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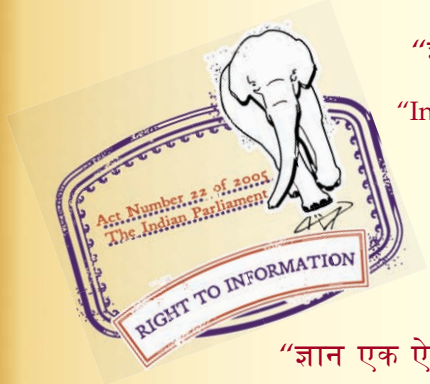

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Indian Standard

METHODS OF TEST FOR DAIRY INDUSTRY

PART I RAPID EXAMINATION OF MILK

Eighth Reprint SEPTEMBER 2007
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BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 110002

Gr 8 *July* 1960

Indian Standard

METHODS OF TEST FOR DAIRY INDUSTRY

PART I RAPID EXAMINATION OF MILK

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CONTENTS

AMENDMENT NO. 4 NOVEMBER 1997
TO
IS 1479 (PART 1) : 1960 METHODS OF TEST FOR DAIRY INDUSTRY

PART 1 RAPID EXAMINATION OF MILK

(*Page* 31, *clause* **17.1**) — Insert the following at the end:

'e) addition of urea,

f) addition of vegetable oils/foreign fat.'

(*Page* 32, *clause* **17.6**) — Insert the following new clauses after **17.6**:

'**17.7 Detection and Determination of Urea in Milk** — Urea content in natural milk varies from 20 mg/100 ml to 70 mg/100 ml. However, urea content above 70 mg/100 ml in milk indicates milk containing 'added urea'. The adulteration of milk by 'added urea' which serves to increase the total solids content of milk, is detected and determined by the following test.

17.7.1 *Detection of Urea in Milk*

17.7.1.1 *Reagents*

i) *P. Dimethyl amino benzaldehyde solution*

P. Dimethyl amino benzaldehyde (DMAB) in ethyl alcohol (1.6 percent, *m/v*) containing 10% (*v/v*) of concentrated hydrochloric acid.

ii) *Phosphate buffer* p*H 7.0*

3.403 g of Anhydrous potassium dihydrogen orthophosphate and 4.355 g of anhydrous dipotassium monohydrogen orthophosphate are dissolved in distilled water and the volume is made to 1 litre.

iii) *Trichloroacetic acid (TCA) 24 percent m/v*

24 g of TCA is dissolved in distilled water and the total volume is made to 100 ml.

iv) *Diluting reagent*

Equal volumes of 24 percent TCA and phosphate buffer (*p*H 7.0) are mixed to make the diluting reagent.

17.7.1.2 *Procedure*

Take 5 ml of milk sample and add 5 ml of 1.6 percent DMAB reagent and mix well. For comparison, prepare a control, that is, milk sample containing no added urea. A distinct yellow colour if observed in milk sample indicates milk containing 'added urea'. The control, however, would show a slight yellow colour due to the presence of natural urea in milk.

17.7.2 *Determination of Urea in Milk*

17.7.2.1 *Preparation of standard urea solution*

Weigh 100 mg of urea and dissolve in phosphate buffer (1 mg/ml of phosphate, *p*H 7.0) and make up the volume to 100 ml.

17.7.2.2 *Procedure*

Take 10 ml of milk sample and mix with 10 ml of Trichloroacetic acid (TCA) to precipitate the proteins and then filter. Treat 5 ml of the filtrate with 5 ml of P. Dimethyl amino benzaldehyde (DMAB) reagent to develop the colour. Prepare a blank by taking 5 ml of diluting reagent and treating it with 5 ml of DMAB reagent. Measure the optical density of the yellow colour at 425 nm in a spectrophotometer.

17.7.2.3 *Calculation*

Prepare standard curve by taking known concentrations of urea and treating with DMAB as above. Calculate the amount of urea in milk from the standard curve.

17.8 Detection of Vegetable Oils/Foreign Fat in Milk

17.8.1 *Isolation of Fat Using Modified Gerber Method*

Take milk butyrometer with open mouth on both the ends and insert the lock stopper on the neck of the butyrometer. Pipette 10.75 ml of milk sample in the butyrometer and add 10 ml of 85 percent (*v/v*) sulphuric acid and 1 ml of isoamyl alcohol. Insert the rubber stopper on the other end, mix and centrifuge for 5 minutes. Read the fat percentage from the butyrometer column.

17.8.2 *Determination of Butyro-Refractometer (B.R.) Reading*

Remove the rubber stopper and take out a little quantity of fat from the stem of the butyrometer with the help of a syringe. Apply the fat between the prisms of the butyro-refractometer maintained at 40°C. Note down the observed B.R. reading.

17.8.3 *Calculation*

Calculate B.R. reading of the isolated fat as follows:

Corrected B.R. = Observed B.R. + 0.08 × Observed B.R.

17.8.4 *Interpretation*

Butyro-refractometer reading of milk exceeding 45.0, indicates the presence of vegetable oils/foreign fat.

(FAD 57)

Printed at Prabhat Offset Press, New Delhi-2

Indian Standard

METHODS OF TEST FOR DAIRY INDUSTRY

PART I RAPID EXAMINATION OF MILK

0. FOREWORD

0.1 This Indian Standard (Part I) was adopted by the Indian Standards Institution on 25 March 1960, after the draft finalized by the Dairy Industry Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 Milk is one of the most important commodities entering trade and required in every day life as an article of food. The basic public health and economic considerations require that consumers should be provided with pure milk, free from pathogenic bacteria. To maintain quality standards, control operations have to be performed at all stages of the production of milk which include maintenace of sanitary conditions of byres; cleanliness of utensils and of the milking machines, if used; and care during storage and handling. Wilful adulteration at the producing or supplying centre, by carriers or by middlemen, has to be prevented. The only means of ensuring quality, when the purchaser cannot exercise a direct control over the production, transport and handling of milk, is by subjecting representative samples to chemical and bacteriological analysis. The need for having uniform methods for assessing the quality of milk at the time of purchase or sale is, therefore, obvious.

0.3 It may not be often necessary nor is it possible to subject all the samples of milk to a regular and elaborate chemical and bacteriological analysis. At milk collecting centres, rapid examination of milk has to be carried out for routine check with the object of ensuring purity of milk, its hygienic quality and fitness for acceptance as raw milk or for further processing. In order to provide uniform methods for this purpose, such of the methods as can be rapidly employed have been compiled as Part I of this standard.

0.4 Besides performing rapid tests, it is often necessary to carry out accurate determinations of various constituents for specific purposes. Methods for these chemical tests will be covered in Part II of this standard, while elaborate methods for bacteriological examination will be covered in Part III.

The Sectional Committee has further proposed to examine all the prevailing methods and available instruments for the determination of freezing point of milk so as to adopt one of them that would be most suitable in the conditions prevailing in India. The method of determining the freezing point of milk will be covered in Part IV of the

standard. It is also proposed to cover the methods of analysis of other ancillaries in dairy plant control like washing solutions, sterilizing solutions, water, etc, in Part V.

