Federation of Malysia

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MS 1351 (1994) (English): SPECIFICATION FOR COCONUT CREAM POWDER

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MALAYSIAN STANDARD

MS 1351 : 1994

SPECIFICATION FOR COCONUT CREAM POWDER

SIRIM STANDARDS & INDUSTRIAL RESEARCH INSTITUTE OF MALAYSIA

This Malaysian Standard which had been approved by the Food and Agricultural Industry Standards Committee and endorsed by the Board of the Standards and Industrial Research Institute of Malaysia (SIRIM) was published under the authority of the SIRIM Board in August, 1994.

SIRIM wishes to draw attention to the fact that this Malaysian Standard does not purport to include all the necessary provisions of a contact.

The Malaysian Standards are subject to periodical review to keep abreast of progress in the industries concerned. Suggestions for imporvements will be recorded and in due course brought to the notice of the Committees charged with the revision of the standards to which they refer.

The following references relate to the work on this standard:
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CONTENTS

Committee representation

The Food and Agricultural Industry Standards Committee under whose supervision this Malaysian Standard was prepared, comprises representatives from the following Government Ministries, trade, commerce and manufacturer associations and scientific and professional bodies.

Department of Agriculture
Federal Agricultural Marketing Authority
Federation of Malaysian Consumers Associations
Federation of Malaysian Manufacturers
Malaysian Agricultural Research and Development Institute
Ministry of Agriculture
Malaysian Oil Palm Growers' Council
Rubber Research Institute of Malaysia
Universiti Pertanian Malaysia

The Working Group on Oils and Fats which prepared this Malaysian Standard consists of the following representatives:

Encik S. Krishnan (Chairman)	Incorporated Society of Plantewrs
Encik Lim Pang Hon/Encik Tan Kong Sing	Federation of Malaysian Manufacturers
Encik Tang Loon Boon	Federal Agricultural Marketing Authority
Puan Rozita Baharuddin/Cik Pushpalatha Pillai/ Cik Aminah Abd. Manap	Palm Oil Registration and Licensing Authority
Puan Noraini Sudin/Puan Siew Wai Lin	Palm Oil Research Institute of Malaysia
Encik Yong Moh Lim/Encik Pan Kum Wan	Federal Land Development Authority
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Encik Low Thing	Malayan Edible Oil Manufacturers' Association
Puan Saleha A. Jalil/Puan Radziah Mohd. Daud (Secretary)	Standards and Industrial Research Institute of Malaysia

Co-opted members

Encik Lee Swee Heng	Federal Flour Mills Bhd.
Dr. Tony Ng Kock Wai	Institute for Medical Research

A Panel on Coconut Cream Powder comprising the following members was set up by the Working Group to assist with the preparation of the Malaysian Standard:

Encik Chuah Eng Chong (Chairman)	Malaysian Agricultural Research and Development Institute
Encik Tan Sing Ming	Agriculture Department
Puan Teng Yit Chan	Malaysian Industrial Development Authority
Encik Husine Shahar	S & P Food Industries (M) Sdn. Bhd.
Encik Tee Seng Biung	Kapar Coconut Industries Sdn. Bhd.
Encik Abdul Razak Akil	Santara Industries Sdn. Bhd.
Representative	Ministry of Health
Puan Radziah Mohd. Daud (Secretary)	Standards and Industrial Research Institute of Malaysia

FOREWORD

This Malaysian Standard was prepared by the Working Group on Oils and Fats, under the authority of the Food and Agricultural Industry Standards Committee. A Panel on Coconut Cream Powder was set up by the Working Group to assist in the preparation of this Malaysian Standard.

It was felt necessary to prepare this Malaysian Standard so that various quality requirements can be followed by the manufacturers in order to produce a consistent quality product, thereby, enhancing the export trade of the product.

In the preparation of this standard, the following publications were referred to:-

1. Food Regulations 1985, Ministry of Health, Malaysia.

2. FAO Food and Nutrition Paper 14/4, Manuals of food quality control - Vol. 4: Microbiological analysis : F7. Rome 1979.

3. FDA Bacteriological Analytical Manual (1984). 6th. edition.

4. Association of Official Analytical Chemists (AOAC) (1990). Official Methods of Analysis. 15th Edition (K. Helrich, ed.); AOAC: Virginia, USA.

5. International Standard ISO 7002:1986(E) 'Agricultural food products - Layout for a standard method of sampling from a lot'.

6. BS 4285: Section 3.9:1987, Microbiological examination for dairy purposes : Detection of *Salmonella.*

7. Product specification from Red V Company, Philippines.

8. International Standard ISO 2446:1976(E) 'Milk - Determination of fat content (Routine method)'.

Due consideration has also been given to local conditions or practice and market conditions.

SPECIFICATION FOR COCONUT CREAM POWDER

1. Scope

1.1 This Malaysian Standard specification prescribes the requirements and methods of sampling and analysis for coconut cream powder.

2. Description

2.1 Coconut cream powder shall be the soluble white powder resulting from the removal of water from coconut milk. Coconut cream powder may contain permitted food conditioner and permitted antioxidant.

3. Requirements

3.1 Coconut cream powder shall be reasonably uniform in composition. The colour shall be white and free from brown or yellow colour typical of over-heated product and free from any other unnatural colour. It shall be substantially free from brown specks. The product in the dry form or on reconstitution shall be clean and free from rancid, cheesy, soapy or other objectionable flavours and odours.

3.2 Coconut cream powder shall also comply with the requirements prescribed in Table 1 and Table 2.

Table 1. Requirements for coconut cream powder

Item	Properties	Requirements	Method of test (Appendix)
1.	Fat, % by weight, min.	60	B
2.	Protein, % by weight, min.	10	*MS 1194
3.	Moisture, % by weight, max.	2.5	C
4.	Ash, % by weight, max.	2.5	D
5.	pH	6 - 7	E
6.	Free fatty acids (as lauric acid), % by weight, max.	0.1	F

* MS 1194, Methods for determination of crude protein in foods and feeds.

Table 2. Microbiological limits for coconut cream powder

Item	Properties	Limits	Method of test (Appendix)
1.	Total plate count/g, max.	10^4	G
2.	Coliform count (MPN)/g, max.	50	)
3.	E. coli (MPN)/g.	absent	) H
4.	Salmonella/25 g.	absent	I
5.	Moulds/g, max.	50	)
6.	Yeasts/g, max.	50	) J

3.3 Any food additive which may be added during manufacture shall comply with the requirements of the Food Regulations 1985.

4. Hygiene

4.1 The product shall be processed and handled under hygienic conditions in premises licensed in accordance with the public health legislations currently enforced in Malaysia.

5. Packaging and labelling

5.1 Coconut cream powder shall be packed under hygienic condition in suitable clean packages sealed in such a manner so as to prevent contamination.

5.2 The containers shall be marked legibly and indelibly with the following information:-

5.2.1 Name of the product;

5.2.2 Name and address of the manufacturer and/or packer, or the owner of the rights of manufacture or packing or the agent of any of them;

5.2.3 Minimum net weight;

5.2.4 List of ingredients in descending order of proportions used by weight in the product;

5.2.5 Code number identifying batch and/or date of manufacture;

5.2.6 Date marking;

5.2.7 Country of origin;

5.2.8 Preparation instructions.

5.3 Each container may, by arrangement with the Standards and Industrial Research Institute of Malaysia be marked with the certification mark of SIRIM, provided the product conforms to the requirements of this Malaysian Standard.

6. Legal requirement

6.1 The product in all other aspects shall comply with the requirements of the legislations currently in force in Malaysia.

7. Sampling and tests

7.1 Sampling and tests shall be carried out as prescribed in the relevant appendices.

7.2 Unless otherwise specified, chemicals used shall be of a recognized analytical reagent grade, that is, they shall not contain impurities which affect the results of the test. Distilled water or water of equivalent purity shall be used. Solutions shall be freshly prepared and filtered where necessary.

Appendix A

Sampling

A1. Definition of terms

A1.1 **Lot**; batch : An identified quantity of some commodity, manufactured or produced under conditions that are presumed uniform.

NOTE 1. Uniform conditions have several features, for example products supplied by one producer always using the same production process, where production is stable and the quality characteristic is distributed according to the normal distribution or a close approximation to the normal distribution. Specialized subdivisions etc can occur.

NOTE 2. Consequently the term lot (or batch) means inspection lot/batch for sampling ie a quantity of material or a collection of items : a population from which a sample is to be drawn and inspected may differ from a collection of units designated as a lot, for example for production shipment.

A2. General requirements of sampling

In drawing, preparing, storing and handling samples, the following precautions and directions shall be observed and the general rules for making a representative sample shall be considered.

A2.1 The sampling device shall be clean and dry when use.

