + | p | – | | Orthorhombic system |
| Equilin | 1.534 | 1.677 | 1.705 | – | | | 40° | Plates |
| Estradiol benzoate | 1.586 | 1.603 | 1.633 | + | p | – | large | |
| Estradiol dipropionate | 1.506 | — | 1.598 | – | | | | 2E = 46° |
| Estrone, phase 1 | 1.520 | 1.642 | 1.692 | – | i | | 60° | Ext. angle = 12°; r>v |
| Estrone, phase 2 | 1.511 | 1.621 | 1.697 | – | | | 75° | r>v weak |
| Estrone, phase 3 | 1.594 | 1.628 | 1.647 | – | p | | 73° | Metastable crystals, 6-sided plates |
| Ethisterone | 1.576 | 1.625 | 1.645 | – | | | 67° | 2E = 127° |
| Fluorometholone | 1.562 | 1.568 | 1.704 | + | | | 26° | r > v strong |
| Hydrocortisone | 1.531 | 1.550 | 1.638 | + | p | n | | Tiny rods & plates |
| Hydrocortisone acetate | 1.543 | 1.589 | 1.627 | – | p, i | ± | 83° | Monoclinic; r>v |
| Methylprednisolone acetate | 1.562 | 1.575 | 1.700 | + | | | 38° | v > r |
| Methylprednisolone sodium succinate | 1.552 | — | 1.561 | + | | | very small | |
| Methyltestosterone | 1.555 | ca 1.565 | 1.620 | + | p | – | medium | |

*(Continued)*

**955.57 Optical crystallographic properties of some crystalline drugs[a]—*Continued.***

| Compound | $\alpha$ | $\beta$ | $\gamma$ | Optic Sign | Extinction | Elongation | 2V | Remarks |
|---|---|---|---|---|---|---|---|---|
| | | | Steroids—*Continued* | | | | | |
| Prednisone | 1.587 | 1.590 | 1.651 | + | p | | very small | Bx. ac. common |
| Progesterone, alpha | 1.542 | 1.554 | 1.663 | + | | | 40° | Platy fragments with brilliant interference colors |
| Progesterone, beta | 1.529 | 1.575 | 1.676 | + | p | − | 68° | Crystals acicular |
| Testosterone | 1.548 | 1.565 | 1.670 | + | p | − | medium | |
| Triamcinolone acetonide | $1.546_{\epsilon}$ | | $1.595_{\omega}$ | − | | | | |
| Triamcinolone diacetate | 1.517 | 1.567 | 1.592 | − | | | 69° | |
| | | | Sulfonamides | | | | | |
| Succinylsulfathiazole | 1.578 | 1.676 | 1.710 | − | i | | 58° | Rods |
| Sulfacetamide | 1.559 | 1.564 | 1.727 | + | s | | 21° | |
| Sulfadiazine | 1.596 | 1.675 | 1.830 | + | p, i | ± | 76° | |
| Sulfadiazine[b] | 1.615 | 1.663 | >1.734 | | p | ± | | Rods |
| Sulfaguanidine | 1.606 | 1.663 | 1.734 | | | | | Op. ax. fig. |
| Sulfaguanidine. $H_2O$ | 1.586 | 1.649 | 1.731 | + | p, i | ± | 86° | |
| Sulfallantoin® (sulfanilamide+allantoin-addition product) | 1.513 | 1.590 | >1.690 <1.733 | | | | | Op. ax. fig. |
| Sulfamerazine | 1.568 | 1.657 | 1.687 | − | p | ± | 58° | |
| Sulfamerazine[b] | 1.587 | — | 1.675 | | | | | |
| Sulfamethazine | 1.584 | 1.623 | >1.778 | | p | − | | Rods |
| Sulfamidazole® (sulfanilamide+sulfathiazole-double crystal)[c] | 1.661 | 1.678 | >1.733 | + | | | small | Bx. ac. fig. |
| Sulfanilamide phase B (anhyd.) | 1.555 | 1.672 | 1.85 | + | p | − | | Stable form com. prepns |
| Sulfanilamide.HCl | 1.540 | 1.655 | 1.690 | | p | − | | Rods |
| Sulfapyridine[b] | 1.680 | 1.733 | >1.733 | | | | | Op. ax. fig. |
| Sulfapyridine, phase I | 1.670 | 1.736 | 1.813 | + | p, i | ± | 88° | Tabular to equant; stable form com. prepns |
| Sulfapyridine sodium. $H_2O$ | 1.590 | — | 1.700 | | p | − | | |
| Sulfathiazole, phase I | 1.674 | 1.685 | >1.733 | + | | | small | $\alpha$ & $\beta$ common |
| Sulfathiazole, phase II | 1.598 | 1.741 | 1.780 | − | p, i | ± | 52° | Lath shaped |
| Sulfathiazole[b] | 1.695 | $n_i$1.733 | >1.733 | | | | | |
| Sulfathiazole sodium.1½$H_2O$ | 1.596 | — | 1.621 | | | | | |
| Sulfisoxazole | 1.605 | 1.642 | 1.697 | + | p | ± | large | Plates & rods |

*(Continued)*

[b] The second set of optical properties in each case represents intermediate data which are quite commonly found in some commercial samples. They probably represent an anhydrous form or merely a different common orientation of the crystal.

[c] Equimolecular proportions.

**955.57 Optical crystallographic properties of some crystalline drugs[a]—*Continued.***

| Compound | $\alpha$ | $\beta$ | $\gamma$ | Optic Sign | Extinction | Elongation | 2V | Remarks |
|---|---|---|---|---|---|---|---|---|
| | | | Sympathomimetic Amines | | | | | |
| *dl*-Amphetamine.HCl | 1.508 | 1.582 | 1.611 | | p | − | | Rods and plates |
| *dl*-Amphetamine phosphate, dibasic | 1.549 | 1.589 | 1.665 | + | | | large | Small platy crystals; bx. ac. figs. common |
| *dl*-Amphetamine sulfate | 1.520 | 1.531 | 1.614 | + | | − | very small | |
| Dextroamphetamine.HCl | 1.560 | 1.592 | 1.622 | + | p, i | ± | very large | Large plates & rods; op. ax. figs. occasional |
| Dextroamphetamine phosphate, dibasic | 1.546 | 1.583 | 1.664 | + | p | ± | medium | Plates with truncated corners |
| Dextroamphetamine sulfate | 1.501 | 1.545 | 1.603 | + | | − | small | 6–8-sided plates |
| *l*-Ephedrine.HCl | 1.530 | 1.603 | 1.638 | − | p | − | 70° | Elongated prisms & rods |
| *l*-Ephedrine sulfate | 1.540 | 1.565 | 1.587 | | p | | large | 6-sided plates & rods |
| Epinephrine | 1.548 | 1.597 | >1.735 | + | p | − | medium | Thin, blade-like, 6-sided crystals in rosettes; bx. ac. figs. common |
| Hydroxyamphetamine.HBr | 1.560 | 1.680 | 1.734 | − | | | | Irregular fragments |
| *p*-Hydroxyephedrine.HCl | 1.507 | 1.604 | 1.668 | − | p | ± | | Rectangular rods; bx. ob. figs. common |
| *p*-Hydroxymethamphetamine sulfate | 1.516 | 1.552 | 1.645 | + | s | + | | Rhombohedral or 6-sided plates |
| Isoxsuprine.HCl | 1.508 | 1.648 | 1.670 | − | p, i | | 40° | |
| Levamfetamine succinate | 1.572 | 1.587 | 1.650 | + | p, i | | 52° | Bx. ac. figs. common |
| Mephentermine sulfate | 1.530 | 1.585 | 1.596 | − | p, i | | 46° | |
| Methamphetamine.HCl | 1.530 | 1.537 | 1.615 | + | | | | Irregular fragments; op. ax. figs. frequent |
| *dl*-Methamphetamine.HCl | 1.535 | 1.540 | 1.620 | + | p | − | small | Small 6-sided platy or rod-like crystals; no figs. |
| Naphazoline nitrate | 1.560 | 1.619 | >1.740 | + | s, i | | | 6-sided plates & irregular fragments; bx. ac. figs. common |
| Phendimetrazine.HCl | 1.535 | 1.602 | 1.607 | − | | | fairly small | Small, rounded crystals |
| Phendimetrazine tartrate | 1.544 | 1.594 | 1.614 | − | p | − | fairly large | Platy crystals; $\alpha$ lengthwise, $\beta$ and $\gamma$ crosswise |
| Phenylpropanolamine.HCl | 1.563 | 1.618 | 1.650 | − | | | large | |
| Phenylpropylmethylamine.HCl | 1.577 | — | 1.603 | | p | − | | Small rod-like fragments; no figs. |
| Pseudoephedrine.HCl | 1.543 | — | 1.632 | | p | − | | |
| Racephedrine.HCl | 1.570 | 1.608 | 1.630 | − | | | | Irregular fragments; op. ax. figs. occasional |
| *dl*-Synephrine base (Desoxyepinephrine) | 1.546 | 1.604 | ca 1.725 | + | s, i | | large | Platy crystals, often diamond-shaped; op. ax. figs. common |
| Synephrine.HCl | 1.549 | 1.605 | 1.664 | + | p, i | | large | Large plates; bx. ac. figs. frequent |
| *dl*-Synephrine (+) tartrate (neutral salt) | 1.516 | $n_i$1.620 | 1.689 | + | i | + | large | Rods & plates; partial op. ax. figs. common |
| Tolazoline.HCl | 1.586 | 1.604 | 1.703 | + | i | | | 6-sided plates; inclined op. ax. figs. common |
| Tuaminoheptane sulfate | $1.458_{\omega}$ | | $1.468_{\epsilon}$ | + | p | | | Irregular-shaped plates & fibrous flakes; figs. frequent |

*(Continued)*

**955.57 Optical crystallographic properties of some crystalline drugs[a]—*Concluded.***

| Compound | $\alpha$ | $\beta$ | $\gamma$ | Optic Sign | Extinction | Elongation | 2V | Remarks |
|---|---|---|---|---|---|---|---|---|
| | | | | Tranquilizers | | | | |
| Azacyclonol.HCl | 1.638 | 1.647 | 1.674 | + | | | medium | |
| Chlordiazepoxide.HCl | 1.634 | ca 1.710 | >1.780 | + | | | large | 6-sided prisms; $n_\beta$ rare |
| Chlorpromazine.HCl | 1.584 | — | >1.735 | + | i | | | |
| Ethinamate | 1.530 | 1.536 | 1.546 | + | | | 76° | Recrystallized from dil. alcohol |
| Glutethimide | 1.572 | 1.585 | 1.590 | − | p, i | + | large | 6-sided rods and plates |
| Meprobamate | ca 1.515 | — | ca 1.544 | | p | + | | Fibers and irregular thin platy fragments with wavy extinction |
| Thiopropazate.2HCl | 1.589 | 1.609 | 1.700 | | | | | Irregular platy fragments |