0.5 Different methods of analysis are used in different countries for the same purpose and the Sectional Committee, after examining the various methods that are available and at present in use in India and abroad, has recommended the methods included in this standard as the most suitable under the conditions prevailing in this country.

0.6 In the formulation of this standard, considerable assistance has been derived from the following publications:

B.S. 809 : 1949 SAMPLING OF MILK AND MILK PRODUCTS. British Standards Institution.

B.S. 1741 : 1951 METHODS FOR THE CHEMICAL ANALYSIS OF LIQUID MILK. British Standards Institution.

STANDARD METHODS FOR THE EXAMINATION OF DAIRY PRODUCTS, 10TH ED. American Public Health Association, 1960.

LABORATORY MANUAL OF THE MILK INDUSTRY FOUNDATION, WASHINGTON, 1959.

Full use has also been made of the valuable information received from the National Dairy Research Institute, Karnal.

0.7 The Indian Standard Determination of Fat in Whole Milk, Evaporated (Unsweetened) Milk, Separated Milk, Skim Milk, Buttermilk and Cream by the Gerber Method (IS : 1224-1958) and the Indian Standard Specification for Density Hydrometers for Use in Milk (IS : 1183-1957*) are necessary adjuncts to this standard.

0.7.1 Wherever a reference to any Indian Standard mentioned in **0.7** or otherwise appears in this standard, it shall be taken as a reference to the latest version of the standard.

0.8 This standard is one of a series of Indian Standards on methods of testing milk and equipment used for the tests. Other standards published so far in the series† are:

*IS : 1183-1957 SPECIFICATION FOR DENSITY HYDROMETERS FOR USE IN MILK

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*Since revised.

†Since the publication of this Standard the following standards have been published up to the time of this reprint:

IS : 1479 (PART II)-1961 METHODS OF TEST FOR DAIRY INDUSTRY: PART II CHEMICAL ANALYSIS OF MILK

IS : 1479 (PART III)-1962 METHODS OF TEST FOR DAIRY INDUSTRY: PART III BACTERIOLOGICAL ANALYSIS OF MILK

IS : 1479 (PART IV)-1962 METHODS OF TEST FOR DAIRY INDUSTRY: PART IV DETERMINATION OF FREEZING POINT DEPRESSION OF MILK BY HORTVET METHOD

IS : 1479 (PART V)-1962 METHODS OF TEST FOR DAIRY INDUSTRY: PART V METHODS FOR DAIRY PLANT CONTROL

IS : 1223-1958 SPECIFICATION FOR APPARATUS FOR THE DETERMINATION OF FAT IN WHOLE MILK, EVAPORATED (UNSWEETENED) MILK, SEPARATED MILK, SKIM MILK, BUTTERMILK AND CREAM BY THE GERBER METHOD

IS : 1224-1958 DETERMINATION OF FAT IN WHOLE MILK, EVAPORATED (UNSWEETENED) MILK, SEPARATED MILK, SKIM MILK, BUTTERMILK AND CREAM BY THE GERBER METHOD

0.9 All quantities and dimensions in this standard are given in the metric system.

0.10 In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with †IS : 2-1949 Rules for Rounding Off Numerical Values. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

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1. SCOPE

1.1 This standard (Part I) specifies rapid methods that are normally required for assessing the quality of raw milk supplies intended for processing and manufacture. The specific test(s) to be employed would depend upon the purpose of the analysis.

1.1.1 This part does not include such methods of chemical and bacteriological analysis as are used for the detailed examination of milk.

2. QUALITY OF REAGENTS

2.1 Unless otherwise specified, pure chemicals and distilled water (*see* *IS : 1070-1957) shall be employed in tests.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect the experimental results.

3. SAMPLING OF MILK

3.1 General — Sampling of milk shall be done by an experienced person. It is not possible to lay down a single sampling procedure for milk which will be applicable in all cases. The method of sampling will vary according to the purpose for which the sample is collected and the tests which are to be carried out. Proper sampling, however, requires the most careful attention to the recommended procedures in this standard.

*Since revised and split into various parts.
†Since revised.

3.1.1 Samples may be required for chemical or bacteriological examination. All precautions shall be taken to prevent contamination and adulteration.

3.1.2 For chemical examination, the sampling equipment shall be clean and dry.

3.1.3 Samples for bacteriological examination shall be collected by a person trained in the technique of sampling for bacteriological work.

3.1.3.1 For bacteriological purposes, all equipment including plungers, sample bottles, rubber stoppers, shall be sterile and the samples shall be collected under aseptic conditions. Equipment shall be sterilized by one of the following methods:

a) Heating in a hot air oven for not less than 2 hours at 160°C, or

b) Autoclaving for not less than 15 minutes at 120°C.

NOTE 1 — Under field conditions, equipment may be sterilized by immersion for at least 5 minutes in boiling water. Equipment treated by this method shall be used immediately.

NOTE 2 — Rubber stoppers shall be sterilized in an autoclave as in (b). Treatment by immersion in boiling water for not less than 10 minutes would be satisfactory if they are used immediately.

3.1.4 The sample collected for chemical analysis should be representative of the entire batch of milk that is being sampled. Since milk fat is of lower density than the other constituents of milk, it tends to rise to the surface. Thorough mixing of milk with a proper instrument which will reach the entire depth of the liquid is essential to ensure a representative sample of the entire batch. In small batches, it should be possible to accomplish mixing by pouring the entire quantity of milk from one container to another, three or four times. Larger batches of milk shall be thoroughly agitated by a hand stirrer, or by mechanical means. Milk churns easily at 26·5 to 29·5°C and agitation near this temperature shall be avoided.

3.2 Sampling from Individual Container — Pour the milk from one container to another, three or four times. Where this is not practicable, mix thoroughly with a plunger (*see* **3.7.1**). In mixing the milk, the plunger shall be allowed to fall to the bottom of the container and brought to the top of the milk as rapidly as possible not less than 10 times. The position of the plunger shall also be moved from place to place to ensure that the whole of the milk at the bottom of the vessel is thoroughly agitated and mixed with the upper layer. Any milk fat adhering to the neck and under the shoulder of the can shall be well mixed with the remainder of the milk. After thorough mixing, a sample shall be drawn immediately.

3.3 Sampling from Several Containers — The samples shall be taken after pouring the contents of the containers into a vat and mixing.

When this is not possible, a composite sample is taken in the following manner from the containers after milk has been agitated and mixed. First, the milk shall be distributed as equally as possible among a number of containers. The cans shall not be filled, but the same quantity shall be placed in each. After mixing the contents of each can thoroughly, an equal volume of milk shall be taken from each. These portions shall be placed in another vessel, thoroughly mixed as described in **3.2** and a sample then taken.