A2.2 Precautions shall be taken to protect the samples, the product being sampled, the sampling instrument and the bags for samples from any other forms of contamination.

A2.3 The samples shall be placed in clean and dry laminated aluminium bags. The bags shall be of such size that can hold the samples.

A2.4 Each bag shall be sealed airtight after filling, and marked with full details of sampling, date of sampling, name of sampler, batch or code number, name of manufacturer and other particulars of the consignment.

A2.5 All the samples shall be analysed as soon as possible. If necessary, they shall be stored in such a manner that there is no deterioration of the product.

A2.6 Sampling shall be done by an authorised person as agreed upon between the purchaser and the vendor.

A3. Sampling from bulk consignment

A3.1 Samples shall be tested from each lot for ascertaining conformity of the product to the requirements of the standard.

A3.2 The number of bags to be selected from the lot shall depend on the size of the lot and shall be at least one-half the square root of N, where N is the number of bags in the lot, provided that a minimum of 3 bags shall be selected. In case of doubt, the number of bags to be selected shall be increased to 10 per cent of the bags in the lot.

A3.3 The bags shall be chosen at random from the lot and for this purpose a random number table, shall be used. If such table is not available, the following procedure shall be adopted:-

Arrange all the bags in the lot in a systematic manner and starting from any bag count serially and withdraw the rth bag where

$$r = \frac{N}{n}$$

N being the number of bags in the lot and n the number of bags to be chosen according to A2.3. If r comes out to be a fractional number, its value shall be taken as equal to its integral part.

A3.4 When the lot size is less than 10, one composite sample prepared by taking equal quantities of the product from different parts of each bag in the lot shall be tested.

A3.5 Preparation of sample

Equal quantities of the product representative of each bag, selected according to A3.2, shall be drawn, using an appropriate sampling device and mixed up together so as to form a composite sample weighing not less than 0.75 kg.

A4. Sampling products packed in small retail containers

A4.1 Samples shall be tested from each lot for ascertaining conformity of the product to the requirements of the standard.

A4.2 The number of cartons shall be selected at random from a lot according to Table 3.

Table 3. Scale of sampling (for products packed in small retail containers)

No. of cartons in the lot	No. of cartons to be selected
2 to 8	2
9 to 27	3
28 to 64	4
65 to 125	5
126 to 216	6
217 to 343	7
344 to 512	8
513 to 729	9
730 to 1000	10
1001 and over	10 + 1 for each additional 200 units or fraction thereof over 1000

A4.3 From each of the cartons selected, draw at random one package for testing the chemical and microbiological requirements.

Appendix B

Determination of fat

B1. Roese - Gottlieb method

B1.1 Apparatus

Usual laboratory equipment and the following items.

B1.1.1 *Fat extraction flask or tube*

B1.1.2 *Centrifuge*

B1.1.3 *Flask or metal dish*

B1.1.4 *Steam bath*

B1.1.5 *Oven*, maintained at 102 $\pm$ 2°C [or vacuum oven, maintained at 70 - 75°C under pressure < 50 mm Hg (6.7 kPa)].

B1.1.6 *Desiccator*

B1.2 Reagents

B1.2.1 *Ammonium hydroxide* (20 - 28% NH_3)

B1.2.2 *Alcohol* (95% v/v ethanol)

B1.2.3 *Ether* (ethyl ether, peroxide free)

B1.2.4 *Petroleum ether* (boiling range 30 - 60°C)

B1.3 Procedure

B1.3.1 Weigh 1 g of the well mixed sample into the fat extraction flask or tube (B1.1.1) and add 9 ml of warm distilled water, stopper, shake to dissolve and cool to a room temperature. Add 1.25 ml ammonium hydroxide (B1.2.1) and mix thoroughly. Add 25 ml ether (B1.2.3), stopper with cork or stopper (synthetic rubber) unaffected by usual fat solvents and shake very vigorously for 1 min.

B1.3.2 Cool, if necessary; add 25 ml petroleum ether (B1.2.4) and repeat vigorous shaking. Centrifuge the flask at about 600 rpm or let it stand until upper layer is practically clear. Decant ether solution into suitable flask or metal dish (B1.1.3). Wash the lip and stopper of extraction flask or tube with a mixture of equal parts of the two ethers and add the washings to weighing flask or dish.

B1.3.3 Repeat extraction of the liquid remaining in the flask or tube twice, using 15 ml of each solvent each time and adding water if necessary, but omitting rinsing with mixed solvents after final extraction.

B1.3.4 Evaporate the solvents completely on steam bath (B1.1.4) at the temperature that does not cause spattering or bumping (boiling chips may be added).

B1.3.5 Dry the fat to constant weight in the oven (B1.1.5) at 102 ± 2°C. Cool the flask and dish in a desiccator (B1.1.6) and weigh.

B1.3.6 Remove the fat completely from the container with 15 - 25 ml warm petroleum ether, dry, and weigh as before. Correct the weight of fat by blank determination on reagents used. If the blank is 0.5 mg, purify or replace reagents. Difference between duplicate determinations obtained simultaneously by the same analyst should be ≤ 0.03 g fat/100 g product.

B1.4 Calculation

$$\% \text{ fat} = \frac{(W_1 - W_2 - B)}{W} \times 100$$

where,

B is the blank determination;

W is the weight (g) of sample;

W_1 is the weight (g) of flask or dish and fat;

W_2 is the weight (g) of flask or dish.

or alternatively.

B2. Gerber method

B2.1 Principle

Separation of the fat of the milk in a butyrometer by centrifuging after dissolving the protein with sulphuric acid, the separation being aided by the addition of a small quantity of amyl alcohol. The butyrometer is graduated to give a direct reading of fat content.

B2.2 Reagents

B2.2.1 *Sulphuric acid*

Requirements

The sulphuric acid shall have a density at 20°C of 1.816 ± 0.004 g/ml, which corresponds to approximately 90.4 ± 0.8% (m/m) H_2SO_4. The acid shall be colourless, or not darker in colour than pale amber, shall be free from suspended matter and shall be found suitable for use when tested as specified in suitability test.

Suitability test

A sulphuric acid may satisfy the specific requirements for density and appearance and yet be unsuitable for the Gerber method. Therefore, check the suitability of the acid before use by means of the following comparative test with a standard sulphuric acid.

B2.2.1.1 *Standard sulphuric acid*

Add sulphuric acid of analytical reagent quality (for example 98% (m/m) H_2SO_4, ρ_{20} 1.84 g/ml) to distilled water, or water of at least equal purity, to obtain a solution with a density within the range specified the requirements.

NOTE. Approximately 1 l of standard sulphuric acid is obtained by adding 908 ml of 98% (m/m) sulphuric acid to 160 ml of water, checking the density of the diluted acid with a suitable hydrometer and adjusting the density, if necessary by adding a small volume of water or 98% (m/m) acid.

B2.2.1.2 *Comparison procedure*

Determine in duplicate the fat content of four samples of coconut milk with average fat content by the Gerber method described, using butyrometers whose scale errors are less than 0.01% and standard amyl alcohol. In one of each pair of duplicates use 10 ml of the sulphuric acid under test and in the other use 10 ml of the standard sulphuric acid. Keep the butyrometers in a random order from the shaking stage onwards. Take the readings to the nearest 0.01% fat (read by at least two persons). The mean fat content of the four milk samples obtained with the sulphuric acid under test shall not differ by more than 0.015% fat from the mean value obtained using the standard sulphuric acid.

B2.2.2 *Amyl alcohol*

Composition

At least 98% (V/V) of the amyl alcohol shall consist of the primary alcohols 3-methylbutan-1-ol and 2-methylbutan-1-ol, the only permissible major impurities being 2-methyl-propan-1-ol and butan-1-ol. It shall be free from secondary pentanols, 2-methylbutan-2-ol, 2-furaldehyde, gasoline (petrol) and derivatives of benzene. Not more than a trace of water shall be present.

Appearance

The amyl alcohol shall be clear and colourless.

Density

The amyl alcohol shall have a density at 20°C of 0.808 or 0.818 g/ml.

2-furaldehyde and other organic impurities

When 5 ml of the amyl alcohol is added to 5 ml of the sulphuric acid (B2.2.1), no more than a yellow or light-brown colour shall develop,

Distillation range

When the amyl alcohol is distilled at a pressure of 1013 mbar*, not less than 98% (V/V) shall distil below 132°C and not more than 5% (V/V) below 128°C. There shall be no solid residue after distillation.

NOTE. If the atmospheric pressure during the distillation is lower or higher than 1013 mbar, the specified temperatures should be respectively decreased or increased by 0.03°C C/mbar.

Suitability test

An amyl alcohol may satisfy the requirements stated previously yet be unsuitable for the Gerber method. Therefore, check the suitability of the amyl alcohol before use by means of the following comparitive test with a standard amyl alcohol.