**955.58 Table of refractive indices for drugs, arranged according to ascending value of the lowest index[a]**

| $\alpha$ | $\beta$ | $\gamma$ | Compound |
|---|---|---|---|
| | | | **Alkaloids and Related Amines** |
| 1.490 | 1.701 | >1.734 | Berberine |
| 1.499 | 1.572 | 1.597 | Alphaprodine.HCl |
| 1.500 | 1.535 | >1.733 | Berberine.HCl.$2H_2O$ |
| 1.508 | 1.516 | 1.628 | Phenmetrazine.HCl |
| 1.509 | 1.566 | 1.613 | Cetylpyridinium chloride |
| 1.512 | 1.595 | 1.688 | Brucine sulfate |
| 1.513 | — | 1.619 | Ethylhydrocupreine.HCl |
| 1.522 | 1.574 | 1.612 | Isobucaine.HCl |
| 1.526 | 1.597 | 1.639 | $O^6$-Monoacetylmorphine.HCl |
| 1.529 | 1.538 | 1.646 | Syrosingopine |
| 1.530 | 1.603 | 1.638 | *l*-Ephedrine.HCl |
| 1.536 | 1.617 | >1.673 | Phensuximide |
| 1.537 | — | 1.556 | Ethoheptazine citrate |
| 1.540 | 1.590 | 1.635 | Morphine.HCl.$H_2O$ |
| 1.543 | 1.636 | 1.684 | Codeine |
| 1.545 | 1.581 | 1.618 | Meperidine.HCl |
| 1.545 | 1.595 | 1.653 | Levallorphan tartrate |
| 1.545 | 1.617 | 1.661 | Cinchonine.HCl.$2H_2O$ |
| 1.545 | 1.620 | 1.632 | Morphine sulfate.$5H_2O$ |
| 1.548 | 1.563 | 1.688 | Yohimbine |
| 1.550 | 1.583 | 1.595 | Atropine |
| 1.550 | 1.734 | >1.734 | Hydrastine |
| 1.555 | — | 1.60 | Atropine sulfate |
| 1.555 | 1.590 | 1.655 | Arecoline.HBr |
| 1.555 | $n_i$1.733 | >1.733 | Papaverine.HCl |
| 1.556 | 1.607 | 1.627 | Diacetylmorphine |
| 1.558 | 1.581 | 1.585 | Methylphenidate.HCl |
| 1.559 | 1.580 | 1.676 | Codeine.HCl |
| 1.560 | — | 1.575 | Aconitine |
| 1.560 | 1.565 | 1.589 | Benzethonium chloride |
| 1.561 | 1.642 | 1.661 | Codeine sulfate |
| 1.562 | — | 1.581 | Hyoscyamine |
| 1.562 | — | >1.65 | Brucine |
| 1.562 | 1.604 | 1.660 | Cinchonidine sulfate |
| 1.565 | 1.607 | 1.670 | Quinidine sulfate |
| 1.567 | 1.585 | 1.623 | Scopolamine.HBr |
| 1.568 | 1.636 | 1.638 | Dextropropoxyphene napsylate |
| 1.568 | 1.659 | sl >1.800 | Methaqualone.HCl |
| 1.57 | 1.61 | 1.69 | Yohimbine.HCl |
| 1.570 | 1.596 | 1.618 | Cocaine.HCl |
| 1.570 | 1.608 | 1.630 | Racephedrine.HCl |
| 1.570 | 1.685 | 1.690 | Cinchonine |
| 1.575 | 1.590 | 1.627 | Pentazocine |
| 1.577 | — | 1.594 | Pentazocine.HCl |
| $1.578_\omega$ | | $1.613_\epsilon$ | Diacetylmorphine.HCl.$H_2O$ |
| 1.580 | 1.615 | 1.617 | Methscopolamine bromide |
| 1.580 | 1.625 | 1.645 | Morphine.$H_2O$ |
| 1.580 | 1.665 | 1.690 | Quinidine |
| 1.586 | 1.622 | 1.698 | Desipramine.HCl |
| 1.590 | 1.610 | 1.669 | Quinine.HCl |
| 1.600 | — | 1.620 | Phenylbutazone |
| 1.600 | — | 1.635 | Diphenylhydantoin |
| 1.610 | 1.625 | 1.675 | Cinchonidine |
| 1.610 | 1.626 | 1.668 | Strychnine.HCl.$2H_2O$ |
| 1.612 | 1.637 | 1.644 | Morphine.HCl, anhyd. |
| 1.617 | 1.660 | >1.690 | Strychnine |
| 1.620 | 1.625 | 1.630 | Quinine |
| 1.625 | 1.690 | >1.690 | Papaverine |
| 1.638 | 1.658 | 1.701 | Apomorphine.HCl |
| | | | **Antibiotics** |
| 1.474 | 1.484 | 1.513 | Carbomycin |
| 1.483 | 1.488 | 1.515 | Erythromycin estolate |
| 1.484 | 1.492 | 1.516 | Erythromycin oxalate.$2H_2O$ |
| 1.490 | 1.515 | 1.567 | Erythromycin ethylsuccinate |
| 1.496 | 1.506 | 1.510 | Erythromycin ethylcarbonate |
| 1.498 | 1.507 | 1.563 | Erythromycin stearate |

[a] *See* **52.023** for symbols.

***(Continued)***

**955.58 Table of refractive indices for drugs, arranged according to ascending value of the lowest index[a] —*Continued.***

| $\alpha$ | $\beta$ | $\gamma$ | Compound |
|---|---|---|---|
| | | **Antibiotics—*Continued*** | |
| 1.506 | — | 1.528 | Erythromycin gluceptate |
| 1.512 | 1.523 | 1.532 | Erythromycin.$2H_2O$ |
| 1.512 | 1.583 | 1.682 | Nystatin |
| 1.518 | ca 1.572 | >1.780 | Fumagillin |
| 1.522 | 1.548 | 1.566 | Dihydrostreptomycin.3HCl |
| 1.523 | 1.608 | 1.659 | Chloramphenicol |
| 1.523 | 1.622 | 1.630 | Penicillin G benzathine |
| 1.527 | — | 1.569 | Chloramphenicol palmitate |
| 1.528 | 1.536 | 1.550 | Erythromycin.HI.$H_2O$ |
| 1 538 | 1.646 | sl >1.787 | Tetracycline.$3H_2O$ |
| n 1.541 | | | Neomycin sulfate |
| 1.541 | ca 1.553 | 1.573 | Gramicidin |
| 1.541 | 1.585 | 1.656 | Penicillin O chloroprocaine |
| 1.545 | — | 1.593 | Penicillin O potassium |
| 1.545 | 1.570 | 1.685 | Penicillin G procaine |
| 1.546 | 1.635 | 1.730 | Oxytetracycline.HCl |
| 1.550 | — | 1.603 | Penicillin G potassium |
| 1.550 | 1.609 | 1.620 | Penicillin G sodium |
| 1.552 | 1.558 | 1.566 | Dihydrostreptomycin sulfate |
| 1.553 | — | 1.584 | Tyrocidine.HCl |
| 1.556 | ca 1.590 | 1.619 | Penicillin G hydrabamine |
| 1.565 | — | 1.629 | Novobiocin sodium |
| 1.567 | — | 1.613 | Penicillin G dibenzylamine |
| 1.575 | — | 1.610 | Penicillin G *l*-ephedrine |
| 1.583 | — | 1.630 | Cycloserine |
| 1.583 | 1.590 | 1.648 | Penicillin G *l*-ephenamine |
| 1.601 | 1.608 | 1.632 | Penicillin G.HI diethylaminoethyl ester |
| 1.603 | 1.685 | 1.714 | Tetracycline.HCl |
| 1.608 | 1.638 | 1.654 | Novobiocin acid, form 2 |
| 1.634 | 1.646 | >1.700 | Oxytetracycline.$2H_2O$ |
| 1.635 | 1.706 | 1.730 | Chlortetracycline.HCl |
| | | **Antihistamines** | |
| 1.505 | 1.585 | 1.617 | Anthallan®.HCl |
| 1.509 | 1.564 | 1.683 | Dexchlorpheniramine maleate |
| ca 1.525 | 1.563 | 1.598 | Doxylamine succinate |
| 1.533 | $n_i$1.668 | sl <1.734 | Chlorpheniramine maleate |
| 1.548 | 1.574 | 1.665 | Pheniramine maleate |
| 1.553 | 1.625 | >1.734 | Chlorothen.HCl |
| 1.566 | 1.614 | 1.653 | Pyrrobutamine phosphate |
| 1.577 | 1.631 | 1.672 | 2(4-Morpholinyl) ethyl benzhydryl ether.HCl |
| 1.580 | 1.655 | 1.705 | Tripelennamine.HCl |
| 1.583 | 1.603 | 1.645 | Chlorothen citrate |
| 1.585 | 1.600 | 1.668 | *p*-Fluorotripelennamine.HCl |
| 1.587 | 1.635 | 1.734 | Phenbenzamine.HCl |
| 1.588 | 1.654 | >1.695–<1.734 | Methapyrilene.HCl |
| 1.590 | — | 1.680 | Thenyldiamine.HCl |
| 1.590 | 1.610 | 1.665 | Chlorcyclizine.HCl |
| 1.602 | 1.625 | 1.630 | Diphenhydramine.HCl |
| 1.604 | 1.675 | 1.733 | Methaphenilene.HCl |
| 1.610 | 1.660 | 1.665 | Chlorcyclizine.2HCl |
| 1.612 | 1.679 | 1.691 | Thonzylamine.HCl |
| 1.617 | 1.654 | 1.734 | Bromothen.HCl |
| 1.617 | 1.691 | 1.733 | Promethazine.HCl |
| 1.620 | 1.647 | 1.738 | Cyproheptadine.HCl |
| 1.667 | 1.675 | >1.733 | Promethazine.HBr |
| 1.690 | — | 1.737 | Pyrathiazine.HCl |
| | | **Barbiturates** | |
| 1.445 | 1.548 | 1.580 | Barbital |
| 1.454 | 1.518 | 1.556 | Butethal |
| 1.465 | — | 1.565 | Pentobarbital |
| 1.465 | 1.529 | 1.532 | Butabarbital sodium |
| 1.467 | 1.533 | 1.560 | Amobarbital |
| 1.473 | 1.519 | 1.549 | Hexethal |