3.3.1 Alternatively, where facilities exist for accurate measurements, a composite sample may be obtained by taking the same proportion of the milk therein from each container in a consignment after thorough mixing, collecting this in another vessel and taking a sample as described in **3.2**.

3.4 Sampling Bulk Units — When milk of uniform quality is supplied in bulk units (for example, cans filled from storage tanks), the number of random units to be sampled shall be as follows:

Total Number of Units	*Number of Units to be Selected*
1	1
2-5	2
6-20	3
21-60	4
61-100	5
Over 100	5 plus *one for each additional* 100 *units* or fraction thereof

3.4.1 The testing laboratory, may, within its discretion, instruct the person who draws the sample to submit:

a) separate samples from each unit selected, or

b) one or more composite samples consisting of aliquot portions from each unit selected.

The latter course should only be applied where the product is likely to be of fairly uniform composition, for example, where the consignment to be sampled is produced from a quantity of properly bulked milk, and where variations in composition from unit to unit are, therefore, small. Where there is a possibility of wide variations between different units, for example, a consignment of milk from and individual producer, every selected unit shall be separately sampled.

3.5 Sampling from Storage Tanks and Rail and Road Milk Tankers — The method of sampling of milk from storage tanks and rail and road tankers is largely governed by storage/transport conditions.

It is, therefore, difficult to lay down any rigid procedure for the sampling, but the following is recommended:

a) In all cases, the milk in the tank/tanker shall be thoroughly mixed by a sufficiently large plunger, a mechanical agitator or by compressed air; the uniformity of the samples being determined, when necessary, by mixing till such time as complete agreement is obtained between samples taken at the manhole and at the outlet cock in respect of fat and total milk solids.

NOTE — When a plunger (*see* **3.7.1**) is used for mixing the milk in rail or road milk tankers, a convenient and satisfactory method is to insert the plunger in the manhole, the operator sitting astride or standing on top of the tanker. The plunger is thrust foreward and pulled back, thrust downwards and pulled back and thrust backwards and pulled back. The cycle of operations should be repeated for at least 15 minutes.

b) After proper mixing of the milk, the sample may be taken from the tank, removed through the stopcock in the tank door, or from a valve in the discharge line from the tank as it is being emptied.

3.6 Composite Milk Samples for Fat Test — Suppliers of milk are often paid for milk on the basis of fat test (*see* **15**). The determination of fat contents of the suppliers' daily deliveries is laborious and expensive. Composite samples of the suppliers' milk are taken over a period and then tested. The volume of the individual composite sample shall be not less than 175 ml and it shall be collected during the agreed period by placing into the patron's composite sample bottle proportionate amounts of the suppliers' daily delivery. For preserving the composite sample, 0·1 ml of 36 percent formaldehyde for 25 ml of milk may be used. The bottle containing the composite milk sample shall be tightly stoppered to prevent evaporation and kept in a locker, away from light, till required for analysis. The sample shall be analysed on the same day as the last portion of milk is transferred to the composite sample bottle.

NOTE — Each time when fresh sample of milk is added, the sample shall be mixed by rotating the bottle to prevent the formation of solid cream layer or cream plug.

3.7 Appliances for Sampling — The following appliances are required for sampling:

a) Plungers (*see* Fig. 1 and 2),

b) Sampling dippers (*see* Fig. 3), and

c) Sampling tube.

They shall preferably be made of stainless steel, but adequately tinned iron may also be used. If solder is employed, it shall be capable of withstanding a sterilizing temperature of 180°C. All surfaces shall be smooth and free from crevices or projections.

3.7.1 *Plungers*—Plungers shall have sufficient area to produce adequate disturbance of the product, and sufficiently light in weight for the operator to be able to move them rapidly through the liquid. In view of the differing shapes and sizes of containers, no specific design of plunger can be recommended for all purposes. A form of plunger recommended as being suitable for the mixing of milk in buckets or in cans (*see* Fig. 1) consists of a disc 150 mm in diameter, perforated with six holes each 12·5 mm in diameter on a pitch circle of 100 mm diameter, the disc being fixed centrally to a metal rod, the other end of which forms a loop handle. The length of the rod, including the handle, should be approximately one metre. A suitable plunger (*see* Fig. 2) for use with road and rail tanks has a rod not less than 1·8 m in length and is fitted with a disc 300 mm in diameter perforated with twelve holes each 30 mm in diameter on a pitch circle of 225 mm diameter.

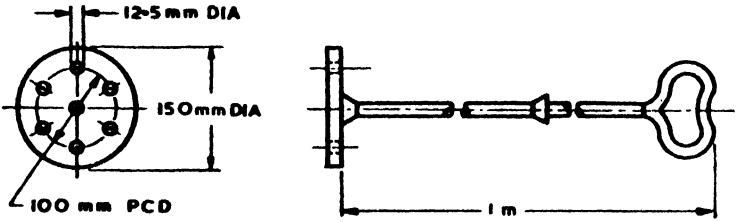

FIG. 1 PLUNGER FOR CANS AND BUCKETS

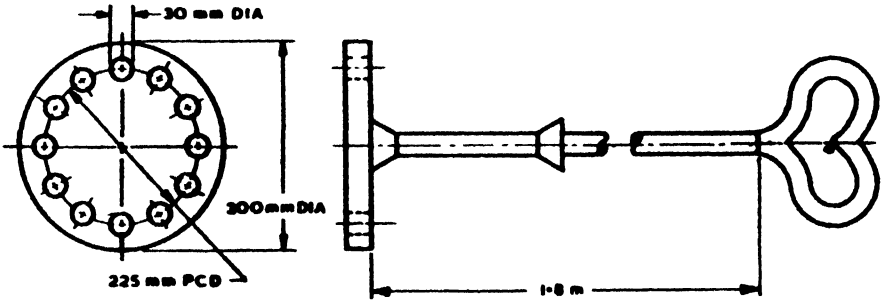

FIG. 2 PLUNGER FOR ROAD AND RAIL TANKS

3.7.2 *Sampling Dippers*—Sampling dippers (*see* Fig. 3) shall be fitted with a solid handle at least 150 mm long. The capacity of the sampling dipper shall be not less than 80 ml. It is advantageous to have a cup and have the handle bent over. The tapered form of the cup permits

nesting of the sampling dippers. The arc of the inside bottom corner of the sampling dipper shall be defined to assure proper cleaning. The body of the sampling dipper shall be of one-piece construction with no seams, overlaps, rivets, or sharp corners.

NOTE — When approximate quantity of milk is to be measured, dipping measures of convenient quantities may also be used.

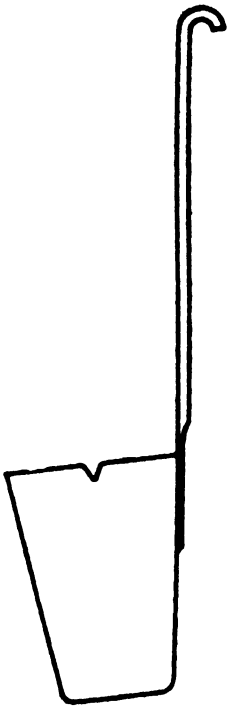

FIG. 3 SAMPLING DIPPER

3.7.3 *Sampling Tube* — Straight seamless metal tube about 600 mm long, of 6 mm inside diameter and about 1·6 mm thickness may be used for sampling, where convenient.