B2.2.2.1 *Standard amyl alcohol*

Distil an amyl alcohol satisfying the requirements stated previously, using a suitable fractionation column, and collect a fraction within a boiling range of 2°C between 128°C and 131.5°C (see above note). Apply the following tests to the fraction:

(a) When analysed by gas-liquid chromatography, at least 99% (V/V) shall consist of 3-methylbutan-1-ol and 2-methylbutan-1-ol and butan-1-ol shall be present.

(b) When fractionally distilled, the first 10% and the last 10% collected, when compared using the procedure described in B2.2.3.2, shall give values for the fat content of milk that do not differ by more than 0.015% fat.

If the fraction satisfies both these tests it can be regarded as standard amyl alcohol. The standard amyl alcohol can be used for several years, provided that it is kept in the dark in a cool place.

* 1 mbar = 0.1 kPa.

B2.2.2.2 *Comparison procedure*

Determine in duplicate the fat content of four samples of whole milk average fat content by the Gerber method described, using butyrometers whose scale errors are less than 0.01% and standard sulphuric acid B2.2.1.1. In one of each pair of duplicates use 1 ml of the amyl alcohol under test, and in the other use 1 ml of the standard amyl alcohol (B2.2.2.1).

Keep the butyrometers in a random order from the shaking stage onwards. Take the readings to the nearest 0.01% fat (read by at least two persons).

The mean fat content of the four milk samples obtained with the amyl alcohol under test shall not differ by more than 0.015% fat from the mean value obtained using the standard amyl alcohol.

NOTE. Instead of the specified amyl alcohol an artificial amyl alcohol or an amyl alcohol substitute, coloured if desired, may be used, provided that its use has been demonstrted by experiment not to lead to any significant differences in the results of the determination.

B2.3 Apparatus

B2.3.1 *Pipette*

B2.3.1.1 The pipette shall be of the single graduation line, bulb type, and its capacity shall be defined as the volume, in millilitres, of water at 27°C delivered by the pipette when emptied as described in the annex.

The capacity of the pipette, determined by the method described in the annex, shall not differ from the nominal capacity, established according to B2.3.1.3, by more than 0.03 ml.

B2.3.1.2 In each country, the appropriate capacity of the pipette shall be established by carrying out comprative determinations using the Gerber method described and the reference method (ISO/R 1211). For these comparative determinations, butyrometers whose scale errors are less than 0.01% shall be used, and the butyrometers read to the nearest 0.01% fat.

B2.3.1.3 If the value for fat is to be expressed in grams of fat per 100 ml of milk, the basis of comparison with the reference method shall be stated.

B2.3.2 *Butyrometer and stopper*, as described in ISO/R 488.

NOTE 1. Use a butyrometer whose scale range is appropriate for the expected fat content of the sample.

NOTE 2. With corrugated-neck butyrometers, either lock stoppers or solid single-ended or double-ended rubber stoppers may be used.

With plain-neck butyrometers, lock stoppers should preferably be used.

B2.3.3 *Automatic measure*, or *safety pipette*, to deliver 10.0 ± 0.2 ml of sulphuric acid (B2.2.1).

B2.3.4 *Automatic measure*, or *safety pipette*, to deliver 1.0 ± 0.05 ml of amyl alcohol (B2.2.2).

B2.3.5 *Protected stand*, for shaking the butyrometers (B2.3.2).

B2.3.6 *Centrifuge*, in which the butyrometers can be spun, provided with a speed indicator which indicates the number of revolutions per minute with a maximum tolerance of ± 50 rev/min, and preferably of the vertical-loading type rather than the horizontal-loading type.

The design of the centrifuge shall be such that the temperature of the butyrometer contents after the centrifuging (see B2.5.6) is between 30 and 50°C.

The centrifuge shall be capable of producing within 2 min, when fully loaded, a relative centrifugal acceleration of 350 ± 50 g at the outer end of the butyrometer stopper. This acceleration is produced by centrifuges with the following effective radius (horizontal distance between the centre of the centrifuge spindle and the outer end of the butyrometer stopper) operated at the speed indicated.

Effective radius mm	Revolutions per minute ± 70 rev/min
240	1140
245	1130
250	1120
255	1110
260	1100
265	1090
270	1080
275	1070
300	1020
325	980

NOTE. The relative centrifugal acceleration produced in a centrifuge is given by the following formula

$$1.12 \times 10^{-6} R N^2$$

where,

R is the effective horizontal radius, in millimetres;

N is the speed, in revolutions per minute.

B2.3.7 *Water bath for butyrometers*, capable of being maintained at 65 ± 2°C and such that the butyrometers (B2.3.2) can be supported in a vertical position with their scales completely immersed.

B2.3.8 *Thermometer*, suitable for insertion in the water bath (B2.3.7).

B2.3.9 *Water bath*, if necessary, for the preparation of the sample (see B2.4.1).

B2.4 Preparation of test sample

B2.4.1 Weigh approximately about 10 g sample and add 90 ml of distilled water. Adjust the temperature of the sample to 20 to 30°C using a water bath if necessary. Mix the milk thoroughly if necessary, a suitable mixing device may be used to assist the dispersal of the fat. When a uniform distribution of the fat has been achieved, quickly adjust the temperature of the milk to approximately 27°C (in tropical countries), where the milk pipette is calibrated at this temperature). Allow the milk to stand after the final temperature adjustment, to let air bubbles rise. Normally 3 to 4 min is sufficient but, if a mixing device has been used, up to 2 h may be required, followed by a further temperature adjustment.

NOTE 1. If, after the preparation of the test sample, white particles are visible on the walls of the sample bottle, or liquid fat is visible on the surface of the sample, a reliable value for fat content cannot be expected.

B2.4.2 Immediately after the preparation of the test sample, the procedure described in clause B2.5, as appropriate, should be started and completed without interruption.

B2.5 Procedure

WARNING - Take suitable precautions, such as the wearing of a face-visor, against the accidental release of sulphuric acid.

B2.5.1 Measure 10 ± 0.2 ml of sulphuric acid (B2.2.1) into the butyrometer (B2.3.3), in such a way that the acid does not wet the neck of the butyrometer or entrap any air.

B2.5.2 Gently invert the bottle containing the prepared test sample (B2.4) three or four times and immediately measure the required volume of milk into the butyrometer in the following manner.

Suck the milk into the pipette (B2.3.1) until the milk level is slightly above the graduation line and wipe the out-side of the delivery jet free from milk. With the pipette held vertically, the graduation line at eye level and the tip of the jet touching the inside of the neck of the inclined sample bottle, allow the milk to flow from the pipette until the top of the milk meniscus (not the bottom of the meniscus, which is difficult to see).

Remove the jet from contact with the sample bottle and then, with the butyrometer in a vertical position and the pipette held at an angle of about 45° with the tip of the jet just below the bottom of the neck of the butyrometer, allow the milk to flow gently down the inside of the butyrometer to form a layer on top of the acid, preventing as far as possible any mixing with the acid. When the out-flow has ceased, wait 3 s, touch the tip of the pipette against the bottom of the neck and then remove the pipette. Care should be taken not to wet the neck of the butyrometer with milk.

B2.5.3 Measure 1 ± 0.05 ml of the amyl alcohol (B2.2.2) into the butyrometer, using the automatic measure or safety pipette (B2.3.4). Do not wet the neck of the butyrometer with the amyl alcohol and avoid at this stage mixing the liquids in the butyrometer.

B2.5.4 Securely stopper the butyrometer without disturbing its contents. When a double-ended stopper is used, screw it in until the widest part is at least level with the top of the neck. When a lock stopper is used, insert it until the rim is in contact with the neck of the butyrometer.

B2.5.5 Shake and invert the butyrometer, suitably protected in case of breakage or loosening of the stopper, until its contents are thoroughly mixed, and until the protein is completely dissolved, i.e. until no white particles remain.

B2.5.6 Immediately place the butyrometer in the centrifuge, bring the centrifuge to the operating speed required to give a relative centrifugal acceleration of 350 ± 50 g within 2 min, and then maintain this speed for 4 min.

B2.5.7 Remove the butyrometer from the centrifuge and, if necessary, adjust the stopper to bring the fat column on to the scale. Place the butyrometer, stopper downwards, in the water bath (B2.3.7) at 65 ± 2°C for not less than 3 min and not more than 10 min; the water level shall be above the top of the fat column.

B2.5.8 Remove the butyrometer from the water bath and carefully adjust the stopper to bring the bottom of the fat column, with the minimum movement of the column, to the top edge of a graduation line, preferably a main graduation line. When a solid rubber stopper is used, the adjustment should preferably be done by slightly withdrawing the stopper and not by forcing it farther into the neck. When a lock stopper is used, insert the key and apply sufficient pressure to raise the fat column to the required position.