*(Continued)*

**955.58 Table of refractive indices for drugs, arranged according to ascending value of the lowest index[a] —*Continued.***

| $\alpha$ | $\beta$ | $\gamma$ | Compound |
|---|---|---|---|
| | | | Barbiturates—*Continued* |
| 1.477 | — | 1.523 | Pentobarbital sodium |
| 1.477 | 1.573 | 1.624 | Probarbital |
| 1.487 | 1.557 | 1.563 | Secobarbital |
| 1.490 | $n_i$1.500 | 1.525 | Secobarbital sodium |
| n 1.505 | | | Amobarbital sodium |
| 1.506 | 1.544 | 1.672 | Vinbarbital |
| 1.508 | $n_i$1.521 | 1.577 | Butalbital |
| 1.512 | 1.532 | 1.615 | Barbital sodium |
| 1.515 | 1.546 | 1.621 | Cyclobarbital |
| 1.516 | 1.572 | 1.625 | Allobarbital |
| 1.519 | 1.583 | 1.634 | Sigmodal® |
| 1.520 | 1.575 | 1.626 | Cyclopal® |
| 1.520 | 1.581 | 1.600 | Aprobarbital |
| 1.524 | 1.577 | 1.603 | Butallylonal |
| 1.532 | — | 1.629 | Probarbital sodium |
| 1.534 | 1.634 | — | Thiopental |
| 1.546 | 1.608 | 1.634 | Hexobarbital |
| 1.551 | 1.578 | 1.645 | Alphenal |
| 1.557 | 1.620 | 1.667 | Phenobarbital |
| 1.594 | 1.610 | 1.651 | Mephobarbital |
| | | | Hallucinogens |
| 1.517 | 1.612 | 1.679 | 3,4-Methylenedioxyamphetamine.HCl |
| 1.518 | 1.622 | 1.632 | 4-Methyl-2,5-dimethoxyamphetamine.HCl |
| 1.527 | 1.554 | 1.672 | Psilocybin |
| 1.537 | — | 1.629 | 3,4-Methylenedioxyamphetamine sulfate |
| 1.540 | 1.596 | 1.676 | *d*-Lysergic acid diethylamide tartrate |
| 1.564 | 1.586 | 1.598 | 3,4-Methylenedioxyamphetamine carbonate |
| 1.572 | 1.618 | 1.654 | Phencyclidine.HCl |
| 1.572 | 1.620 | 1.654 | Phencyclidine.HBr |
| | | | Steroids |
| 1.506 | — | 1.598 | Estradiol dipropionate |
| 1.510 | 1.542 | 1.572 | Dehydrocholic acid |
| 1.511 | 1.621 | 1.697 | Estrone, phase 2 |
| 1.512 | 1.552 | 1.621 | Cortisone acetate |
| 1.517 | 1.567 | 1.592 | Triamcinolone diacetate |
| 1.520 | 1.532 | 1.566 | Cholesterol |
| 1.520 | 1.642 | 1.692 | Estrone, phase 1 |
| 1.529 | 1.550 | 1.630 | Desoxycorticosterone acetate |
| 1.529 | 1.575 | 1.676 | Progesterone, beta |
| 1.531 | 1.550 | 1.638 | Hydrocortisone |
| 1.534 | 1.677 | 1.705 | Equilin |
| 1.542 | 1.554 | 1.663 | Progesterone, alpha |
| 1.543 | 1.589 | 1.627 | Hydrocortisone acetate |
| $1.546_\epsilon$ | | $1.595_\omega$ | Triamcinolone acetonide |
| 1.548 | 1.565 | 1.670 | Testosterone |
| 1.552 | — | 1.561 | Methylprednisolone sodium succinate |
| 1.552 | 1.572 | 1.625 | Cortisone |
| 1.553 | 1.572 | 1.648 | Dexamethasone |
| 1.554 | — | 1.667 | Betamethasone |
| 1.555 | ca 1.565 | 1.620 | Methyltestosterone |
| 1.562 | 1.575 | 1.700 | Methylprednisolone acetate |
| 1.562 | 1.568 | 1.704 | Fluorometholone |
| 1.576 | 1.625 | 1.645 | Ethisterone |
| 1.586 | 1.603 | 1.633 | Estradiol benzoate |
| 1.587 | 1.590 | 1.651 | Prednisone |
| 1.594 | 1.611 | 1.73 | Diethylstilbestrol, trans |
| 1.594 | 1.628 | 1.647 | Estrone, phase 3 |
| | | | Sulfonamides |
| 1.513 | 1.590 | >1.690–<1.733 | Sulfallantoin® |
| 1.540 | 1.655 | 1.690 | Sulfanilamide.HCl |
| 1.555 | 1.672 | 1.85 | Sulfanilamide, phase B (anhyd.) |
| 1.559 | 1.564 | 1.727 | Sulfacetamide |

*(Continued)*

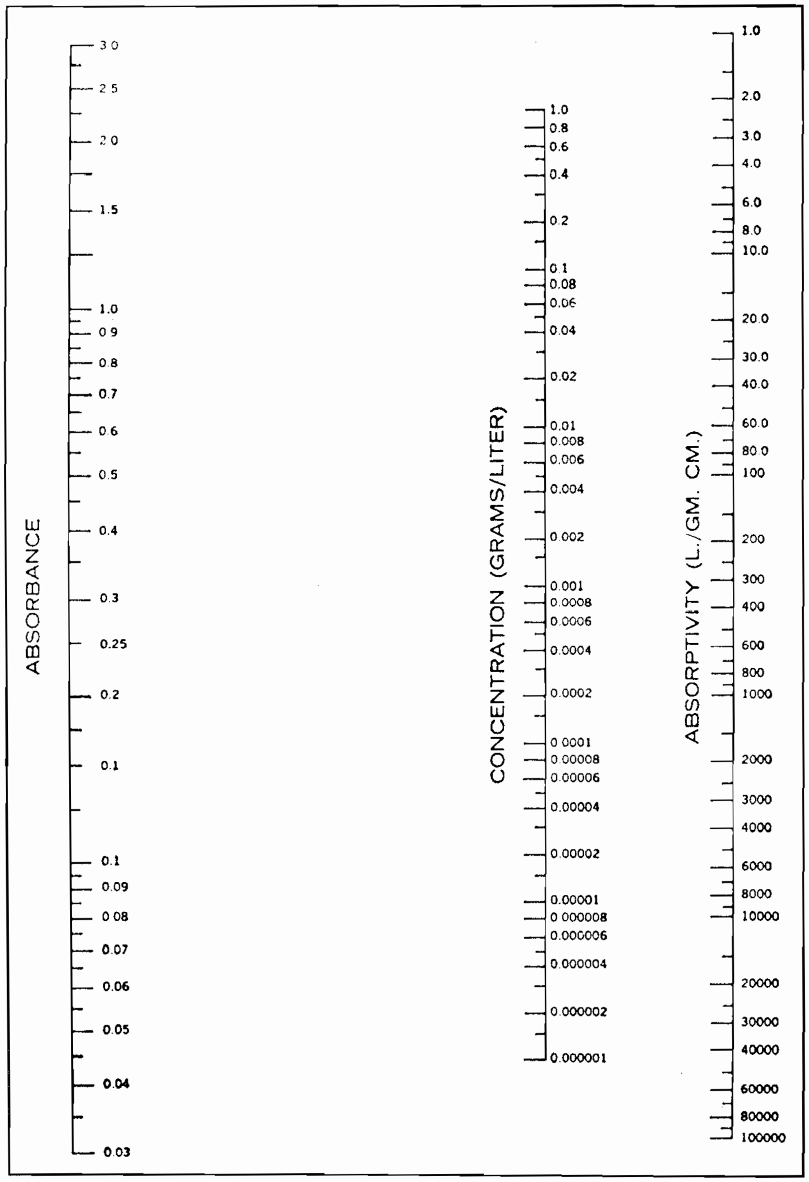
**955.58 Table of refractive indices for drugs, arranged according to ascending value of the lowest index[a] —*Concluded.***