3.8 Sample Bottles — The sample bottles shall be made of good quality glass, suitable for sterilization. The sample bottle shall be wide mouthed, round with sloping sides on the pattern of the milk bottles. Bottles used for collecting samples for chemical analysis shall be provided with well-fitting caps, or bark corks. Bottles for collecting samples for bacteriological examination shall be glass stoppered.

3.8.1 The capacity of the sample bottle shall be 100, 150 or 250 ml. The size of the sample bottle selected for taking a sample shall be such that after containing the quantity of milk required for analysis, only a small space would be left for efficient mixing of the sample as a larger space would allow the fat to churn during transit.

3.8.1.1 When samples are collected for bacteriological examination, it is desirable to avoid air-space by filling the bottles to the top, leaving however, sufficient space to allow for expansion of the rubber stopper.

3.8.2 Bark corks shall not be used for closing milk sample bottles for bacteriological examination.

3.8.3 Sample bottles containing milk which are to be examined for flavour subsequently shall be closed with grease-proof, non-absorbent stopper so that no deleterious odour or taste is imparted.

NOTE — Alternatively, for collecting samples for chemical analysis suitable plastic bottles of above capacities may also be used.

3.9 Labelling of Samples — Each sample container shall be sealed air-tight after filling and marked with particulars regarding the purpose of sampling, the name of the supplier or other particulars of the stock, the date and time of sampling, the nature of preservatives, if any, added and any other relevant information. Samples for bacteriological examination shall be marked distinctively.

3.10 Transport of Samples — Milk samples which are to be examined for flavour (*see* **4**) shall be protected from light and shall not be exposed to odours which may be absorbed during transport.

a) *Samples for Chemical Examination* — It is desirable that samples of milk for chemical examination are delivered for testing on the same day they are taken. The samples shall be stored in a refrigerator at a temperature of 0 to 5°C. Where this is not possible, adequate precautions shall be taken to prevant deterioration and exposure to high temperature and light during transit. In some cases, formaldehyde may be added as a preservative to prevent deterioration, provided it does not interfere with the subsequent analysis. When formaldehyde has been added, this fact and the quantity added shall be indicated on the lable.

NOTE — If any sample is to be used for cryoscopic examination, mercuric chloride shall be permissible as a preservative, provided the bottle is properly labelled as ' POISON '.

b) If the tests are to determine the bacteriological quality, the milk samples shall be chilled immediately and maintained at a temperature not exceeding 4·5°C. If the interval between sampling and examination exceeds 4 hours, the time of sampling

and examination should be recorded on the analytical report.

Generally the samples should be examined within 4 hours of collection. The results of analysis of any sample, the temperature of which has exceeded 7·0°C during a storage period of 4 hours, may be unreliable. At a storage temperature of 0·0 to 4·5°C no detectable increase in bacterial counts will occur within 24 hours.

4. ORGANOLEPTIC TEST AND TEMPERATURE

4.0 Judging the quality of milk by its taste and smell requires considerable skill which could only be acquired by practice. Organoleptic tests are used in all dairies and an experienced person can pick out bad samples with a high degree of accuracy.

4.1 Adopt the following procedure on the receiving platform:

a) Smell the milk in the container immediately after removing the lid. In case of foul or abnormal smell, reject the milk or hold over for subjection to confirmatory test.

b) Observe the colour of the milk. If abnormal in colour, it should be regarded with suspicion.

c) Examine the milk for the following taints:

1) Those due to developed acidity. This is the most important factor to be examined when grading milk by organoleptic test.

2) Those due to feed, or exposure of milk to the atmosphere of the stable.

3) Extraneous matter which might gain access to milk after milking.

4) Oxidized flavour due to exposure of milk to light or metallic contamination from untinned containers.

d) Determine the temperature of milk with a standard thermometer. Bulk raw milk, when received from a chilling station in the factory shall not have a temperature more than 7°C.

5. DETERMINATION OF DENSITY

5.1 Determine the density of the sample by the procedure prescribed in IS : 1183-1957*.

6. SEDIMENT TEST

6.0 Sediment test on raw milk reveals the extent to which visible insoluble matter has gained entrance to the milk and the extent to which such

*Since revised.

material has not been removed from milk by single service strainers. Despite the limitations as to the interpretation that may be applied when visible sediment is or is not detected in milk, the sediment test presents a simple, rapid and a quantitative measure of indicating the cleanliness of milk with respect to visible dirt. The test is carried out by allowing a measured quantity of milk to pass through a fixed area of a filter disc and comparing the sediment left with the prepared standards.

6.1 Apparatus

a) *Sediment Tester* — with filtering surface 2·5 cm in diameter.

b) *White Lintine Cotton Discs* — 32 mm in diameter, exposed filtration area 28 mm in diameter.

c) *Sampling Dipper* — of 500 ml capacity for sampling from milk cans or weigh vats.

d) *Sieves* — two, one coarse corresponding to 850-micron IS Sieve and the other fine corresponding to 425-micron IS Sieve [*see* IS:460-1962 Specification for Test Sieves (*Revised*)].

e) *Sediment Disc Ratings* — showing 0·0, 0·2, 0·5, 1·0, 2·0, 3·0 mg sediment, or higher concentration as required, per 500 ml of milk.

6.1.1 Since sediment encountered in different localities may vary in composition, density, colour and other physical characteristics thus giving rise to variations in the appearance of the discs, it is recommended that the standard sediment discs be prepared as follows:

Make a uniform mixture of oven-dried (100°C) materials which meet the following screening specifications:

Cow or buffalo dung passing through fine sieve [*see* **6.1** (d)]	53 parts
Cow or buffalo dung passing through the coarse sieve but retained on the fine one	10 parts
Garden soil passing through the fine sieve	27 parts
Charcoal passing through the fine sieve	8 parts
Charcoal passing through the coarse sieve but retained on the fine one	2 parts
Total	100 parts

Accurately weigh 0·1 g of the above mixture and transfer to a 1 000-ml flask, using 50 percent sugar solution to wash all fine particles down into the flask. Make the volume up to the mark with more of the sugar solution after most of fine particles have been wetted by shaking the half-filled flask thoroughly several times. After the volume is made

up to the mark, shake the contents of the flask vigorously every 5 minutes for sufficient time (for thirty minutes to one hour) to saturate particles thoroughly. When particles have been thoroughly wetted, it will be noted that the sugar solution will hold them evenly in suspension and the mixture is ready for use in making the standard discs.