Note the scale reading coincident with the bottom of the fat column and then, taking care that the fat column does not move, as quickly as possible note the scale reading coincident with the lowest point of the fat meniscus at the top of the nearest half of a smallest scale division. While readings are being taken, hold the butyrometer vertically with the point of reading at eye level. Record the difference between the two readings.

NOTE. If the fat is turbid or dark in colour, or if there is white or black material at the bottom of the fat column, the value for fat content will not be reliable.

B2.5.9 Repeat the procedure given in B2.5.6, B2.5.7 and B2.5.8, and obtain a second value for fat content. If the second value does not exceed the first value by more than half a smallest scale division, the second value shall be recorded as the fat content of the milk.

B2.5.10 If the second value exceeds the first value by more than half a smallest scale division, repeat the procedure described in B2.5.6, B2.5.7 and B2.5.8 and obtain a third value for the fat content. If the third value does not exceed the second value by more than half a smallest scale division, the third value shall be recorded as the fat contents of the milk.

B2.5.11 If the third value exceeds the second value by more than half a smallest scale division, repeat the procedure described in B2.5.6, B2.5.7 and B2.5.8 and obtain a third value for the fat content. The fourth value shall be recorded as the fat content of the milk, but if this value exceeds the third value by more than half a smallest scale division, it shoudl be regarded as of doubtful accuracy.

B2.5.12 Calculate the fat content as described in B2.6.1. The requirements of B2.6.2, B2.6.3 and B2.6.4 apply.

NOTE. If, after the several centrifugings, the fat is turbid or dark in colour, or if there is white or black material at the bottom of the fat column, the value for fat content will not be accurate.

B2.5.13 If a check of the value obtained is desired, replace the butyrometer in the water bath (B2.3.7) at 65 $\pm$ 2°C for not less than 3 min and not more than 10 min, and then remove it from the bath and again take readings as described in B2.5.8.

B2.6 Expression of results

B2.6.1 *Method of calculation*

The fat content of the milk is

$$B - A$$

where,

A is the reading at the bottom of the fat column;

B is the reading at the top of the fat column.

The fat content shall be expressed in grams of fat per 100 g of milk or in grams of fat per 100 ml of milk, according to whether the capacity of the milk pipette was chosen with the former or latter method of expressing the results in view.

B2.6.2 *Repeatability*

If two determinations are carried out simultaneously, or in rapid succession, by the same analyst, the difference between the results should not exceed the value corresponding to one smallest scale division of the butyrometer. When butyrometers with scale errors less than 0.01% are used (for example see B2.3.1.2) the difference between the results of two determinations obtained as described should not exceed the value corresponding to half a smallest scale division.

B2.6.3 *Correction of results*

If the value obtained is outside the range in which the particular milk pipette used gives results in agreements with the reference method, the appropriate correction (B2.3.1.2) may be applied if desired.

B2.6.4 *Special precision*

For the comparative determinations and for other special purposes which require a Gerber value for fat that is as precise as possible, use a butyrometer whose scale errors are less than 0.01%, and read the butyrometer to the nearest 0.01% fat. If necessary, apply a correction as described in B2.6.3.

B2.7 Test report

The test report shall show the method used and the result obtained, including :

(a) its method of expression;

(b) the capacity of the milk pipette;

(c) the scale of the butyrometer;

(d) indication of whether the result is uncorrected or corrected as described in B2.6.3 and whether the procedure of B2.6.4 has been followed;

(e) any observation that indicates that the result is of doubtful accuracy.

The report shall also mention any operating conditions not specified in this standard, or conditions regarded as optional, and any circumstances that may have influenced the result.

The report shall include all details required for complete identification of the sample.

Annex to B2.0 Gerber method

Procedure for checking the capacity of the pipette

1. Carry out the following operations at room temperature and with the water and the pipette at room temperature.

2. Suck distilled water into the throughly cleaned milk pipette until the water level is a few millimetres above the graduation line, then wipe the outside of the delivery jet free from water. With the pipette held vertically and the graduation line at eye level, allow water to flow from the pipette until the lowest point of the meniscus is coincident with the graduation line. Remove any water adhering to the tip of the jet by momentarily bringing the tip of the jet into contact with the inside of an inclined glass beaker.

3. With the pipette held vertically and the tip of the jet touching the inside of an inclined weighing bottle (previously weighed), allow the water to flow freely from the pipette until visible outflow ceases. Then, after 3 s, remove the weighing bottle from contact with the jet, stopper the bottle, weigh it an calculated the mass of water delivered by the pipette. Record the temperature of the water to the nearest 0.1°C. Using appropriate tables for use in the calibration of volumetric glassware, calculate the capacity of the pipette as the volume, in millilitres, of water at 20.0°C (27.0°C in tropical countries) delivered by the pipette.

Appendix C

Determination of moisture

C1. Apparatus

Usual laboratory equipment and the following items.

C1.1 *Round, flat-bottom metal dish* ($\geq$ 5 cm diameter and provided with tight-fitting slip-in cover).

C1.2 *Vacuum oven*, maintained at 90°C.

C1.3 *Desiccator*

C2. Procedure

C2.1 Weigh 1 - 1.5 g sample into the metal dish (C1.1). Loosen cover and place the dish in vacuum oven (C1.2). Dry to constant weight (about 5 h) under pressure $\leq$ 100 mm of Hg. During drying, admit slow current of air into oven (about 2 bubbles/second), dried by passing through sulphuric acid. Stop vacuum pump and carefully admit dried air into the oven.

C2.2 Press cover tightly into dish, remove from the oven. Cool the dish in a desiccator and weigh.

C3. Calculation

$$\% \text{ moisture} = \frac{(W_1 - W_2)}{W_3} \times 100$$

where,

W_1 is the weight (g) of dish and sample before drying;

W_2 is the weight (g) of dish and sample after drying;

W_3 is the weight (g) of sample.

Appendix D

Determination of ash

D1. Apparatus

Usual laboratory equipment and the following items.

D1.1 *Suitable silica dish*

D1.2 *Muffle furnace*, maintained at 550 ± 2°C.

D1.3 *Desiccator*

D1.4 *Hot plate*

D2. Procedure

D2.1 Place a silica dish (D1.1) into the muffle furnace (D1.2) for at least 15 min. Remove the dish from the furnace, cool in a desiccator (D1.3) and weigh to the nearest mg. Record the dish weight (W1).

D2.2 Place 1 g sample into the dish and spread it evenly over the bottom, reweigh the dish. Record the dish and sample weight (W2).

D2.3 Place the dish on a hot plate (D1.4) in a fume cupboard and slowly increase the temperature until fuming ceases and the sample becomes thoroughly charred.

D2.4 Place the dish in the muffle furnace at 550 ± 2°C for 16 h (overnight).

D2.5 Cool the dish in a desiccator (D1.3) and weigh to the nearest mg. Record the dish and ash weight (W3).

D3. Calculation

$$\% \text{ ash, w/w} = \frac{(W_3 - W_1)}{(W_2 - W_1)} \times 100$$

where,

W_1 is the weight (g) of dish;

W_2 is the weight (g) of dish and sample;

W_3 is the weight (g) of dish and ash.

Appendix E

Determination of pH

E1. Apparatus

Usual laboratory equipment and the following items.

E1.1 Erlenmeyer flask

E1.2 Potentiometer

E2. Procedure

E2.1 Weigh 10.0 g sample into clean, dry Erlenmeyer flask (E1.1) and add 100 ml recently boiled water at 25°C. Dissolve the sample. Determine the pH using electrode and potentiometer (E1.2) standardized by buffer solutions of pH 4.0 and pH 9.0 both at 25°C.

Appendix F

Determination of free fatty acids

F1. Apparatus

Usual laboratory equipment and the following items.

F1.1 *Rotary flask*, 300 ml.

F1.2 *Rotary evaporator.*

F1.3 *Conical flask*, 150 ml.

F2. Reagents

F2.1 *Neutralized mixed solvent* (Equal volume of 95% ethanol and diethyl ether).

F2.2 *Phenolphthalein indicator*, 1% in 95% alcohol.

F2.3 *Sodium hydroxide solution*, 0.1N, accurately standardized.

F2.4 *Petroleum ether*, b.p. 40 - 60°C.

F3. Procedure

F3.1 Extraction of oil from coconut cream powder

Soak 15 g (or more, depends on types of analysis or amount of oil required) of sample in 200 ml petroleum ether (F2.4) and leave overnight. Filter into a rotary flask (F1.1). Evaporate petroleum ether using a rotary evaporator (F1.2) at 60°C under vacuum. The oil is ready for analysis.

F3.2 Determination of free fatty acids

Weigh 3 g oil (F3.1) into a conical flask (F1.3). Add 40 ml neutralized mixed solvent (F2.1). Swirl until the sample dissolves. Add 1 ml phenolphthalein indicator solution (F2.2). Titrate with alkali (F2.3) shaking vigorously to the end point pink colour which persists for 15 s.