| α | β | γ | Compound |
|---|---|---|---|
| | | Sulfonamides—*Continued* | |
| 1.568 | 1.657 | 1.687 | Sulfamerazine |
| 1.578 | 1.676 | 1.710 | Succinylsulfathiazole |
| 1.584 | 1.623 | >1.778 | Sulfamethazine |
| 1.586 | 1.649 | 1.731 | Sulfaguanidine. $H_2O$ |
| 1.587 | — | 1.675 | Sulfamerazine[b] |
| 1.590 | — | 1.700 | Sulfapyridine sodium. $H_2O$ |
| 1.596 | — | 1.621 | Sulfathiazole sodium. 1½$H_2O$ |
| 1.596 | 1.675 | 1.830 | Sulfadiazine |
| 1.598 | 1.741 | 1.780 | Sulfathiazole, phase II |
| 1.605 | 1.642 | 1.697 | Sulfisoxazole |
| 1.606 | 1.663 | 1.734 | Sulfaguanidine |
| 1.615 | 1.663 | >1.734 | Sulfadiazine[b] |
| 1.661 | 1.678 | >1.733 | Sulfamidazole® |
| 1.670 | 1.736 | 1.813 | Sulfapyridine, phase I |
| 1.674 | 1.685 | >1.733 | Sulfathiazole, phase I |
| 1.680 | 1.733 | >1.733 | Sulfapyridine[b] |
| 1.695 | $n_i$1.733 | >1.733 | Sulfathiazole[b] |
| | | Sympathomimetic Amines | |
| $1.458_\omega$ | | $1.468_\epsilon$ | Tuaminoheptane sulfate |
| 1.501 | 1.545 | 1.603 | Dextroamphetamine sulfate |
| 1.507 | 1.604 | 1.668 | *p*-Hydroxyephedrine.HCl |
| 1.508 | 1.582 | 1.611 | *dl*-Amphetamine.HCl |
| 1.508 | 1.648 | 1.670 | Isoxsuprine.HCl |
| 1.516 | 1.552 | 1.645 | *p*-Hydroxymethamphetamine sulfate |
| 1.516 | $n_i$1.620 | 1.689 | *dl*-Synephrine (+) tartrate (neutral salt) |
| 1.520 | 1.531 | 1.614 | *dl*-Amphetamine sulfate |
| 1.530 | 1.537 | 1.615 | Methamphetamine.HCl |
| 1.530 | 1.585 | 1.596 | Mephentermine sulfate |
| 1.530 | 1.603 | 1.638 | *l*-Ephedrine.HCl |
| 1.535 | 1.540 | 1.620 | *dl*-Methamphetamine.HCl |
| 1.535 | 1.602 | 1.607 | Phendimetrazine.HCl |
| 1.540 | 1.565 | 1.587 | *l*-Ephedrine sulfate |
| 1.543 | — | 1.632 | Pseudoephedrine.HCl |
| 1.544 | 1.594 | 1.614 | Phendimetrazine tartrate |
| 1.546 | 1.583 | 1.664 | Dextroamphetamine phosphate, dibasic |
| 1.546 | 1.604 | ca 1.725 | *dl*-Synephrine base |
| 1.548 | 1.597 | >1.735 | Epinephrine |
| 1.549 | 1.589 | 1.665 | *dl*-Amphetamine phosphate, dibasic |
| 1.549 | 1.605 | 1.664 | Synephrine.HCl |
| 1.560 | 1.592 | 1.622 | Dextroamphetamine.HCl |
| 1.560 | 1.619 | >1.740 | Naphazoline nitrate |
| 1.560 | 1.680 | 1.734 | Hydroxyamphetamine.HBr |
| 1.563 | 1.618 | 1.650 | Phenylpropanolamine.HCl |
| 1.570 | 1.608 | 1.630 | Racephedrine.HCl |
| 1.572 | 1.587 | 1.650 | Levamfetamine succinate |
| 1.577 | — | 1.603 | Phenylpropylmethylamine.HCl |
| 1.586 | 1.604 | 1.703 | Tolazoline.HCl |
| | | Tranquilizers | |
| ca 1.515 | — | ca 1.544 | Meprobamate |
| 1.530 | 1.536 | 1.546 | Ethinamate |
| 1.572 | 1.585 | 1.590 | Glutethimide |
| 1.584 | — | >1.735 | Chlorpromazine.HCl |
| 1.589 | 1.609 | 1.700 | Thiopropazate.2HCl |
| 1.634 | ca 1.710 | >1.780 | Chlordiazepoxide.HCl |
| 1.638 | 1.647 | 1.674 | Azacyclonol.HCl |

[b] The second set of optical properties in each case represents intermediate data which are commonly found in some commercial samples. They probably represent an anhydrous form or merely a different common orientation of the crystal.

**963.37 Nomograph relating absorbance, concentration, and absorptivity (1 cm cell)**

(A straight edge placed at known values on two appropriate axes (i.e., absorbance and absorptivity) will intersect the corresponding value on the third axis (i.e., concentration).)




Reprinted from *The Analyzer* **4**, No. 2 (1963)
Beckman Instruments, Inc., Fullerton, Calif.

# Appendix: Guidelines for Collaborative Study Procedure to Validate Characteristics of a Method of Analysis

(Incorporates symbols, terminology, and recommendations accepted by consensus by the participants at the IUPAC Workshop on Harmonization of Collaborative Analytical Studies, Geneva, Switzerland, May 4–5, 1987.)

This document has been prepared from the point of view of AOAC administrative arrangements. In AOAC, the Associate Referee is the individual scientist responsible for choosing the method, conducting intra- and interlaboratory tests, evaluating the results, and recommending approval of a method of analysis. Other organizations who may utilize this document may wish to substitute their own terminology. Although the directions were developed for chemical studies, some parts may be applicable to all types of collaborative studies.

### Summary Statement of AOAC Recommendation for the Design of a Collaborative Study:

1. *Minimum number of materials:* 5 (only when a single level specification is involved for a single matrix may this minimum be reduced to 3).
2. *Minimum number of laboratories:* 8 reporting valid data for each material (only in special cases involving very expensive equipment or specialized laboratories may the study be conducted with a minimum 5 laboratories, with the resulting expansion in the confidence interval for the statistical estimates of the method characteristics).
3. *Minimum number of replicates:* 1, if within-laboratory repeatability parameters are not desired; 2, if these parameters are required. Replication should ordinarily be attained by blind replicates or split levels (Youden pairs).

### Guide to Collaborative Study Procedure

(Section numbers correspond to Outline; not all section numbers are included in guide.)



## 1. Preliminary Work (Within One Laboratory)

### *1.1 Determine Purpose and Scope of the Study and Method*

Determine purpose of the study (e.g., to determine attributes of a method, proficiency of analysts, reference values of a material, or to compare methods), the type of method (empirical, screening, practical, reference, definitive), and the probable use of the method (enforcement, surveillance, monitoring, acceptance testing, quality control, research). Also, on the basis of the relative importance of the various method attributes (bias, precision, specificity, limit of determination), select the design of the interlaboratory study. The directions in this document pertain primarily to determining the precision characteristics of a method, although many sections are also appropriate for other types of studies.

### *1.2 Alternatives for Method Selection*

1.2.1 Sometimes obvious (only method available)
1.2.2 Critical literature review (reported within-laboratory attributes are often optimistic)
1.2.3 Survey of laboratories to obtain candidate methods; comparison of within-laboratory attributes of candidate methods (sometimes choice may still not be objective)
1.2.4 Selection by expert (AOAC-preferred procedure—selection by Associate Referee with concurrence of General Referee)
1.2.5 Selection by committee (ISO-preferred procedure—often time-consuming)
1.2.6 Development of new method or modification of existing method when an appropriate method is not available. (Proceed as a research project.) (This alternative is time-consuming and resource-intensive; use only as a last resort).

### *1.3 Optimize Either New or Available Method*

1.3.1 Practical principles

(a) Do not conduct collaborative study with an unoptimized method. An unsuccessful study wastes a tremendous amount of collaborators' time and creates ill will. This applies especially to methods that are formulated by committees and have not been tried in practice.

(b) Conduct as much experimentation within a single laboratory as possible with respect to optimization, ruggedness, and interferences. Analysis of the same material on different days provides considerable information on variability that may be expected in practice.

1.3.2 Alternative approaches to optimization

(a) Conduct formal ruggedness testing for identification and control of critical variables. See Youden and Steiner (8.1, pp. 33–36, 50–55). The actual procedure is even simpler than it appears. (This is an extremely efficient way for optimizing a method.)

(b) Use Deming simplex optimization to identify critical steps. See Dols and Armbrecht (8.3).

(c) Conduct trials by changing one variable at a time.

### *1.4 Develop Within-Laboratory Attributes of Optimized Method*

(Some items can be omitted; others can be combined.)

1.4.1 Determine calibration function (response vs concentration in pure or defined solvent) to determine useful measurement range of method. For some techniques, e.g., radioimmunoassay, linearity is not a prerequisite. Indicate any mathematical transformations needed.

1.4.2 Determine analytical function (response vs concentration in matrix, including blank) to determine applicability to commodity(ies) of interest.

1.4.3 Test for interferences (specificity):

(a) Test effects of impurities, ubiquitous contaminants, flavors, additives, and other components expected to be present and at usual concentrations.

(b) Test nonspecific effects of matrices.

(c) Test effects of transformation products, if method is to indicate stability, and metabolic products, if tissue residues are involved.

1.4.4 Conduct bias (systematic error) testing by measuring recoveries of analyte added to matrices of interest and to extracts, digests, or other treated solutions thereof. (Not necessary when method itself defines the property or component.)

1.4.5 Develop performance specifications for instruments and suitability tests for systems (which utilize columns or adsorbents) to ensure satisfactory performance of critical steps (columns, instruments, etc.) in method.

1.4.6 Conduct precision testing at the concentration levels of interest, including variation in experimental conditions expected in routine analysis (ruggedness).

In addition to estimating the "classical" repeatability standard deviation, $s_r$, the initiating laboratory may estimate the total within-laboratory variability expected by determining the variability at different days and with different calibration curves, by the same or different analysts within a single laboratory. This total within-laboratory estimate includes both between-run (between-batch) and within-run (within-batch) variability.

1.4.7 Delineate the range of applicability to the matrices or commodities of interest.

1.4.8 Compare the results of the application of the method with existing tested methods intended for the same purposes, if other methods are available.

1.4.9 If any of the preliminary estimates of the relevant performance of these characteristics are unacceptable, revise the method to improve them, and retest as necessary.

1.4.10 Have method tried by analyst not involved in its development.

1.4.11 Revise method to handle questions raised and problems encountered.

### *1.5 Prepare Description of Method*

*Note:* A collaborative study of a method involves practical testing of the written version of the method, in its specific style and format, by a number of laboratories on identical materials.

1.5.1 Prepare method in format and style given in the *Handbook for AOAC Members* (1989), or other recognized manual, e.g., *ISO Guide 18* (8.4).

1.5.2 Clearly specify requirements for chromatographic materials, enzymes, antibodies, and other performance-related reagents.

1.5.3 Clearly describe and explain every step in the analytical method so as to discourage deviations. Use imperative directions; avoid subjunctive and conditional expressions as options as far as possible.

1.5.4 Edit method for completeness, credibility (e.g., buffer pH consistent with specified chemicals, volumes not greater than capacity of container), continuity, and clarity.

1.5.5 Check for inclusion of performance specifications and systems suitability tests (1.4.5), defined critical points, and convenient stopping points. Incorporate physical or chemical constants of working standard solutions, e.g., absorptivities, half-scale deflections, recoveries, etc., or properties of operating solutions and chromatographic materials, e.g., pH, volumes, resolution, etc., and any other indicators (e.g., sum equals 100%) that suggest analysis is proceeding properly.

1.5.6 If time and resources are available, conduct pilot study involving 3 laboratories.

### *1.6 Invite Participation*

1.6.1 *Selection of candidate laboratories.* Laboratories invited to participate should have personnel experienced in the basic techniques employed; experience with the method itself is not a prerequisite for selection. Lists of possible participants can be developed through personal contacts, technical societies, trade associations, or literature search, and advertisements in the AOAC's news publication "The Referee."

1.6.2 *Letter of invitation.* Address a formal letter to the individual responsible for assignment of laboratory effort. State reason for selecting that laboratory (e.g., as a volunteer or has responsibility or familiarity with the problem or method), estimated number of person-hours required for performance, number of materials to be sent, number of analyses to be required, expected date for material distribution, and target date for completion of the study. *Emphasize the importance of management support in assigning the necessary time for the project.* Enclose a copy of the method and a return form or card (with postage affixed, if domestic), requiring only a check mark for acceptance or refusal of the invitation, a signature, and space for address corrections, telephone number, and date.