On the basis of 0·1 g per 1 000 ml, 10 ml of the sugar solution contains 1 mg of the sediment. Make test discs with one of the usual sediment testers, using varying volumes of the sediment suspension. Place 150 ml or more of filtered skimmed milk in the sediment tester and add varying volumes of the sediment suspension. After forcing the milk through the disc, run through a small quantity of filtered skimmed milk to obtain a more even distribution of the sediment on the disc.

Remove the discs from the tester, mount them permanently on a stiff paper, allow to dry, and then make permanent by spraying with a strong disinfectant such as corrosive sublimate. Below each mounted standard disc on the paper, note the quantity of dried material that the dirt or filth on the disc represents.

6.2 Procedure — Take a milk sample from well-stirred cans or vats of milk with the help of the sampling dipper [*see* **6.1**(c)]. Measure the quantity of milk used with reasonable accuracy. Filter the milk through a properly adjusted, firm lintine cotton disc (rough side facing milk) held in the sediment tester so that a filtration area of 28 mm in diameter is exposed. Compare the sediment disc with the prepared sediment standard discs and record the sediment score.

6.3 Interpretation — For the purpose of comparison, it is convenient to use about five prepared standard discs so as to classify the milk with respect to its sediment content in accordance with the specific requirement of the dairy, or the milk collection depot. For the former, five dics showing 0·0, 0·2, 0·5, 1·0 and 2·0 mg may suffice. Under rural conditions, discs showing 0·0, 0·5, 2·0, 5·0 and 7·0 mg sediment may be more convenient to start with. In either case, no attempt shall be made to estimate the degree of sediment in milk in more than five classes, for example, Excellent, Good, Fair, Bad and Very Bad.

No attempt shall be made to grade as sediment any hair, flies, pieces of hay or straw, or any large particles of dirt. These shall be reported separately.

The presence of appreciable sediment in unprocessed milk supplies indicates careless or insanitary dairy farm practice. However, the lack of sediment is not always indicative of ideal conditions, since visible sediment may be readily removed by straining at the dairy farm. Milk that has been divested of visible sediment by straining retains essentially the bacterial contamination incidental to the original sediment.

10.3 Procedure—Place 5 ml of milk in a test-tube and add an equal quantity of the alizarin solution. Mix the contents of the test-tube by inverting several times. Note the colour of the mixture and presence of flakes or clots. Also note whether the flakes, if any, are small or large.

10.4 Interpretation—The general interpretation of the results is as indicated for the alcohol test (*see* **9.4**). For acidity of 0·14 percent upwards, the gradation in size of the flakes and colour is approximately as follows:

Colour	*Size of Flakes*	*Approximate Acidity (Percent Lactic Acid)*
Lilac	—	Up to 0·14
Pale Red	—	0·14 to 0·17
Reddish-Brown to Brown	Small flakes	0·17 to 0·20
Brownish-Yellow to Yellow	Large flakes	Over 0·20

10.4.1 If acidity has not developed and yet coagulation occurs, it indicates the presence of rennet producing bacteria (sweet curdling). Milk from animals suffering from mastitis is alkaline in reaction and when mixed with alizarin-alcohol solution, a violet or purple colour is produced. From the practical point of view, it is of little material difference whether milk clots through the production of acid or the production of rennin by bacteria as in either case it is unstable to heat.

11. TEN-MINUTE RESAZURIN TEST

11.0 General—This test provides a rapid measure of the sanitary condition and keeping quality of milk. Resazurin reduction occurs in two stages, the first an irreversible change from the blue resazurin to the pink resorufin, and the second a reversible change from the pink resorufin to the colourless dihydroresorufin. The first stage of reduction or colour change from blue to pink is fairly easily brought about so that the quality of milk is assessed in much shorter time. Taking advantage of the two-stage reduction, several procedures have been proposed for reading the end point of resazurin test.

With fresh milk the observed change in resazurin reduction is due to the bacteria present and the leucocyte content. The reduction brought about by leucocytes, however, diminishes with the age of milk. Reduction can be assumed to be brought by the leucocytes if the colour in the down graded milk sample (for example, milk from animals suffering from mastitis) remains unchanged for a longer time than observed normally.

11.1 The test is intended as a platform test for detecting milk of poor keeping quality and shall be carried out on samples collected for becteriological analysis (*see* **3.1.3.1**).

11.2 Apparatus

a) *Sterile Test-Tubes Without Rims* — 150 × 16 mm, internal diameter 13·5 ± 0·5 mm accurately marked at 10 ml. If not used directly after sterilization, they shall be kept in closed boxes protected from dust.

b) *Sterilized Rubber Stoppers* — for closing the test-tubes. The stoppers are sterilized by immersing in a boiling water-bath for not less than 10 minutes.

c) *Sterile 1-ml Pipettes* — straight-sided, blow-out delivery pipettes for measuring the dye solution (*see* **11.3.1**) may preferably comply with the following specification:

Overall length	300 mm
External diameter	7·5 to 8·5 ml
Internal diameter	2·6 to 3·0 mm
Graduation	One mark only at 1 ml level
Distance of graduation from tip	140 to 180 mm
Distance of graduation from top	120 to 160 mm

The pipettes shall also be calibrated to deliver one millilitre of water at 27°C when the contents are blown out with the tip touching the side of the vessel, three seconds allowed for drainage and the accumulated drop then blown out. No pipette should have an error of more than ± 2 percent, that is, the amount delivered should be between 0·98 and 1·02 ml.

d) *Sterile 10-ml Pipettes* — straight-sided, blow-out type.

e) *Sampling Dippers* — These shall be sterilized by keeping in boiling water for 30 minutes. Water shall be changed at frequent intervals when a series of samples are to be examined.

f) *Pipette Case* — of metal.

g) *Water-Bath* — maintained at 37·5 ± 0·5°C, fitted with a cover to exclude light, and containing a metal rack, designed to hold test-tubes when immersed in water. The water-bath shall preferably be thermostatically controlled. The level of water in the bath shall be maintained above the level of the milk in the tubes. The interior of the bath shall be completely dark.

h) *Clock or Watch*

j) *Hot Air Oven Autoclave or Steam-Sterilizer*

k) *Wire Baskets* — for holding test-tubes.

m) *Glass Marking Pencil*

n) *Bunsen Burner, or Spirit Lamp*

p) *Glass Still* — for preparing distilled water.

q) *Comparator with Standard Resazurin Disc* — The comparator may be provided with artificial daylight source of illumination.

r) *Sterile 50-ml Measuring Flask or Cylinder*

11.3 Reagents

11.3.1 *Sterile Standard Resazurin Solution* — Prepare 0·05 percent (*w/v*) stock solution by dissolving resazurin in glass distilled, sterilized water. Preserve in a tightly stoppered amber-coloured bottle in a refrigerator. Prepare a 0·005 percent bench solution by diluting with sterile water. It shall be prepared fresh after every 8 hours. When actually not in use, keep it in a cool dark place.