F4. Calculation

F4.1 The percentage of free fatty acids in the sample is expressed as follows:

$$\text{Free fatty acids (as lauric acid), \%} = \frac{N \times T \times 100}{W}$$

where,

N is the normality of sodium hydroxide solution;

T is the volume (ml) of 0.1 N sodium hydroxide solution;

W is the weight (g) of sample.

Appendix G

Determination of total plate count

G1. Apparatus

Usual laboratory equipment and the following items.

G1.1 *Autoclave*, at 121 ± 1°C.

G1.2 *Flasks or test tubes.*

G1.3 *Petri dishes*, glass or plastic, sterile.

G1.4 *Blender or stomacher*

G1.5 *Incubator*, at 37 ± 1°C

G1.6 *Colony counter.*

G1.7 *Pipettes*, 1, 5 ml and 10 ml, graduated, sterile.

G1.8 *Water bath*, at 45 ± 1°C.

G2. Media

Basic materials

For uniformity of results, it is recommended that either dehydrated culture medium components of uniform quality and analytical grade chemicals, or dehydrated complete media be used. The water used shall be distilled water or water of at least equivalent purity. If the dehydrated complete medium is used, prepare according to the manufacturer's instructions.

G2.1 Buffered peptone water (BPW)

Potassium dihydrogenorthophosphate	1.5 g
Disodium hydrogenorthophosphate dodecahydrate	9.0 g
Sodium chloride	5.0 g
Peptone	10.0 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water and adjust the pH to 7.2 ± 0.2. Dispense in portions of 225 ml into flasks of 500 ml capacity and of 9 ml into test tubes (G1.2). Sterilize the medium in an autoclave (G1.1) for 15 min at 121 ± 1°C.

G2.2 Plate count agar (PCA)

Dehydrated yeast extract	2.5 g
Pancreatic digest of casein	5.0 g
Glucose	1.0 g
Agar	15.0 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water and adjust pH to 7.0 ± 0.1. Dispense in appropriate flasks (G1.2). Sterilize the medium in an autoclave (G1.1) for 20 min at 121 ± 1°C. If to use immediately, cool the medium to 45 ± 1°C. Before use, melt the medium completely in boiling water and keep the flasks in water bath (G1.8) at 45 ± 1°C.

G3. Procedure

G3.1 Preparation of food homogenate

Weigh 25 g of the sample aseptically into a sterile blender jar or into a stomacher bag (G1.4) and add 225 ml of BPW (G2.1). Blend the food.

G3.2 Dilution

G3.2.1 Mix the food homogenate by shaking and pipette 1.0 ml into a tube containing 9 ml of the BPW (G2.1), mix carefully by aspirating 10 times with a pipette.

G3.2.2 From the first dilution, transfer with the same pipette 1.0 ml to 2nd dilution tube containing 9 ml of the BPW (G2.1), mix with a fresh pipette.

G3.2.3 Repeat using a 3rd, 4th tube or more until the required number of dilutions are made.

G3.2.4 Shake all dilutions carefully.

G3.3 Pour plating

G3.3.1 Pipette 1.0 ml of the food homogenate and of each dilution of the homogenate into each of the appropriately marked duplicate dishes (G1.3).

G3.3.2 Pour into each Petri dish 15 ml of the PCA (G2.2) (kept at 45 ± 1°C in a water bath) within 15 min of the time of original dilution.

G3.3.3 Mix the sample dilution and agar medium thoroughly and uniformly, and allow to solidify.

G3.4 Incubation

Incubate the prepared dishes, inverted, at 37 ± 1°C for 72 ± 3 h.

G3.5 Counting the colonies

Following incubation, count all colonies on dishes containing 30 -300 colonies and record results per dilution counted.

G3.6 Calculation

G3.6.1 When the dishes examined contain no colonies, the result is expressed as : less than 1×10^1 bacteria per g or ml.

G3.6.2 When the dishes (dilution 1 in 10) contain less than 30 colonies, the result is expressed as : less than 3×10^2 ($30 \times 10 = 3 \times 10^2$).

G3.6.3 When the colonies are more than 30, count the colonies in both plates of a dilution and calculate the average, retaining only two significant digits and multiply by the inverse of the corresponding dilution to obtain the number of bacteria per g.

Example:

1/100 dish 1	:	175 colonies
dish 2	:	208 colonies
calculation	:	175 + 208 = 383 ÷ 2 = 191 -> 190 = 190 x 100
Results	:	1.9×10^4 bacteria per g.

Appendix H

Determination of coliform and *E. coli* (Most probable number, MPN)

H1. Apparatus

Usual laboratory equipment and the following items.

H1.1 *Test tube or universal bottle.*

H1.2 *Durham tubes.*

H1.3 *Pipette*, 1 ml (total-flow), sterile.

H1.4 *Incubator*, at 37 ± 1°C.

H1.5 *Wire loop.*

H1.6 *Autoclave*, at 121 ± 1°C.

H1.7 *Water bath*, at 44°C.

H2. Media and reagents

Basic materials

For uniformity of results, it is recommended that either dehydrated culture medium components of uniform quality and analytical grade chemicals, or dehydrated complete media be used. The water used shall be distilled water or water of at least equivalent purity. If the dehydrated complete medium is used, prepare according to the manufacturer's instructions.

H2.1 *Ringer's solution (quarter strength)*

Sodium chloride	2.25 g
Potassium chloride	0.105 g
Calcium chloride ($6H_2O$)	0.12 g
Sodium bicarbonate	0.05 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water by stirring. Adjust the pH to 7.0 ± 0.1. Sterilize the medium in an autoclave (H1.6) for 15 min at 121°C ± 1°C.

H2.2 *MacConkey broth*

Peptone	20.0 g
Lactose	10.0 g
Bile salts	5.0 g
Sodium chloride	5.0 g
Bromocresol purple	0.01 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water by stirring. Adjust the pH to 7.4 ± 0.2. Transfer the medium in 10 ml portions to test tubes or universal bottles (H1.1) containing inverted Durham tubes (H1.2). Sterilize the medium in an autoclave (H1.6) for 15 min at 121 ± 1°C).

The Durham tubes shall not contain gas bubbles after sterilization.

H2.3 *Tryptone water*

Tryptone	10.0 g
Distilled water	1,000 ml

Dissolve the ingredient in distilled water by stirring. Adjust the pH to 6.9 ± 0.2. Sterilize the broth in an autoclave (H1.6) for 15 min at 121 ± 1°C.

H2.4 *Kovacs' reagent (Indole reagent)*

P-Dimethylaminobenzaldehyde	5.0 g
Hydrochloric acid, concentrated	25.0 ml
Amyl alcohol	75.0 ml

Dissolve aldehyde in alcohol by gently warming in a water bath (about 50° to 55°C). Cool and add the acid. Protect from light and store at about 4°C. The reagent shall be light yellow to light brown.

H3. Procedure

H3.1 Preparation of food homogenate

Homogenize 20 g of sample with 80 ml of quarter strength Ringer's solution (H2.1). This gives 1 in 5 dilution. Add 1 ml of the 1 in 5 homogenate to 9 ml of the quarter strength Ringer's solution to give a 1 in 50 dilution using a clean sterile pipette (H1.3) for the dilution.

H3.2 Inoculation

H3.2.1 Determine out the presumptive coliform count by the Multiple Tube Dilution Method (Most Probable Number - MPN method), using MacConkey broth (H2.2) as follows:-

MacConkey Broth	No. of Tubes Used	Vol. of homogenate (1 in 5 dilution) inoculated
i. 10 ml double strength	5	10 ml
ii. 10 ml double strength	5	1 ml
iii. 10 ml single strength	5	0.1 ml (1 ml of 1 in 50 dilution)

H3.2.2 After inoculation, invert the tubes to remove any air bubbles from the Durham tubes.

H3.3 Incubation

Incubate the tubes at 37 ± 1°C and examine after 24 and 48 h.

H3.4 Take tubes showing both acid and gas production as presumptive positive for coliforms.

H3.5 Using Table 4, compute the MPN of coliform on the basis of the number of tubes that are positve for the various dilutions.

H3.6 Calculation (MPN)

Calculate the MPN of coliform count as :

from Table 4 and 5

$$\text{MPN of coliform per g of sample} = \frac{\text{MPN value}}{100}$$

H3.7 Determination of *E. coli*

Perform the Eijkman test and test for indole production as follows:-

H3.7.1 *Eijkman test*

Transfer a loopful from each of the positive MacConkey broth (H3.4) with a wire loop (H1.5) from the determination of coliform count into a fresh MacConkey broth (H2.2) previously warmed to approximately 44°C. Incubate the tubes in a circulating water bath (H1.7) for 24 h at 44°C. Examine for acid and gas production.