With large studies, involving several analysts per laboratory, several familiarization materials, receipt of items at different times, or similar recurrent situations, acceptance of the invitation should be followed by a letter suggesting that a study coordinator be appointed. The study coordinator should be responsible for receiving and storing the materials, assigning the work, dispensing materials and information related to the study, seeing that the method is followed as written, accumulating the data, assuring that the data are correctly reported, and submitting the report within the deadline.

(A file of letters previously used for these purposes is available).

### *1.7 Instructions and Report Forms*

1.7.1 Carefully design and prepare instructions and forms, and scrutinize them before distribution. A pilot study (1.5.6) is also useful for uncovering problems in these documents.

1.7.2 Send instructions and report forms immediately on receipt of acceptance, independent of materials, if selection of laboratories is not to be based on performance in pilot or training studies. The instructions should include in bold face or capital letters a statement: "THIS IS A STUDY OF THE METHOD, NOT OF THE LABORATORY. THE METHOD MUST BE FOLLOWED AS CLOSELY AS PRACTICABLE, AND ANY DEVIATIONS FROM THE METHOD AS DESCRIBED, NO MATTER HOW TRIVIAL THEY MAY SEEM, MUST BE NOTED ON THE REPORT FORM."

1.7.3 Include instructions on storage and handling, markings, and identifications to be noted, any special preparation for analysis, and criteria for use of practice or familiarization materials, if included (*see* 1.8). Precode the form for each laboratory and provide sufficient space for as much sequential data as may be required for proper evaluation of the results, including a check of the calculations.

Indicate the number of significant figures to be reported.

When recorder tracing reproductions are required to evaluate method performance, request their submission both in the instructions and as a check item on the form. Provide instructions with regard to labeling of recorder tracings, such as identification with respect to item analyzed, axes, date, submitter, experimental conditions, and instrument settings.

Include in the report form a signature line for the analyst and lines for a printed or typed version of the name and address for correct acknowledgment. Provide for a review by the laboratory supervisor. An example of a completed form is helpful. A questionnaire may be included or sent after completion of the analyses in which the questions can be designed to reveal if modifications have been made at critical steps in the method.

1.7.4 Request a copy of the calibration curve or other relationship between response and concentration or amount of analyte so that if discrepancies become apparent after examining all of the data, it can be determined whether the problem is in the calibration or in the analysis.

### *1.8 Familiarization or Practice Materials*

If deemed necessary, supply as far ahead as practicable, familiarization materials, with instructions, before actual materials are sent. When familiarization materials have been submitted, supply forms for reporting progress toward satisfactory performance.

## 2. Design of the Collaborative Study

### *2.1 General Principles*

2.1.1 The purpose of the collaborative study is to provide a realistic estimate of the attributes of a method, particularly the systematic and random deviations, to be expected when the method is used in actual practice. A collaborative study usually provides information on the best performance to be expected.

2.1.2 The design should attempt to identify and to include the possible sources of significant variability that may occur in actual practice, including between days, between runs, and between calibration curves, if these are significant factors. (Within-laboratory performance, 1.4.6, particularly on different days and with different calibration curves may provide a clue with respect to between-laboratory performance and is required information for quality control.) The best measure of within-laboratory variability is obtained by using blind replicates or split levels (Youden pairs). The design must take into account how the data will be analyzed statistically.

2.1.3 Present materials sent for analysis as unknowns (blind) and coded in a random pattern. If necessary to conserve analyst time, an indication of the potential range of concentration or amount of analyte may be provided. If spiking solutions are used, provide one coded solution for each material. All spiking solutions should be identical in appearance and volume. Do not provide a single solution from which aliquots are to be removed for spiking. Any information with regard to concentration (e.g., utilizing factorial aliquots or serial dilutions of the same spiking solutions) or known replication is likely to lead to an underestimate of the variability.

2.1.4 The study must be extensive enough to assure sufficient data surviving in the face of possible loss of materials during shipment, inability of collaborators to participate after acceptance, and a maximum outlier rate of 2/9 (2 laboratory failures in a 9-laboratory study, when a minimum design is used).

2.1.5 Improper preparation of reference standards and standard solutions can cause a significant portion of the analytical error. A decision must be made whether such error is to be considered separately or as part of the method, i.e., will the analysts procure their own standards and prepare their own standard solutions or will standards be provided by the Associate Referee. The decision depends primarily on the availability of the standard. If the standard is readily available, the analysts should prepare their own. If the standard is not readily available, the standard may be supplied, but physical constants, e.g., absorptivity, of working standard solutions should be incorporated into the description as a check on proper preparation of the solution.

2.1.6 Obtain the necessary administrative and operational approvals. Review by potential users of the method is also desirable.

### *2.2 Laboratories*

Laboratories must realize the importance of the study. A large investment is being made in testing the method and this probably will be the only collaborative study of the method that will be performed. Therefore, it is important to have a fair and thorough evaluation of the method.

2.2.1 *Type*. The most appropriate laboratory is one with a responsibility related to the analytical problem. Laboratory types may be representative (selection of laboratories that will be using the method in practice), reference (assumed to be "best"), or the entire population of laboratories (usually certified or accredited) that will be using the method. Final selection of participants should be based on a review with the General Referee and other Associate Referees of each laboratory's capabilities and past performance in collaborative studies, followed up, if possible, by telephone conversations or by personal visits. Selection may also be based on performance with familiarization materials. Sometimes only laboratories with dedicated or very specialized instruments must be used. If the study is intended for international consideration, laboratories from different countries should be invited to participate.

2.2.2 *Number of laboratories:* Minimum of 8 laboratories submitting valid data (to avoid unduly large confidence bands about the estimated parameters). Only in special cases of very expensive equipment or specialized laboratories may the study be conducted with a minimum of 5 laboratories. Fewer laboratories widen the confidence limits of the mean and of the variance components (*see* design considerations 2.4.1 and 2.4.2). The optimum number of laboratories, balancing logistics and costs against information obtained, often is 8–10. However, larger studies are not discouraged.*

2.2.3 *Analysts:* Most designs require only 1 analyst per laboratory. If analyst-within-laboratory variability is a desired variance component, multiple analysts should be requested from all participating laboratories. Ordinarily 2 analysts from the same laboratory cannot be substituted for different laboratories, unless standard solutions, reagents, chromatographic columns and/or materials, instrument calibrations, standard curves, etc., are prepared independently, and no consultation is permitted during the work. Different laboratories from the same organization may be used as separate laboratories if they operate independently with their own instruments, standards, reagents, and supervision.

### *2.3 Test Materials*

2.3.1 *Materials must be homogeneous. This is critical.* Establish homogeneity by testing a representative number of laboratory samples taken at random before shipment. (A collaborator who reports an outlying value will frequently claim receipt of a defective laboratory sample.) The penalty for inhomogeneity is an increased variance in the analytical results that is not due to the intrinsic method variability.

2.3.2 *Code laboratory samples* at random so that there is no preselection from order of presentation.

2.3.3 *Concentration range:* Choose analyte levels to cover concentration range of interest. If concentration range of interest is a tolerance limit or a specification level, bracket it and include it with materials of appropriate concentration. If design includes the determination of absence of analyte, include blank (not detectable) materials as part of range of interest.

2.3.4 *Number of materials:* Minimum number of materials is 5. However, when a single-level specification is involved, this may be reduced to an absolute minimum of 3.

2.3.5 *Nature of materials:* Materials should be representative of commodities usually analyzed, with customary and extreme values for the analyte.

2.3.6 *Size of laboratory samples:* Furnish only enough material to provide the number of test portions specified in the instructions. If additional test portions are required, the collaborator must request them, with an explanation.

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* In some cases Associate Referees are unable to obtain more than 5 participants. In such cases a study may be conducted with 5 laboratories, but it must be realized that the reliability of the resulting estimates of performance parameters is reduced considerably. Furthermore, in such cases the number of test materials in the study should be increased to provide some additional assurance, but the analysis of more test materials is not a substitute for fewer laboratories. Methods adopted on the basis of such a design may not be acceptable to other organizations that have adopted the IUPAC-87 recommendations.

2.3.7 *Interferences:* If pertinent, some materials, but not all, should contain contaminants and interferences in concentrations likely to be encountered, unless they have been shown to be unimportant through within-laboratory testing (*see* 1.4.4). The success of the method in handling interference on an intralaboratory basis will be demonstrated by passing systems suitability tests.

2.3.8 *Familiarization materials:* With new, complex, or unfamiliar techniques, provide material(s) of stated composition for practice, on different days, if possible (*see* 1.8). The valuable collaborative materials should not be used until the analyst can reproduce the stated value of the practice samples within a given range. However, it should be pointed out that one of the assumptions of analysis of variance is that the underlying distribution of results is independent of time (i.e., there is no drift). The Associate Referee must be satisfied that this assumption is met.

### *2.4 Replication*

When within-laboratory variability is also of interest, as is usually the case, independent replication can be ensured by applying at least one of the following procedures (listed in suggested order of desirability; the nature of the design should not be announced beforehand):

2.4.1 *Split levels (Youden pairs):* A pair of materials of slightly different composition obtained either naturally or by diluting (or by fortifying) one portion of the material with a small amount of diluent (or of analyte). Both portions are supplied to the participating laboratories under a random code number and each portion should be analyzed only once; replication defeats the purpose of the design.

2.4.2 *Split levels for some materials and blind duplicates for other materials* in the same study (obtain only single values from each portion supplied).

2.4.3 *Blind duplicate laboratory samples, randomly coded. Note:* Triplicate and higher replication are relatively inefficient when compared with duplicate test samples because replication provides additional information only on individual within-laboratory variability, which is usually the less important component of error. It is more effective to utilize resources for the analysis of more levels and/or materials rather than for increasing the number of replicates for the individual materials.
PRACTICAL PRINCIPLE: With respect to replication, the greatest net marginal gain is always obtained in going from 2 to 3 as compared to going from 3 to 4, 4 to 5, etc.

2.4.4 *Independent materials.* (*Note:* Unrelated independent materials may be used as a split level in the calculations of the precision parameters or for plotting, but the more they differ, particularly with respect to concentration, the less reliable the information they provide on within-laboratory variability.)

2.4.5 *Use of known replicates* is a common practice. It is much preferable to use the same resources on blind replicates or split levels. (*See Note* of 2.4.3.)