NOTE — Resazurin powder shall conform to the following requirements:

i) It shall contain sodium resazurate equivalent to 60 ± 3 percent free resazurin.

ii) Apart from traces of sodium resorufate, no other dyestuff shall be present.

iii) The remaining part shall consists of sodium carbonate and/or sodium acetate and moisture only.

iv) It shall give a colourless water-clear solution on reduction in alkaline solution.

v) At a concentration of 1 in 220 000 in fresh normal mixed cow milk of 3·4 percent fat, it shall give a Tintometer disc reading of not less than 6.

11.4 Procedure — Start the test as soon as possible after a group of samples has been taken and at least within 30 minutes.

Shake the sample container at least 25 times, each shake being an up and down movement with an excursion of about 30 cm, the whole process of shaking not exceeding 12 seconds. After shaking, take 10 ml for the test in the test-tube. Place the tubes in numerical order, with the thumb and fingers of the left hand, taking care not to touch the mouth of the tube. Measure one millilitre of the resazurin solution with a sterile pipette, insert the pipette about half an inch into the mouth of the tube and expel the solution by blowing. Replace the stopper, mix by inverting the tube twice in four seconds and return to the rack. When resazurin has been added to a batch of not more than five tubes, place immediately in the water-bath and note the time. The delivery jet of the pipette shall not touch the milk in the tube. Any pipette becoming contaminated shall be immediately discarded. Use a fresh sterile pipette for every group of five samples.

At the end of 10 minutes ±30 seconds, remove the tubes from the water-bath and immediately match the colour with the resazuring disc in the comparator, recording the results for the tubes in numerical order. For comparing, place a control tube of mixed milk without dye in the left section of the comparator and the incubated tube in the right section. The comparator and stand are placed on a bench at such a height that the operator is able to look down on the two apertures. The disc is then revolved until the sample is matched and the disc reading noted. When the colour falls between two disc numbers, it shall be recorded as the half value, for example, a reading between 3 and 4 shall be recorded as 3·5. Tubes giving a reading between 0 and 1 — streaky pink or very pale pink — are recorded as 0·5.

NOTE — It is an advantage for two persons to work in a team when a number of samples are to be taken rapidly — one to take the sample and the other to handle the containers and check the identity of the samples. Similarly, at the time of reading, one person to watch the tubes and another to record.

11.5 Precautions — The following precautions are necessary to get consistent results:

a) All testers should be trained to correctly match the colours in the comparator.

b) The control and experimental test-tubes shall be of the same type and thickness of glass.

c) Control sample used shall be from the same consignment as milk tested to compensate for the natural colour of milk.

d) Resazurin solution, milk, and milk to which resazurin has been added, shall not be exposed to direct sunlight in the laboratory.

e) The water-bath shall be kept covered during the test.

f) The temperature of the water-bath shall be checked before commencing each batch of tests.

11.6 Interpretation — The results shall be interpreted as follows:

Disc Reading	*Keeping Quality*
4 or higher	Satisfactory
3·5 to 1	Doubtful
0·5 to 0	Unsatisfactory

12. HALF-HOUR METHYLENE BLUE REDUCTION (M.B.R.) TEST

12.0 General — The length of time taken by milk to decolourize methylene blue is a fairly good measure of its bacterial content, and hence of its sanitary and keeping quality.

12.1 Apparatus — same as in **11.2.**

12.2 Reagent

12.2.1 *Methylene Blue Solution* — Prepare a standard solution of methylene blue by dissolving one of the good quality methylene blue thiocyanate tablets in 200 ml of cold, sterile, glass-distilled water in a sterile flask. It is preferable to allow the mixture to stand several hours to ensure complete solution. Depending on the nature of the methylene blue tablets used, sometimes the stock solution is further diluted with 800 ml of sterile glass-distilled water. A concentration of 1 part of methylene blue thiocyanate in 300 000 parts of milk is used to obtain satisfactory results. The solution shall be stored in a sterile glass-stoppered amber-coloured bottle in a dark place, and at no time exposed to light. The solution remains stable in the dark for a considerable time but no stock solution more than two months old shall be used.

The amount of methylene blue required for a day's work shall be poured off from the stock bottle into a suitable glass container. On no account shall the pipette used for transferring the methylene blue solution to the tubes of milk be introduced into the stock bottle. Moreover, if at any time during the filling of the tubes the methylene blue solution should become contaminated with milk carried into it by a pipette which has inadvertently come into contact with the milk, the methylene blue soultion shall be immediately discarded and replaced by a fresh stock.

12.3 Procedure — Thoroughly mix the sample of the milk by inverting and shaking the sample bottle as described in **11.4** and then pour the milk in the test-tube up to the 10-ml mark. While doing this, remove the stopper or cap of the bottle under aseptic conditions, the pouring-lip of the bottle and the mouth of the test-tube being flamed and then pour the milk rapidly into the tube up to the 10-ml mark. While pouring into the tube, take care to leave one side of the interior unwetted with milk. Add one millilitre of methylene blue solution to the tube from a pipette taking care that the pipette does not come into contact with any of the milk in the tube or with the wetted side of the interior of the tube. If this occurs, discard the pipette immediately. During delivery, hold the tip of the pipette against the dry side of the tube about 1 to 2 cm above the level of the milk and expel the methylene blue solution by blowing with the mouth or by means of a jet in the pipette. After the lapse of 3 seconds, blow out the solution remaining in the tip of the pipette and withdraw the pipette. Close the tube with a sterile rubber stopper held and inserted by sterile forceps or by the tips of the fingers on the extreme upper end. On no account allow the fingers to come into contact with the mouth of the test-tube or with the end of the stopper which comes into contact with the test-tube. Invert the tube slowly once or twice so that the whole column of contained air rises above the level of the milk and then within 5 minutes, place the tube in the water-bath.

Put up the following control tubes with each batch:

a) 10 ml of mixed milk + 1 ml of tap water, and

b) 10 ml of mixed milk + 1 ml of methylene blue solution.

The milk for the control tubes shall consist of a mixture of milks, preferably from several producers so as to have an average fat content and colour. Fit the control tubes (a) and (b) with stoppers and immerse for 3 minutes in boiling water in order to destroy the natural reducing system present in the milk. Comparison of the experimental tubes with control tube (b) will show when decolourization begins and comparison with control tube (a) will show when it is complete.

Inspect the tube after 30 minutes. Regard the milk as decolourized when the whole column of milk is completely decolourized or is completely decolourized up to within 5 mm of the surface. If a trace of colour persists at the bottom of the tube and does not extend upwards for more than 5 mm, it may be ignored. Record the time at which decolourization is observed. Where a tube is found not to be decolourized within 30 minutes, the sample satisfies the test.

12.4 Precautions — The following precautions shall be taken:

a) It is important that the methylene blue solution when not in use should be kept in the dark: it shall at no time be exposed to sunlight.

b) It is essential that the interior of the water-bath during the progress of the tests shall be completely dark since sunlight, diffused daylight and even artificial light catalyse the reduction of methylene blue.

c) The sterilization of the rubber stoppers for the test-tube and their subsequent satisfactory manipulation can be facilitated by employing a simple rack for holding a large number of rubber stoppers immersed in a suitable vessel of boiling water.

d) The precautions against the contamination of the milk sample described in the method for carrying out the test shall be carefully observed.