H3.7.2 *Indole test*

Transfer a loopful from each of the positive MacConkey broth from the determination of coliform count into a tube of tryptone water (H2.3) previously warmed at 44°C. Incubate the tubes in a circulating water bath (H1.7) for 24 h at 44°C. Test for indole by adding 0.2 - 0.3 ml Kovacs' reagent (H2.4). Appearance of distinct red colour in the upper layer is positive test.

H3.8 Using Table 4, compute the MPN of *E.coli* from tubes showing both positive Eijkman and Indole tests.

H3.9 **Calculation (MPN)**

Calculate the MPN of *E.coli* as:

from Table 4 and 5

$$\text{MPN of } E.coli \text{ per g of sample} = \frac{\text{MPN value}}{100}$$

Table 4. Most Probable Number (MPN) per 100 ml of Sample Using 5 tubes with 10, 1 and 0.1 ml volumes (APHA)

Pos* 10,1,0.1	MPN	Pos* 10,1,0.1	MPN	Pos* 10,1,0.1	MPN	Pos* 10,1,0.1	MPN	Pos* 10,1,0.1	MPN
000	0	111	6.1	222	14	333	28	444	62
001	1.8	112	8.1	223	17	334	31	445	69
002	3.6	113	10	224	19	335	35	450	41
003	5.4	114	12	225	22	340	21	451	48
004	7.2	115	14	230	12	341	24	452	56
005	9	120	6.1	231	14	342	28	453	64
010	1.8	121	8.2	232	17	343	32	454	72
011	3.6	122	10	233	20	344	36	455	81
012	5.5	123	12	234	22	345	40	500	23
013	7.3	124	15	235	25	350	25	501	31
014	9.1	125	17	240	15	351	29	502	43
015	11	130	8.3	241	17	352	32	503	58
020	3.7	131	10	242	20	353	37	504	76
021	5.5	132	13	243	23	354	41	505	95
022	7.4	133	15	244	25	355	45	510	33
023	9.2	134	17	245	28	400	13	511	46
024	11	135	19	250	17	401	17	512	64
025	13	140	11	251	22	402	21	513	84
030	5.6	141	13	252	23	403	25	514	110
031	7.4	142	15	253	26	404	30	515	130
032	9.3	143	17	254	29	405	36	520	49
033	11	144	19	255	32	410	17	521	70
034	13	145	22	300	7.8	411	21	522	95
035	15	150	13	301	11	412	26	523	120
040	7.5	151	15	302	13	413	31	524	150
041	9.4	152	17	303	16	414	36	525	180
042	11	153	19	304	20	415	42	530	79
043	13	154	22	305	23	420	22	531	110
044	15	155	24	310	11	421	26	532	140
045	17	200	4.5	311	14	422	32	533	180
050	9.4	201	6.8	312	17	423	38	534	210
051	11	202	9.1	313	20	424	44	535	250
052	13	203	12	314	23	425	50	540	130
053	15	204	14	315	27	430	27	541	170
054	17	205	16	320	14	431	33	542	220
055	19	210	6.8	321	17	432	39	543	280
100	2	211	9.2	322	20	433	45	544	350
101	4	212	12	323	24	434	52	545	430
102	6	213	14	324	27	435	59	550	240
103	8	214	17	325	31	440	34	551	350
104	10	215	19	330	17	441	40	552	540
105	12	220	9.3	331	21	442	47	553	920
110	4	221	12	332	24	443	54	554	1600
								555	2400

* Where multiple tube method is used, results may be reported as most probable number of organisms per ml (or per 100 ml) of sample. This table shows the most probable numbers of organisms corresponding the frequency of positive fermentation test portion. Since more than 1 organism may be responsible for each positive test the probable number of organisms is a logarithmic function of results when the portions are decimally related as illustrated above.

Appendix I

Determination of *Salmonella*

I1. Apparatus

Usual laboratory equipment and the following items.

I1.1 *Oven or incubator* for drying the surface of agar plates preferably at 50 ± 5°C.

I1.2 *Incubator* for maintaining the inoculated liquid media, plates and tubes at 37 ± 1°C.

I1.3 *Incubator or water bath* for maintaining inoculated liquid media at 42°C to 43°C.

I1.4 *Water baths* for heating and cooling solutions and culture media to the appropriate temperatures.

I1.5 *Culture tubes and flasks* for sterilization and storage of culture media, and culture tubes 8 mm in diameter and 160 mm in length for lysine decarboxylation medium (I2.7).

I1.6 *Measuring cylinder* of 100 ml capacity, subdivided in 10 ml, for preparation of the complete media.

I1.7 *Graduated* pipettes with nominal capacity of 10 ml and 1 ml, subdivided respectively in 1.0 ml and 0.1 ml.

I1.8 *Petri dish*, glass or plastic, sterile.

I1.9 *Autoclave*, at 115 ± 1°C, at 121 ± 1°C.

I2. Media and reagents

Basic materials

For uniformly of results, it is recommended that either dehydrated culture medium components of uniform quality and analytical grade chemicals, or dehydrated complete media be used. The water used shall be distilled water or water of at least equivalent purity. If the dehydrated complete medium is used, prepare according to the manufacturer's instructions.

I2.1 *Bismuth sulphite agar*

Beef extract	5.0 g
Peptone	10.0 g
Glucose	5.0 g
Disodium hydrogenorthophosphate dodecahydrate	4.0 g
Iron (11) sulphate	0.3 g
Sodium sulphite	6.15 g
Brilliant green	0.025 g
Ammonium bismuth citrate	1.85 g
Agar	20.0 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water by boiling for approximately 1 min with agitation. Cool to 40 -45°C. The final pH should be 7.7 ± 0.2. Pour in 25 ml portion in Petri dishes (I1.8). Store in a refrigerator and do not use after 72 h. Immediately before use, dry the plates carefully, preferably with the lids off and the agar surfaces downwards, in an oven or incubator (I1.1) for 30 min at 50 ± 5°C.

I2.2 *Brilliant green/phenol red agar*

I2.2.1 Base

Meat extract powder	5.0 g
Peptone	10.0 g
Yeast extract powder	3.0 g
Disodium hydrogenorthophosphate dodecahydrate	1.0 g
Sodium dihydrogenorthophosphate dihydrate	0.5 g
Agar	12.0 g
Distilled water	900 ml

Dissolve the dehydrated base ingredients in distilled water by boiling. Adjust the pH to 7.0 ± 0.1. Transfer the base to tubes or flasks (I1.5) of not more than 500 ml capacity. Autoclave the base in an autoclave (I1.9) for 15 min at 121 ± 1°C.

I2.2.2 Sugar/phenol red solution

Lactose	10.0 g
Sucrose	10.0 g
Phenol red	0.09 g
Distilled water to final volume of	100 ml

Dissolve the ingredients in distilled water. Heat in a water bath (I1.4) for 20 min at 70 ± 1°C. Cool to 55°C and use immediately.

I2.2.3 Brilliant green solution

Dissolve 0.5 g brilliant green in 100 ml distilled water. Store at least for one day in the dark to allow auto-sterilization to occur.

I2.2.4 Complete medium

Base (I2.2.1)	900 ml
Sugar/phenol red solution (I2.2.2)	100 ml
Brilliant green solution (I2.2.3)	1 ml

Under aseptic conditions, add the brilliant green solution to the sugar/phenol red solution cooled to approximately 55°C. Add to the base at 50°C to 55°C and mix.

Transfer the medium in quantities of about 15 ml to sterile Petri dishes (I1.8) and allow to solidify.

Immediately before use, dry the plates as prescribed in I2.1.

If prepared in advance, the undried plates shall not be kept longer than 4 h at room temperature or one day in a refrigerator.

I2.3 *Buffered peptone water (BPW)*

See clause G2.1.

I2.4 *β - galactosidase reagent*

I2.4.1 Buffer solution

Sodium dihydrogenorthophosphate dihydrate	6.9 g
Sodium hydroxide, approximately 4 g/l solution	3 ml (approximately)
Distilled water to a final volume of	50 ml

Dissolve the sodium dihydrogenorthophosphate in approximately 45 ml of water. Adjust the pH to 7.0 ± 0.1 with the sodium hydroxide solution. Add water to a final volume of 50 ml. Store under refrigeration.

I2.4.2 ONPG solution

o - nitrophenyl β-D-galactopyranoside (ONPG)	80 mg
Distilled water	15 ml
Dissolve the ONPG in distilled water at 50°C.	Cool the solution.

I2.4.3 Complete reagent

Buffer solution (I2.4.1)	5 ml
ONPG solution (I2.4.2)	15 ml

Add the buffer solution to the ONPG solution. Store the complete reagent at 4°C but not longer than one month.