2.4.6 Instead of obtaining repeatability parameters through the collaborative study, *use of quality control materials in each laboratory individually,* for its own use, independent of the collaborative study, for a separate calculation of $s_r$, using 2 (or more) replicates from each quality control test, according to the pattern developed for each product.

### *2.5 Other Design Considerations*

2.5.1 The design can be reduced in the direction of less work and less cost, but at the sacrifice of reduced confidence in the reliability of the developed information.

2.5.2 More work (values) is required if more confidence is needed, e.g., greater confidence is required to enforce a tolerance at 1.00 ppm than at 1.0 ppm. (The distinction is a precision requirement of the order of 1% rather than 10%.)

2.5.3 The estimate of the standard deviation and of the corresponding relative standard deviation is a random variable. For example, 30 data points from a single population will permit estimation of the standard deviation *of an individual reading* only to within ±25% with about 95% confidence; 200 data points are required to estimate that standard deviation to within about 10%, with about 95% confidence. The distribution of standard deviations is actually asymmetrical for small numbers of values, e.g., the relative distribution about 1.00 for 30 values (about 95% confidence limits) is 0.78–1.30; for 200, 0.91–1.10.

2.5.4 The validity of extrapolating the use of a method beyond concentrations and components tested can be estimated only on the basis of the slope of the calibration curve (sensitivity) observed as a function of the nature and concentration of the matrix and contaminant components. If the signal is more or less independent of these variables, a reasonable amount of extrapolation may be utilized. The extrapolator assumes the burden of proof as to what is reasonable.

## 3. Preparation of Materials for Collaborative Studies

### *3.1 General Principles*

3.1.1 Heterogeneity between portions from a single test material must be negligible compared to analytical variability, as measured within the Associate Referee's laboratory. *See also* 2.3.1.

3.1.2 The containers must not contribute extraneous analytes to the contents, and they must not adsorb or absorb analytes or other components from the matrix, e.g., water.

3.1.3 If necessary, the materials may be stabilized, preferably by physical means (freezing, dehydrating), or by chemical means (preservatives, antioxidants) which do not affect the performance of the method.

3.1.4 Compositional changes must be avoided, where necessary, by the use of vapor-tight containers, refrigeration, flushing with an inert gas, or other protective packaging.

### *3.2 Materials Suitable for Collaborative Studies*

3.2.1 *A single batch of homogeneous, stable product* such as milk powder, peanut butter, vegetable oil, starch, etc., is the best type of material.

3.2.2 *Reference materials* supplied by standards organizations such as the National Bureau of Standards are excellent, unless they have easily recognizable characteristics (e.g., odor and color of NBS Orchard Leaves). However, they are of limited availability, composition, and analyte level. If available, they are expensive. Sometimes the certification organization may be interested in making reference materials available for the analyte under study, in which case it may assist in providing the material for the study.

3.2.3 *Synthetic materials* may be especially formulated with known amounts of analytes by actual preparation for the study. This procedure is best used for macroconstituents such as drugs or pesticide formulations.

3.2.4 *Spiked materials* consisting of normal or blank materials to which a known amount of analyte has been added may be used. The amount of analyte added should not be excessive in relation to the amount present (e.g., about 2×), and the analyte added should be in the same chemical form as present in the commodities to be analyzed subsequently.

In drug and pesticide residue-type problems, it is often necessary to use spiked materials in order to assess recovery. However, because incurred residues are likely to present different problems from those of spiked residues, collaborative studies should include some incurred test samples to ensure that the method is applicable under these conditions as well.

(a) *Preparation in bulk:* This requires thorough and uniform incorporation of the analyte, often by serial dilution of solids. The danger of segregation due to differences in densities always exists. Fluid materials susceptible to segregation should be prepared under constant agitation. Uniformity should be checked by direct analysis, with an internal standard, or by a marker compound (dye or radioactive label).

(b) *Laboratory samples, individually prepared:* A known amount of analyte is either weighed directly or added as an aliquot of a prepared solution to premeasured portions of the matrix in individual containers. The collaborator is instructed to use each entire portion for the analysis, transferring the contents of the container quantitatively or a substantial weighed fraction of the portion. (This is the preferred alternative to spiked solid materials at trace [ppm] levels, at the expense of considerably more work.)

(c) *Concentrated unknown solutions for direct addition by collaborators to their own commodities:* Should be used only as a last resort when instability of the analyte precludes distribution from a central point. To preclude direct analysis of the spiking solution, supply individual coded solutions to be added in their entirety to portions of the matrix for single analyses by each laboratory. All solutions should have the same volume and appearance. This type of material is analogous to that of 3.2.4(b) except for the source of matrix. This case should be used only for perishable commodities that are altered by all available preservation techniques.

3.2.5 *Materials analyzed by another, presumably accurate, method,* if available, in the Associate Referee's laboratory or by some or all the collaborators.

3.2.6 *Only as an absolutely last resort (usually with unstable materials and preparation of material studies) should the collaborators be permitted to prepare their own materials* for analysis. Since it is impossible to avoid the personal bias introduced by knowledge of the composition of the material, the materials should be prepared in each laboratory by an individual who will not be involved in the analyses.

### *3.3 Blanks*

When the absence of a component is as important as its presence, when determinations must be corrected for the amount of the component or the presence of background in the matrix, or when recovery data are required, provision must be made for the inclusion of blank materials containing "none" (not detected) of the analyte. It is also important to know the variability of the blank and the tendency of the method to produce false positives. There are 2 types of blanks: matrix blanks and reagent blanks. Since laboratories often will utilize reagents from different sources, each laboratory should perform reagent blanks. Matrix blanks, when required, are an intrinsic part of the method, and the number of blanks needed depends on the combined variance of the material ($s_M$) and of the blank ($s_B$). The total variability of a blank corrected value will be $s = (s_M^2 + s_B^2)^{1/2}$.

### *3.4 Limit of Determination*

If the limit of determination is important, it is necessary to provide a design which gives special attention to the number of blanks, and to the necessity for interpreting false positives and false negatives. In all cases, the definition of limit of determination used in the study must be given by the Associate Referee.

### *3.5 Controls*

When separation from interferences is critical to the analysis, appropriate materials incorporating these interferences must be included.

### *3.6 Practical Principle*

Always allow for contingencies and prepare more sets (e.g., 25% more) of laboratory samples than there are collaborators. Some packages may never arrive, some materials may spoil, and some may be lost or the container broken. New laboratories may have to be substituted for those which are unable to complete the promised work. Some sets may have to be analyzed at a later time for different purposes, such as to verify stability on storage.

## 4. Submission of Laboratory Samples

4.1 *Label laboratory samples legibly and without ambiguity.*

4.2 *Pack shipping cartons well and label properly to avoid transportation delays.* If the containers are breakable, pack well to minimize possibility of breakage. If material is perishable, ship frozen with solid $CO_2$, sufficient to last several days longer than anticipated travel time. Notify collaborators of shipping arrangements, including waybill numbers, arrival time, and required storage conditions. Use special transportation services, if necessary. For international delivery, mark as "Laboratory samples—no commercial value" or other designation as required by customs regulations of the country to which the package is being sent. Hazardous materials must be packed and labeled as required by transportation regulations. Animal and plant products sent across international borders may require special certification from health authorities.

4.3 *Include a return slip, to confirm safe receipt, with each package.* If not sent previously, include copy of method, instructions, and report forms.

4.4 *Provide instructions for proper storage* of laboratory samples between unpacking and analysis. Do not use thawed or decomposed test samples without consulting the Associate Referee.

4.5 *When it is important to have instruments calibrated with the same reference material,* supply reference material to collaborators. Provision for supplying reference standards is particularly important when commercial sources of standards have not yet been developed. The inclusion of a working standard solution as an unknown is useful to establish a consensus value for standardization of quality control parameters, such as absorptivity, retention time, and sensitivity (change in signal intensity divided by the change in concentration).

## 5. Obligations of Collaborators

5.1 *Analyze materials at times indicated, according to submitted protocol.* With unstable materials (e.g., with microbial or decomposition problems) analyses must be started at specified times.

5.2 *FOLLOW METHOD EXACTLY (this is critical).* Any deviation, such as the necessity to substitute reagents, columns, apparatus, or instruments, must be recorded at the time and reported. If the collaborator has no intention of following the submitted method, he or she should not participate in the study. If the collaborator wishes to check another method on the same materials, additional laboratory samples should be requested for that purpose, to be analyzed separately.

5.3 *Conduct exactly the number of determinations stated in the instructions.* Any other number complicates the statistical analysis. Too few determinations may require discarding the results from that laboratory for that material or inserting "missing values"; too many values may require discarding the contribution of that laboratory or at least some of the values. If a laboratory cannot follow instructions as to number of analyses to perform, it raises a question as to its ability to follow the method.

5.4 *Report individual values, including blanks.* Do not average or do other data manipulations unless required by the instructions. Undisclosed averaging distorts statistical measures. If blank is larger than determination, report the negative value; do not equate negative values to zero. Follow or request instructions with regard to reporting "traces" or "less than." Descriptive (i.e., nonquantitative) terms are not amenable to statistical analysis and should be avoided. When results are below the limit of determination, report actual calculated result, regardless of its value.

5.5 *Supply raw data, graphs, recorder tracings, photographs, or other documentation* as requested in the instructions.

5.6 *If analytical results appear unreasonable, investigate possible cause immediately,* first by checking for transcription and calculation mistakes, and then by reanalysis, if permitted by the protocol. Call Associate Referee to discuss suspicious values. If Associate Referee indicates a value may be an outlier, review the determination promptly to the extent possible, by recalculation, reanalysis, or preparation of new standards. If time and materials are available, obtain new laboratory samples for repeat analysis.

Since collaborators may have no basis for judging whether a value is an outlier, the results should be communicated to the Associate Referee as soon as the protocol is complete and before time and equipment are reassigned, so that repeat assays may be performed at once, if necessary.

*Note:* The sooner an apparent outlier is investigated, the greater the likelihood of finding a reason for its occurrence. The most frequent causes of correctable outliers are:

5.6.1 Incorrect calculations and arithmetic errors.

5.6.2 Errors in reporting, such as transposition of numbers, misplacement of the decimal point, or use of the wrong units.

5.6.3 Incorrect standards due to weighing or volumetric errors (check physical constants or compare against freshly prepared standard solutions).

5.6.4 Contamination of reagents, equipment, or test materials.

## 6. Statistical Analysis

### *6.1 Initial Review of Data (Data Audit)*

The Associate Referee should first plot the collaborative data material by material (or one value against the other for a split level [Youden pair]), values vs laboratory, preferably in ascending or descending order of reported average concentration. Usually major discrepancies will be apparent: displaced means, unduly spread replicates, outlying values, differences between methods, consistently high or low laboratory rankings, etc.