12.5 Interpretation — The samples which show complete decolourization of blue colour on incubation for 30 minutes or less shall not be suitable for acceptance.

13. DIRECT MICROSCOPIC COUNT

13.0 General — The direct microscopic method consists of examining, under a compound microscope, stained films of a measured volume of milk spread on glass slides over specified area. It enables the rapid estimation of the total bacterial population of a sample of milk and also reveals useful

information for tracing the sources of contamination in milk. The microscope is first calibrated so that the exact area of the microscopic field is known. Then a measured quantity of the milk (0·01 ml) spread over a measured area (one square centimetre) on a clean glass slide so that each microscopic field examined represents a quantitative aliquot of the sample. The milk is allowed to dry, and the film is defatted, fixed and stained with a suitable dye. The average number of bacteria per microscopic field is determined after examining between 5 and 60 fields, depending on the microscopic factor and the number of bacteria per field. Either the total cells or clumps can be counted, but the clump count agrees more closely with results obtained by the agar plate method than does the total cell count.

This method offers the most rapid technique for determining the extent of bacterial contamination in milk and requires 10 to 15 minutes for examining one sample. It has been used for the classification of raw milk samples into different grades on the basis of clump counts, for screening milk samples for 'acceptable' quality, and for obtaining information regarding likely sources of contamination. The microscopic appearance (types and arrangement of cells) of the milk film will give indication of any udder infections as well as the cause of high counts due to utensil contamination or inadequate cooling.

Estimates of bacterial counts by this method may be expected to be relatively more accurate in the case of poor quality milks having high bacterial counts. In the case of low count samples, examination of a large number of fields is necessary to obtain some accuracy in the estimates. Other sources of error in this method are those caused by (a) variable distribution of bacteria in films, especially those prepared from samples of high sanitary quality; (b) inaccurate measurement of 0·01 ml quantities and faulty preparation and staining of slides; (c) failure of this method to distinguish between the dead and living organisms; and (d) mistakes in observation and calculation.

13.1 Apparatus

a) *Microscope Slides* — (optional sizes 2·5 × 7·5 cm, 5·0 × 7·5 cm or 5·0 × 11·25 cm) of clear glass. They may be either — (1) plain or with etched margins to permit unmistakable identification of films; or (2) with clear, circular one square centimetre areas (diameter approximately 11·28 mm) delineated on each, with remaining area etched. The slides should be clean and dry before use and should not contain any finger-prints or other residues on areas where milk films are to be placed.

New slides may be cleaned by soaking in strong cleaning solution (bichromate-sulphuric acid solution prepared by dissolving

50 g sodium bichromate in 200 ml of water in glass or earthen container and then adding cautiously 300 ml of concentrated sulphuric acid of commercial grade), rinsing in flowing tap water and then in distilled water. Used slides should be soaked in hot or boiling alkaline detergent solution until all residues are removed, then rinsed in flowing tap water, dried and properly stored for re-use. Slides may be stored submerged in chloroform or alcohol and drained and dried at the time of use.

b) *Guide Plates* — special glass plates or cardboard guides, convenient size 5·0 × 11·25 cm with 16 or 24 round or square areas, each one square centimetre, inscribed thereon, for use beneath plain slides.

c) *Pipette or Syringe for Transfer of* 0·01 *ml of Milk*:

1) Pipettes (Breed) shall be calibrated to deliver 0·01 ml of milk. They shall be of straight thick wall, capillary tubing with bore of such diameter that the single graduation mark is 40 to 60 mm from tip, and the tip blunt, and formed so as to discharge milk cleanly. Pipette calibrated to contain 0·1395 g (approximately 140 mg) of mercury will discharge 0·01 ml of milk at 20°C.

When not in use, the pipettes may be kept submerged in and the bores filled with suitable soapless detergent or strong cleaning solution. Before use, the bore and exterior are thoroughly rinsed in clean water until free from detergent or cleaning solution. While using the same pipettes for a number of samples, the graduated portion of the instrument is rinsed each time in clean water (at 25 to 35°C) by dipping the tip slightly beneath surface and repeatedly drawing in and expelling water.

2) Alternatively, a metal syringe, capable of transferring rapidly 0·01 ml of milk with semi-automatic, spring-actuated plunger, with stainless steel piston sliding in close fitting, stainless steel measuring tube, with an adjustable set-screw to permit presetting instrument for repeated accurate measurements of 0·01 ml, may be used. Accuracy of adjustment should be determined by analysis before use to assure 0·01 ml deliveries (average 0·010 3 g). It should be cleaned only in soapless detergents and fat solvents.

d) *Needle, Bent-Point* — suitable for spreading milk over an area of one square centimetre.

e) *Drying Device* — level surface at 40 to 45°C, clean, dust-free, insect proof (cabinet or metal box over electric bulb is satisfactory).

f) *Forceps or Slide Holders* — suitable for dipping and holding slides.

g) *Trays or Jars* — equipped with tight-fitting covers, of size convenient for holding solvents and stains and submerging slides held in partitioned racks.

h) *Slide Storage Boxes, Cases or Files*

j) *Compound Microscope* — binocular (preferably) or monocular with 1·8 mm oil-immersion objective, sub-stage and condenser, mechanical stage, and oculars for different magnifications. A substage lamp or separate microscope lamp may be used as a source of artificial illumination in preference to day light, for microscopic examination.

k) *Stage Micrometer* — slide ruled in 0·1 and 0·01 mm.

13.2 Reagents

a) *Xylene* — or any other fat solvent may also be used.

b) *Ethyl Alcohol* — 95 percent.

c) *Immersion Oil* — of refractive index 1·51 to 1·52 at 20°C.

d) *Alcoholic Methylene Blue Chloride Solution* — prepared by adding 0·6 g methylene blue chloride to 100 ml of 95 percent ethyl alcohol. The mixture is shaken for some time, left at room temperature for 24 to 48 hours shaking at intervals until the dye is completely dissolved, and then stored in clean, tightly closed container.

Alternatively, Newman's stain prepared by dissolving methylene blue powder in a mixture of glacial acetic acid, tetrachlorethane and ethyl alcohol in the following proportions may be used:

Methylene blue chloride	1 g
Glacial acetic acid	6 ml
Tetrachlorethane	40 ml
Ethyl alcohol (95 percent)	54 ml

13.3 Procedure

13.3.1 *Determination of Microscopic Factor* — This factor is to be determined for each microscope in the following manner:

Focus on the scale of the stage micrometer with the 16 mm objective. Place a drop of immersion oil on the micrometer scale and focus with the 1·8 mm objective. Move the stage micrometer until one end of the scale arc is at the edge of the field. Count the number of small spaces (0·01 mm each) in the diameter of the field and determine the diameter of the field, which may range from 0·146 to 0·206 mm depending on the length of the draw tube of the

microscope (usually American microscopes are adjusted to 160 mm and Continental microscopes to 170 mm) and the objective and ocular used. The microscopic factor (MF) is given by the formula

$$MF = \frac{10000}{3{\cdot}1416 \times r^2}$$

where r is the radius of the microscopic field.