I2.5 *Indole medium*

Tryptone	10.0 g
Sodium chloride	5.0 g
DL-tryptophan	1.0 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water and adjust the pH to 7.0 ± 0.1. Dispense in 5 ml portions in 16 mm diameter tubes (I1.5). Sterilize the medium in an autoclave (I1.9) for 15 min at 121 ± 1°C.

I2.6 *Kovacs' reagent (Indole reagent)*

See clause H2.4.

I2.7 *Lysine decarboxylation medium*

L-lysine monohydrochloride	5.0 g
Yeast extract	3.0 g
Glucose	1.0 g
Bromocresol purple	0.015 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water by boiling. Adjust the pH to 6.1 ± 0.2. Transfer the medium in quantities of 5 ml to narrow culture tubes (I1.5) approximately 8 mm in diameter and 160 mm in length. Autoclave the medium in an autoclave (I1.9) for 15 min at 121 ± 1°C.

I2.8 *Nutrient agar*

Peptone	5.0 g
Meat extract	3.0 g
Agar	15.0 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water. Adjust the pH to 7.0 ± 0.2. Autoclave the medium in an autoclave (I1.9) for 15 min at 121 ± 1°C. Pour into sterile Petri dishes (I1.8) about 15 ml of the freshly prepared medium. Allow to solidify. Immediately before use, dry the plates as prescribed in I2.1.

I2.9 *Saline solution*

Sodium chloride	8.5 g
Distilled water	1,000 ml

Dissolve the sodium chloride in the water by boiling. Adjust the pH to 7.0 ± 0.1. Transfer such quantities of the solution to flasks or tubes (I1.5) that they will contain 90 ml to 100 ml after sterilization. Sterilize the solution in an autoclave (I1.9) for 20 min at 121 ± 1°C.

I2.10 *Selenite cystine medium*

Solution C

Tryptone	5.0 g
Lactose	4.0 g
Disodium hydrogenorthophosphate dodecahydrate	10.0 g
Sodium hydrogenselenite	4.0 g
Distilled water	1,000 ml

Dissolve the first three ingredients in distilled water by boiling for 5 min. After cooling, add the sodium hydrogenselenite. Adjust the pH to 7.0 ± 0.1. Do not autoclave.

Solution D

L-cystine	0.1 g
N-sodium hydroxide (40 g/l solution)	15.0 ml
Distilled water	85 ml

Dilute L-cystine and N-sodium hydroxide to 100 ml with water. Do not autoclave.

For complete medium, add D to C at the rate of 0.1 ml to 10 ml. Adjust the pH to 7.0 ± 0.1, and dispense in 100 ml portions in flasks. Do not autoclave. Use the medium on the day of preparation.

I2.11 *Semi-solid nutrient agar*

Meat extract	3.0 g
Peptone	5.0 g
Agar	8.0 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water by boiling. Adjust the pH to 7.0 ± 0.1. Transfer the medium to flasks (I1.5) of not more than 500 ml capacity. Sterilize the medium in an autoclave (I1.9) for 15 min at 121 ± 1°C.

Add to sterile Petri dishes (I1.8) about 15 ml of the freshly prepared complete medium. Do not dry the agar plates.

I2.12 *Tetrathionate medium*

I2.12.1 **Base**

Meat extract	5.0 g
Peptone	10.0 g
Sodium chloride	3.0 g
Calcium carbonate	45.0 g
Distilled water	1,000 ml

Add the base ingredients to distilled water and boil until complete dissolution of the soluble components. Adjust the pH to 7.0 ± 0.1. Sterilize the base in an autoclave (I1.9) for 15 min at 121 ± 1°C.

I2.12.2 Sodium thiosulphate solution

Sodium thiosulphate pentahydrate	50.0 g
Distilled water to a final volume of	100 ml

Dissolve the sodium thiosulphate in a part of the water. Dilute to the final volume. Sterilize the solution in an autoclave (I1.9) for 15 min at 121 ± 1°C.

I2.12.3 Iodine solution

Iodine	20.0 g
Potassium iodide	25.0 g
Distilled water to a final volume of	100 ml

Dissolve the potassium iodide in a minimal volume of water and add the iodine. Shake until solution is complete. Dilute to the final volume. Store the solution in a tightly closed opaque container.

I2.12.4 Brilliant green solution

Add brilliant green (0.5 g) to water (100 ml). Store the solution at least for one day in the dark to allow auto-sterilization to occur.

I2.12.5 Ox bile solution

Dissolve desiccated ox bile (10.0 g) in water (100 ml) by boiling. Sterilize the solution in an autoclave (I1.9) for 15 min at 121 ± 1°C.

I2.12.6 Complete medium

Base (I2.12.1)	900 ml
Sodium thiosulphate solution (I2.12.2)	100 ml
Iodine solution (I2.12.3)	20 ml
Brilliant green solution (I2.12.4)	2 ml
Ox bile solution (I2.12.5)	50 ml

Add to the base, under aseptic conditions, the other ingredients in the above-mentioned order. Mix the liquids well after each addition.

Transfer the complete medium in quantities of 100 ml aseptically into sterile flasks (I1.5) of 500 ml capacity. Store it at 4°C in the dark until needed but use it within one week after preparation.

I2.13 *Triple sugar/iron agar (TSI agar)*

Meat extract	3.0 g
Yeast extract	3.0 g
Peptone	20.0 g
Sodium chloride	5.0 g
Lactose	10.0 g
Sucrose	10.0 g
Glucose	1.0 g
Iron (III) citrate	0.3 g
Sodium thiosulphate pentahydrate	0.3 g
Phenol red	0.024 g
Agar	12.0 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water by boiling. Adjust the pH to 7.4 ± 0.2. Transfer the medium in quantities of 10 ml to tubes (I1.5) of diameter 17 mm to 18 mm. Sterilize the medium in an autoclave (I1.9) for 15 min at 121 ± 1°C. Allow to set in sloping position to give a butt of depth 2.5 cm, and a slant of 4.0 to 5.0 cm.

I2.14 *Urea agar*

I2.14.1 Base

Peptone	1.0 g
Glucose	1.0 g
Sodium chloride	5.0 g
Potassium dihydrogenorthophosphate	2.0 g
Phenol red	0.012 g
Agar	15.0 g
Distilled water	1,000 ml

Dissolve the base ingredients in distilled water by boiling. Adjust the pH to 6.8 ± 0.1. Sterilize the base in an autoclave (I1.9) for 15 min at 121 ± 1°C.

I2.14.2 Urea solution

Urea	400 g
Distilled water	1,000 ml

Dissolve the urea in distilled water. Sterilize by filtration and check the sterility.

(For details of the technique of sterilization by filtration, reference should be made to any appropriate textbook on microbiology).

I2.14.3 Complete medium

Base (I2.14.1)	950 ml
Urea solution (I2.14.2)	50 ml

Under aseptic conditions, add the urea solution to the base, previously melted and then cooled to 45°C. Adjust the pH to 6.8 ± 0.2. Transfer the complete medium in quantities of 10 ml into sterile tubes (I1.5). Allow to set in a sloping position.

I2.15 *VP medium*

Peptone	7.0 g
Glucose	5.0 g
Dipotassium hydrogenorthophosphate	5.0 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water and adjust the pH to 6.9 ± 0.1 and filter. Transfer the medium in quantities of 3 ml into test tubes. Sterilize the medium in an autoclave (I1.9) for 15 min at 121 ± 1°C.

I2.16 *Creatine solution*

Dissolve creatine monohydrate (0.5 g) in water (100 ml).

I2.17 *α-Naphthol reagent*

Dissolve α-naphthol (6.0 g) in ethanol, 96% v/v (100 ml).

I2.18 *KOH reagent*

Dissolve potassium hydroxide (40.0 g) in the water (100 ml).

I2.19 *Toluene*

I2.20 *Sera*

Several types of agglutinating sera containing antibodies for one or several antigens, are available commercially, i.e. anti-sera containing antibodies to one or more 'O' groups (monovalent or polyvalent anti-O sera), anti-Vi sera, and anti-sera containing antibodies for one or several H-factors (called monovalent or polyvalent anti-H sera).

Every attempt should be made to ensure that the anti-sera used are adequate to provide for the detection of all *Salmonella* serotypes.

I3. Procedure

I3.1 Preparation of food homogenate

Prepare as described in clause G3.1.

I3.2 Pre-enrichment

I3.2.1 Transfer the food homogenate (25 g sample blended with 225 ml BPW) aseptically to a sterile 500 ml bottle.

I3.2.2 Incubate for 16 - 20 h at 37 ± 1°C.

I3.3 Enrichment

I3.3.1 Transfer 10 ml of each pre-enrichment bottle to 100 ml tetrathionate medium (I2.12) and another 10 ml to 100 ml of selenite medium (I2.10), previously warmed to 42 - 43°C.