Only valid data should be included in the statistical analysis. Valid data are values that the collaborator has no reason to suspect as being wrong. Invalid data may result when: (a) the method is not followed; (b) a nonlinear calibration curve is found although a linear curve is expected; (c) system suitability specifications were not met; (d) resolution is inadequate; (e) distorted absorption curves arise; (f) unexpected reactions occur; or (g) other atypical phenomena materialize. Other potential causes of invalid data are included in 5.6.1–5.6.4.

### *6.2 Statistical Approach*

Perform calculations on each material individually or as split levels, as appropriate. Only if the variances are not significantly different from each other should the results across materials be pooled for analysis of variance. If the relative standard deviations of the results from different materials are not significantly different, it may be convenient to average them over the range tested and to thereby report just a single relative standard deviation. Consultation with a statistician is always desirable.

### *6.3 Outliers*

Collaborative studies seem to have an inherent level of outliers, the number depending on the definition of outliers and the basis for calculation (analytes, materials, laboratories, or determinations). Rejection of more than 2/9 of the data from each material in a study, without an explanation (e.g., failure to follow the method), is ordinarily considered excessive. This corresponds to rejection of more than 1 laboratory from a 5–6 laboratory study or 2 from a 9 laboratory study. For larger studies, a smaller acceptable percentage of rejections may be more appropriate. Determine the probability that the apparent aberrant value(s) is part of the main group of values considered as a normal population by applying the following tests in order:

6.3.1 *Cochran test* for removal of laboratories (or indirectly for removal of extreme individual values from a set of laboratory values) showing significantly greater variability among replicate (within-laboratory) analyses than the other laboratories for a given material. Apply as a 1-tail test at a probability value of 0.01.

To calculate the Cochran test statistic: Compute the within-laboratory variance for each laboratory and divide the largest of these by the sum of all of these variances. The resulting quotient is the Cochran statistic which indicates the presence of a removable outlier if this quotient exceeds the critical value listed in the Cochran table for $P$ = 0.01 (1-tail) and L (number of laboratories), Appendix A-1.

6.3.2 *Grubbs tests* for removal of laboratories with extreme averages. Apply in the following order: single value test (2-tail; $P = 0.01$); then if no outlier is found, apply pair value test (2 values at the highest end, 2 values at the lowest end, and 2 values, one at each end, at an overall $P = 0.01$).

To calculate the single Grubbs test statistic: Compute the average for each laboratory and then calculate the standard deviation (SD) of these L averages (designate as the original s). Calculate the SD of the set of averages with the highest average removed ($s_H$); calculate the SD of the set of averages with the lowest average removed ($s_L$). Then calculate the percentage decrease in SD as follows:

$$100 \times [1 - (s_L/s)] \text{ and } 100 \times [1 - (s_H/s)]$$

The higher of these 2 percentage decreases is the single Grubbs statistic, which signals the presence of an outlier to be omitted if it *exceeds* the critical value listed in the single Grubbs tables at the $P = 0.01$ level, 2-tail, for L laboratories, Appendix A-2.

To calculate the Grubbs pair statistic, proceed in an analogous fashion, except calculate the standard deviations $s_{2L}$, $s_{2H}$, and $s_{HL}$, following removal of the 2 lowest, the 2 highest, and the highest and the lowest averages, respectively, from the original set of averages. Take the smallest of these 3 SD values and calculate the corresponding percentage decrease in SD from the original s. A Grubbs outlier pair is present if the selected value for the percentage decrease from the original s *exceeds* the critical value listed in the Grubbs pair value table at the $P = 0.01$ level, for L laboratories, Appendix A-2.

6.3.3 If the single value Grubbs test signals the need for outlier removal, remove the single Grubbs outlier and recycle back to the Cochran test as shown in the flowchart, Appendix A-3.

If the single value Grubbs test is negative, check for masking by performing the pair value Grubbs test. If this second test is positive, remove the 2 values responsible for activating the test and recyle back to the Cochran test as shown in the flowchart, Appendix A-3, and repeat the sequence of Cochran, single value Grubbs, and pair value Grubbs. Note, however, that no outliers should be removed if such removal results in an overall reduction of more than 2/9 in the number of laboratories whose values are removed.

6.3.4 If no outliers are removed for a given cycle (Cochran, single Grubbs, pair Grubbs), outlier removal is complete. Also, stop outlier removal whenever more than 2/9 of the laboratories are flagged for removal. With a higher removal rate, either the precision parameters must be taken without removal of all outliers or the method must be considered as suspect.

*Note:* The decision as to whether a value(s) should be removed as an outlier ultimately is not statistical in nature. The decision must be made by the Associate Referee on the basis of the indicated probability given by the outlier test and any other information that is pertinent. (However, for consistency with other organizations adhering to the harmonized outlier removal procedure, the estimate resulting from rigid adherence to the prescribed procedure should be reported.)

### *6.4 Bias (Systematic Deviation) of Individual Results*

6.4.1 (Estimated) Bias = mean amount found − amount added (or known or assigned value)

*Note:* Error of a single value = the single value − amount added (true value)

% Recovery = (measured concentration in fortified material − measured concentration in unfortified material) × 100/(known increment in concentration)

The amount added should be a substantial fraction of, or more than, the amount present in the unfortified material.

6.4.2 A true or assigned value is known only in cases of spiked or fortified materials, certified reference materials, or by analysis by another (presumably unbiased) method. Concentration in the unfortified material is obtained by direct analysis or by the method of additions. In other cases, there is no direct measure of bias, and consensus values derived from the collaborative study itself often must be used for the reference point.

6.4.3 Notes:

(a) [Note for Youden Manual] Youden equates "true" or "pure" between-laboratory variability (not including the within-laboratory variability) to the variability in bias (or variability in systematic error) of the individual laboratories. Technically, this definition refers to the average squared difference between individual laboratory biases and the mean bias of the assay.

(b) The presence of random error limits the ability to estimate the systematic error. To detect the systematic error of a single laboratory when the magnitude of such error is comparable to that laboratory's random error, at least 15 values are needed, under reasonable confidence limit assumptions.

### *6.5 Precision (Random Error)*

The relative basis (i.e., relative standard deviation, RSD) is the most useful measure of precision in chemical analytical work because the RSDs are usually independent of concentration or amount of analyte over a reasonable range of concentrations. Therefore, the use of RSD facilitates comparision of variabilities at different concentrations. When the RSD increases rapidly with a decrease in concentration or amount, the rise delineates the limit of usefulness of the method (limit of reliable measurement). The most important types of precision are:

6.5.1 *Reproducibility*—among-laboratories (including within-laboratories) precision, designated as $s_R$.

*Note:* This component is not obtained merely by calculating the standard deviation of all the data (except when there are no replicates) since this term must be corrected by a replication term (Youden, 8.1, p. 19). The correction term must be extracted by an analysis of variance technique (Steiner, 8.1, p. 78-81). However, this crude, overall calculation of the standard deviation of all the data can serve as a check on the arithmetic, since the 2 values are usually fairly close.

6.5.2 *Repeatability*—within-laboratory precision, designated as $s_r$.

6.5.3 *Among-laboratories (not including within-laboratory, variability).* Designated as $s_L$ and used only for calculating $s_R$.

6.5.4 *Relationship among precision components.*

(a) The relationship among the 3 precision parameters is:

$$s_R^2 = s_L^2 + s_r^2$$

The parameters, $s_R^2$, $s_L^2$, and $s_r^2$, must be nonnegative, by definition. The *estimate* of $s_L^2$, however, can be negative. This frequently occurs in practice when $s_r^2$ is so large (poor repeatability) that it swamps out $s_L^2$. A negative estimate of the $s_L^2$ term arises from the fact that $s_L^2$ is calculated from the difference of 2 terms, each of which is calculated independently. If the second term is larger than the first, the difference is negative. When this occurs, $s_L^2$ is set equal to zero, which does result in a biased estimate of $s_R^2$. If this occurs with a number of the materials in the collaborative study, the method is probably unsatisfactory due to poor replication. Otherwise, such an occasional aberration can be tolerated.

(b) When only single determinations are performed on each material (except in the case of the split level design), there is no rigorous basis for calculating $s_r^2$, and within-laboratory variability cannot be estimated directly.

(c) The ISO definitions for repeatability value (r) and reproducibility value (R) (see 8.5) are simple multiples of the above measures of precision expressed as standard deviations. They are shown to be expressible in terms of the corresponding standard deviations below. The ISO definitions use a prediction interval statement: the value below which the absolute difference between 2, and only 2, single test results of identical test material may be expected to lie with a specified probability (usually 95%); in other words, assuming normal distribution, when duplicate measurements are performed, the absolute difference between the results of each of these duplicate measurements is expected to be below r or R in 95% of the cases. The relationship between the 2 definitions is:

$$\text{Repeatability value (r)} = 2 \cdot 2^{1/2} \cdot s_r = 2.8 \cdot \bar{X} \cdot RSD_r/100$$

$$\text{Reproducibility value (R)} = 2 \cdot 2^{1/2} \cdot s_R = 2.8 \cdot \bar{X} \cdot RSD_R/100$$

The coefficient 2.8 is derived from assumptions about the distribution of the sample populations, and $s_r$ and $s_R$ are repeatability and reproducibility standard deviations, respectively; $RSD_r$ and $RSD_R$ are the corresponding relative standard deviations, and $\bar{X}$ is the mean of the laboratory means.

6.5.5 *Confidence limits for precision terms*. Standard deviations and relative standard deviations from actual collaborative studies are merely estimates of "true values." The "confidence interval" (bounded by the confidence limits) is the range within which the true value is expected to lie with a stated degree of confidence (customarily 95%). The confidence intervals of precision terms are rarely given because about 200 values are required to estimate the standard deviation to within approximately 10%; for a small study of about 30 values, the standard deviation can be estimated to only about 25%.

### 6.6 *Incorrect, Improper, or Illusory Values (False Positive and False Negative Values)*

These results are not necessarily outliers (no a priori basis for a decision), since there is a basis for determining their incorrectness (a positive value on a blank material, or a zero (not found) or negative value on a spiked material). There is a statistical basis for the presence of false negative values: In a series of materials with decreasing analyte concentration, as the RSD increases, the percent false negatives increases from an expected 2% at an RSD = 50% to 17% at a RSD = 100%, merely from normal distribution statistics alone.