I3.3.2 Incubate tetrathionate medium at 42 - 43°C for 48 h and selenite medium at 37 ± 1° for 48 h.

I3.4 Plating out

I3.4.1 After 48 h, streak from each enrichment flask (I3.3.1) one Petri dish of each of Brilliant green/phenol red agar (I2.2) and bismuth sulphite agar (I2.1).

I3.4.2 Incubate for 48 h at 37 ± 1°C.

I3.4.3 Examine the plates after 24 and 48 h for typical colonies of *Salmonella*.

I3.5 Confirmation

I3.5.1 *Biochemical confirmation*

I3.5.1.1 Select 5 typical or suspected colonies (see I3.5.1.3) from each plate and streak them on nutrient agar plates (I2.8). Incubate for 20-24 h at 37 ± 1°C.

I3.5.1.2 From isolated colonies on nutrient agar plates (I3.5.1.1) inoculate the following media:-

(a) **TSI agar (I2.13)**

Streak the agar slope surface and stab the butt. Incubate for 24 - 48 h at 37 ± 1°C. Interpret the changes in the medium as follows:-

Butt

Yellow	glucose fermented
Red or unchanged	glucose not fermented
Black	hydrogen sulphide formed
Bubbles or cracks	gas formed from glucose

Slant surface

Yellow	lactose and/or sucrose fermented
Red or unchanged	lactose and/or sucrose not fermented

Typical Salmonella cultures show alkaline (red) slopes with gas formation and acid (yellow) butts, with formation of hydrogen sulphide (blackening of the agar).

(b) **Urea agar (I2.14)**

Streak the agar slope surface. Incubate for 24-48 h at 37 ± 1°C. Rose-pink colour indicates positive reaction. (Splitting of urea liberates ammonia, which changes the colour of phenol red to rose-pink and later to deep cerise).

(c) **Lysine decarboxylation medium (I2.7)**

Inoculate just below the surface of the liquid medium. Incubate for 24 h at 37 ± 1°C. A purple colour after growth indicates a positive reaction. A yellow colour indicates a negative reaction.

(d) **β-galactosidase reagent (I2.4)**

Suspend a loopful of the colony in 0.25 ml of the saline solution (I2.9) in a tube. Add 1 drop of toluene (I2.19) and shake the tube. Put the tube in a water bath for several min at 37 ± 1°C. Add 0.25 ml of β-galactosidase reagent (I2.4) and mix. Put the tube again in the water bath for 24 h at 37 ± 1°C. A yellow colour indicates a positive reaction. The reaction is often apparent after 20 min.

(e) **VP medium (I2.15)**

Suspend a loopful of the colony in a sterile tube containing 3 ml of the medium (I2.15). Incubate for 48 h at 37 ± 1°C. After incubation, transfer 0.2 ml of the culture to a test tube. Add 2 drops of the creatine solution (I2.16), 3 drops of the ethanolic naphthol solution (I2.17) and 2 drops of the KOH reagent (I2.18). Shake after the addition of each reagent and read the reaction within 15 min. A pink to bright red colour indicates a positive reaction.

(f) **Indole medium (I2.5)**

Inoculate a tube (I2.5) containing 5 ml of indole medium with the colony and incubate for 24 h at 37 ± 1°C. Add 0.2 - 0.3 ml of the indole reagent (I2.6). The formation of a red ring indicates a positive reaction. A yellow- brown ring indicates a negative reaction.

I3.5.1.3 Typical Salmonella Colonies

(a) *Brilliant green agar (I2.2)*

Colonies are colourless, pink to fuchsia, translucent to opaque with surrounding medium pink to red. Some *Salmonella* appear as transparent green colonies if surrounded by organisms fermenting lactose or sucrose, since these carbohydrate-fermenting organisms produce colonies and zones that are yellow green or green; less than 1% of the *Salmonella* are atypical in that they ferment lactose and appear as yellow-green or green colonies.

(b) *Bismuth sulphite agar (I2.1)*

Colonies are brown, 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 the surrounding medium.

I3.5.1.4 Biochemical reactions of Salmonella

(a) TSI agar

butt:	yellow (acid formed)	+ (100%)
	black (hydrogen sulphide formed)	+ (91.6%)
	bubbles or cracks (gas formed from glucose)	+ (91.9%)
slant:	red or unchanged (lactose and/or sucrose not fermented)	- (99.2%)

(b) Urea agar

no change of colour	- (100%)
(c) Lysine decarboxylase purple colour	+ (94.6%)
(d) β-galactosidase reaction no change of colour	- (98.5%)
(e) VP reaction no change of colour	- (100%)
(f) Indole test; a yellow brown test	- (98.9%)

I3.5.2 *Serological confirmation*

I3.5.2.1 General

The detection of the presence of *Salmonella* O- , Vi- and H-antigens is carried out by slide agglutination with the appropriate sera on pure colonies (I3.5.1) and after auto- agglutinable strains have been eliminated.

I3.5.2.2 Elimination of auto-agglutinable strains

Place one drop of the saline solution (I2.9) on a carefully cleaned glass slide.

Disperse in this drop part of the colony to be tested, so as to obtain a homogeneous and turbid suspension.

Rock the slide gently for 30 s to 60 s.

Observe the result against a dark background, preferably with the aid of a magnifying glass.

If the bacteria have clumped into more or less distinct units, the strain is considered auto-agglutinable, and shall not be submitted to the following tests as the detection of the antigens is impossible.

I3.5.2.3 Examination for O-antigens

Using one pure colony recognized as non-auto-agglutinable, proceed according to I3.5.2.2 using one drop of the anti-O serum (I2.20) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

Use the poly- and monovalent sera one after the other.

I3.5.2.4 Examination for Vi-antigens

Proceed according to I3.5.2.2, but using a drop of the anti-Vi serum (I2.20) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

I3.5.2.5 Examination for H-antigens

Inoculate the semi-solid nutrient agar (I2.8) with a pure non-auto-agglutinable colony.

Incubate the medium for 18 h to 24 h at 37 ± 1°C.

Use this culture for examination for the H-antigens, proceeding according to I3.5.2.2, but using a drop of the anti-H serum (I2.20) instead of the saline solution.

If agglutination occurs, the reaction is considered positive.

I3.5.3 *Interpretation of biochemical and serological reactions*

Table 5 gives the interpretation of the confirmatory tests (see I3.5.1 and I3.5.2) carried out on the colonies selected.

Table 5. Interpretation of confirmatory tests

Biochemical reactions	Auto-agglutination	Serological reactions	Interpretation
Typical	No	O-, Vi- or H-antigens positive	Strains considered to be *Salmonella*
Typical	No	All reactions negative	May be *Salmonella*
Typical	Yes	Not tested	
No typical reactions	No	O-, Vi- or H-antigen positive	
No typical reactions	No	All reactions negative	Not considered to be *Salmonella*

Appendix J

Determination of moulds and yeast

J1. Apparatus

Usual laboratory equipment and the following items.

J1.1 *Petri dish*, glass or plastic, sterile.

J1.2 *Pipette*, 1 ml, sterile.

J1.3 *Autoclave*, at 121 ± 1°C

J1.4 *Incubator*, at 32°C.

J2. Media

Basic materials

For uniformity of results,it is recommended that either dehydrated culture medium components of uniform quality and analytical grade chemicals, or dehydrated complete media be used. The water used shall be distilled water or water of at least equivalent purity. If the dehydrated complete medium is used, prepare according to the manufacturer's instructions.

J2.1 ***Potato dextrose agar***

Potato extract	4.0 g
Dextrose	20.0 g
Agar	15.0 g
Distilled water	1,000 ml

Dissolve the ingredients in distilled water by boiling. Sterilize the medium in an autoclave (J1.3) for 15 min at 121 ± 1°C. Acidify the medium to pH 3.5 ± 0.1 subsequent to autoclaving by adding 1.0 ml of lactic acid 10% to each 100 ml of sterilized medium at 50°C. The medium must not be heated after the addition of the acid, this would result in hydrolysis of the agar and destroy its gelling properties.

J3. Procedure

J3.1 **Preparation of food homogenate**

Prepare as described in clause G3.1.

J3.2 Dilution

Prepare as described in G3.2.

J3.3 Pour plating

J3.3.1 Pipette 1.0 ml of each dilution into each of appropriately marked duplicate Petri dishes (J1.1).

J3.3.2 Pour into each Petri dish 10 ml of the potato dextrose agar (J2.1) kept at 43-45°C. Mix by rotating the dish without splashing over the edge. Allow to set.

J3.4 Incubation and reporting

J3.4.1 Invert the plates and incubate for 5 days at 32°C. Observe and count any yeast or mould colonies daily up to the fifth day.

J3.4.2 Report as yeast and mould count per g.

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