When false positives and/or false negatives exceed about 10% of all values, analyses become uninterpretable from lack of confidence in the presence or absence of the analyte, unless all positive laboratory samples are reanalyzed by a more reliable (confirmatory) method with a lower limit of determination than the method under study. When the proportion of zeros (not necessarily false negatives) becomes greater than approximately 30%, the distribution can become bimodal and even more uninterpretable (is the analyte present or absent?).

## 7. Final Report

7.1 The final report should contain a description of the materials used, their preparation, any unusual features in their distribution, and a table of all *valid* data, including outliers. When replication is performed, the individual values, not just averages, must be given, unless the method requires averages (e.g., microbiological methods). Values not used for specified reasons, such as decomposition, failure to follow method, or contamination, should not be included in the table since they may be included erroneously in subsequent recalculations. The report should include the statistical parameters calculated with and without specified outliers. Report the standard deviations, means, and the corresponding RSDs. Proofread tables very carefully since errors are of typographical origin. Give the names of the participants and their organizations, if agreement has been obtained for their acknowledgment.

7.2 The final report should be published in a generally accessible publication, or availability of the report from the organization sponsoring the method should be indicated in the published method. Without public documentation, the significance of the study is very limited.

7.3 The report should be sent to all participants, preferably at the mauscript stage, so that clerical and typographical errors may be corrected before publication. If changes in values from the original submission are offered, they must be accompanied by an explanation.

7.4 Example of Table of Statistical Parameters: See Table 1.

## 8. References


8.1 W.J. Youden & E.H. Steiner (1975) *Statistical Manual of the AOAC*. Association of Official Analytical Chemists, 2200 Wilson Blvd., Arlington, VA 22201 USA. The fifth printing (1987) contains several explanatory footnotes.

8.2 *Handbook for AOAC Members* (1989). Availability as in 8.1.

8.3 T. Dols & B. Armbrecht (1976) *J. Assoc. Off. Anal. Chem.* **59,** 1204-1207.

8.4 International Organization for Standardization Guide 18, Geneva Switzerland. Available from American National Standards Institute, 1430 Broadway, New York, NY 10018 USA and other national standards organizations.

8.5 Ibid, ISO 5725-1986.

**Table 1 [x] collaborative tests carried out at the international level in [year(s)] by [organization(s)] in which [y and z] laboratories participated, each performing [k] replicates, gave the following statistical results:**

**Results expressed in [units]**

| Material [Description and listed across the top in increasing order of magnitude of means] |
|---|
| Number of laboratories retained after eliminating outliers |
| Number of outlying laboratories removed |
| Mean |
| True or accepted value, if known |
| Repeatability standard deviation ($s_r$) |
| Repeatability relative standard deviation ($RSD_r$) |
| Repeatability *value*, r(2.8 × $s_r$) |
| Reproducibility standard deviation ($s_R$) |
| Reproducibility relative standard deviation ($RSD_R$) |
| Reproducibility *value*, R(2.8 × $s_R$) |

* * *

The repeatability and reproducibility *values* may also be expressed as a relative value (as a percentage of the determined mean value), when the results so suggest.

If the recovery and precision values are more or less constant for all materials or for groups of materials, an overall average value may be presented. Although such averaging may not have statistical validity, it does have practical value.

**Appendix A-1 Critical Values for the Cochran Test (Abbreviated from ISO 5725-1986, $P = 0.01$)**

L = number of laboratories at a given level (concentration)
r = number of replicates per laboratory

| L | r = 2 | r = 3 | r = 4 | r = 5 | r = 6 |
|---|---|---|---|---|---|
| 3 | 0.993 | 0.842 | 0.883 | 0.834 | 0.793 |
| 4 | 0.968 | 0.864 | 0.781 | 0.721 | 0.676 |
| 5 | 0.928 | 0.788 | 0.696 | 0.633 | 0.588 |
| 6 | 0.883 | 0.722 | 0.626 | 0.564 | 0.520 |
| 7 | 0.838 | 0.664 | 0.568 | 0.508 | 0.466 |
| 8 | 0.794 | 0.615 | 0.521 | 0.463 | 0.423 |
| 9 | 0.754 | 0.573 | 0.481 | 0.425 | 0.387 |
| 10 | 0.718 | 0.536 | 0.447 | 0.393 | 0.357 |
| 11 | 0.684 | 0.504 | 0.418 | 0.366 | 0.332 |
| 12 | 0.653 | 0.475 | 0.392 | 0.343 | 0.310 |
| 13 | 0.624 | 0.450 | 0.369 | 0.322 | 0.291 |
| 14 | 0.599 | 0.427 | 0.349 | 0.304 | 0.274 |
| 15 | 0.575 | 0.407 | 0.332 | 0.288 | 0.259 |
| 16 | 0.553 | 0.388 | 0.316 | 0.274 | 0.246 |
| 17 | 0.532 | 0.372 | 0.301 | 0.261 | 0.234 |
| 18 | 0.514 | 0.356 | 0.288 | 0.249 | 0.223 |
| 19 | 0.496 | 0.343 | 0.276 | 0.238 | 0.214 |
| 20 | 0.480 | 0.330 | 0.265 | 0.229 | 0.205 |
| 21 | 0.465 | 0.318 | 0.255 | 0.220 | 0.197 |
| 22 | 0.450 | 0.307 | 0.246 | 0.212 | 0.189 |
| 23 | 0.437 | 0.297 | 0.238 | 0.204 | 0.182 |
| 24 | 0.425 | 0.287 | 0.230 | 0.197 | 0.176 |
| 25 | 0.413 | 0.278 | 0.222 | 0.190 | 0.170 |
| 26 | 0.402 | 0.270 | 0.215 | 0.184 | 0.164 |
| 27 | 0.391 | 0.262 | 0.209 | 0.179 | 0.159 |
| 28 | 0.382 | 0.255 | 0.202 | 0.173 | 0.154 |
| 29 | 0.372 | 0.248 | 0.196 | 0.168 | 0.150 |
| 30 | 0.363 | 0.241 | 0.191 | 0.164 | 0.145 |
| 35 | 0.325 | 0.213 | 0.168 | 0.144 | 0.127 |
| 40 | 0.294 | 0.192 | 0.151 | 0.128 | 0.114 |

Cochran statistic = (largest individual within-laboratory variance)/(sum of all the within-laboratory variances).

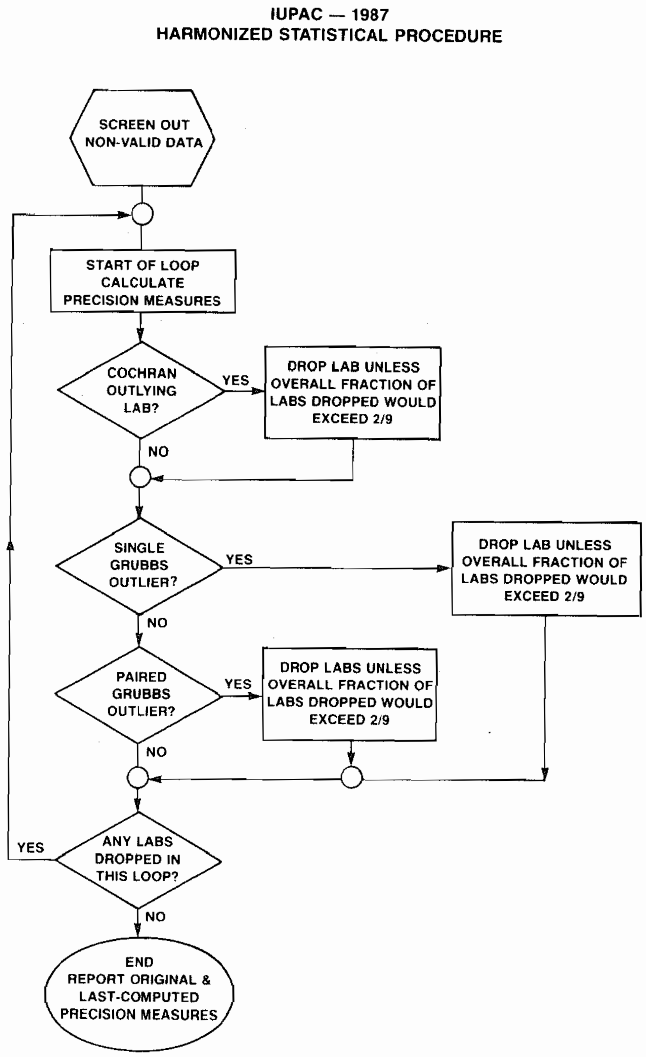
**Appendix A-2 Critical Values for the Grubbs Single Value and Pair Value Tests Expressed as the Percent Reduction in the Standard Deviation Caused by Removal of the Suspect Value(s) (See 6.3.2 for calculating the Grubbs statistics.)**

L = number of laboratories at a given level (concentration)

| L | Single value test | Pair value test |
|---|---|---|
| 4 | 91.3 | 99.7 |
| 5 | 80.7 | 95.4 |
| 6 | 71.3 | 88.3 |
| 7 | 63.6 | 81.4 |
| 8 | 57.4 | 75.0 |
| 9 | 52.3 | 69.4 |
| 10 | 48.1 | 64.6 |
| 11 | 44.5 | 60.5 |
| 12 | 41.5 | 56.8 |
| 13 | 38.9 | 53.6 |
| 14 | 36.6 | 50.8 |
| 15 | 34.6 | 48.3 |
| 16 | 32.8 | 46.0 |
| 17 | 31.2 | 44.0 |
| 18 | 29.8 | 42.1 |
| 19 | 28.5 | 40.4 |
| 20 | 27.3 | 38.9 |
| 21 | 26.2 | 37.4 |
| 22 | 25.2 | 36.1 |
| 23 | 24.3 | 34.9 |
| 24 | 23.4 | 33.7 |
| 25 | 22.7 | 32.7 |
| 26 | 21.9 | 31.7 |
| 27 | 21.2 | 30.8 |
| 28 | 20.6 | 29.9 |
| 29 | 20.0 | 29.1 |
| 30 | 19.5 | 28.3 |
| 35 | 17.1 | 25.0 |
| 40 | 15.3 | 22.5 |

Source: Patrick Kelly, Canada Packers, Toronto, Ontario, Canada. Single critical values calculated from available formulas; pair critical values from simulation and fitting and should be accurate to 0.1% absolute. (Submitted for publication to *Technometrics*.)

## Appendix A-3 Flowchart Complies with IUPAC-1987 Recommendations



# Subject Index

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