In effect, the reciprocal of the MF represents the fraction of one millilitre of milk actually seen in one microscopic field. Thus, if N is the average number of cells counted in one microscopic field, the total counts per millilitre of milk will be NX microscopic factor, using a 10 X ocular with 1·8 mm oil-immersion objective, the microscope can be adjusted to give a microscopic factor in round numbers ranging from 3 000 000 to 5 000 000 depending upon the tube length of the microscope. Once this microscopic factor has been calculated for a definite tube length and combination of objective and ocular, it can be used as a constant factor for determining direct microscopic counts of all milk samples, provided the same adjustment of the microscope is followed throughout.

13.3.2 *Preparation of Milk Film on Microscopic Slides* — Mix the sample of milk thoroughly by shaking. If single 0·01-ml pipette is used, rinse it thoroughly in sterile water (25 to 35°C) between samples. Dip the tip of the pipette into the sample of milk and draw in and expel several times to remove traces of rinse water. Draw milk into the pipette above the graduation mark, wipe the exterior of the pipette with clean, dry paper or cloth towel, and adjust the volume of the sample to exactly 0·01-ml mark. Touch the tip of the pipette to the centre of a one square centimetre area on a slide and expel the entire volume of milk. With a flamed bent-point needle, promptly spread the portion of milk uniformly over the entire one square centimetre area on the slide. Wipe needle between samples on a clean dry tissue or towel.

If a metal syringe is used, rinse the tube as described above and withdraw milk by holding the tip beneath the surface and releasing the plunge fully. Remove excess milk from the exterior of the tip with clean pape tissue or cloth. Holding the instrument nearly vertical, place the tip near the centre of one square centimetre area on the slide and expel the 0·01 ml portion of milk. With the plunger, held fully depressed and with syringe nearly vertical, spread the milk with the tip of extended piston rod over the entire one square centimetre area.

Legibly and indelibly identify each sample on slide, using number or other symbol on margin of slide. After spreading, dry films at 40 to 45°C within five minutes on level surface protected from dust and insects. Rapid heating may cause the film to crack and peel out during later treatments.

13.3.3 *Defatting, fixing and Staining the films* — After the films are dried, submerge the slides in clear xylene (or other suitable fat solvent) for one or two minutes. Drain and air-dry the films. Fix the film in 90 to 95 percent ethyl alcohol for one minute either by submerging the slide in alcohol kept in a jar or by gently pouring the alcohol over the film. Allow the slide to drain and air-dry completely. Dip the slides edgewise in alcoholic methylene blue staining solution (in a jar) for one or two minutes. Slowly remove slides, allowing a few seconds for stain to drain into staining jar. Wash in fresh tap-water in a beaker or large staining jar by raising and lowering edgewise a number of times to remove excess stain without impairing milk films. The cells will be stained deep blue against a faint blue background. Allow stained slides to drain and air-dry gradually.

Alternatively, the slide containing the dried milk film, can be directly dipped in Newman's stain for ½ to 1 minute without the preliminary treatments for defatting and fixing. The composition of Newman's stain is intended to remove fat, fix the cells and stain the organisms in one single operation. If the film is over-stained, rinse in water and decolourize with alcohol slightly. The background will be faintly blue while the cells will be stained deep blue.

NOTE — There are a number of other staining procedures but the two methods described above have been found to be quite satisfactory.

13.3.4 *Microscopic Examination of Stained Films* — Place one drop of immersion oil on film and examine under the oil-immersion objective. Count the single organisms or isolated clumps on a number of microscopic fields. Any isolated single cell, pair of cells or clump of cells is treated as a 'clump'. The fields counted shall be selected at random and represent all parts of the film. The number of fields to be counted is determined by the density of bacterial clumps in the field in the following manner:

If the average number of clumps per field is under 0·5, 0·5 to 1, 1 to 10 and 10 to 30, the number of fields to be counted will be 50, 25, 10 and 5 respectively. If the number of clumps per field is over 30, then the counter is recorded as uncountable. In such cases, if the actual count is required, the milk sample will have to be diluted suitably and then the microscopic count determined.

Calculate the average number of the clumps per field and multiply by the microscopic factor (*MF*) to give the Direct Microscopic Clump Count per millilitre. (Alternatively, the individual cells in each field may be counted and the results reported as total counts per millilitre.)

13.4 Interpretation — The following are tentatively suggested for assessing the bacteriological quality of milk supplies on the basis of Direct

Microscopic Clump Counts:

Direct Microscopic Clump Counts per ml	*Bacteriological Quality of Milk*
a) Less than 500 000	Good
b) 500 001 to 4 000 000	Fair
c) 4 000 001 to 20 000 000	Poor
d) Over 20 000 000	Very poor

13.4.1 A careful study of the types of organisms predominating in the milk film during microscopic examination will reveal valuable information regarding the sources of contamination in milk so that suitable advisory and regulatory measures may be undertaken to improve the quality of milk:

Microscopic Appearance	*Probable Causes of High Counts or Poor Quality*
a) Presence of many cocci and rods in clumps and patches	Improperly cleaned milk utensils
b) Excessive numbers of rod shaped bacteria (particularly spore-formers)	Dusty and dirty environment
c) Large numbers of cocci in pairs or short chains	Improper cooling of milk
d) Large numbers of leucocyte cells (generally exceeding 500 000 per ml) together with long chains of cocci	Mastitis infection

13.5 Preservation of Records — After examination of films and removal of oil (by adding a little xylene and gently removing the dissolved oil), the slides may be preserved in a special dust-free and insect-proof rack or cabinet for future reference.

14. ACIDITY

14.0 General — The titrable acidity test is employed to ascertain if milk is of such a high acidity as to reduce its keeping quality and heat stability. The acidity of milk is not a true measure of lactic acid present but in practice, gives a good indication of the quality of milk. The titrable acidity test really measures the amount of alkali which is required to change the *pH* of milk from its initial value of about 6·6 to 6·8, to the *pH* of the colour change of phenolphthalein added to milk to indicate the end point (*pH* 8·3). In practice, the titration method is employed and measures the buffering capacity of milk and not true acidity.

Due to the opacity of milk, the end point of titration is not sharp and care has to be taken to adjust the conditions to reach the same end-point. Numerous techniques are available but the one recommended in this standard is the simplest.

14.1 Apparatus

a) *White Porcelain Basins* — hemispherical, 60 ml capacity.

b) *Pippette* — 10 ml.

c) *Burette* — with soda-lime guard-tubes.

d) *Measuring Cylinder* — 25 ml.

e) *Stirring Rods* — glass, flattened at one end.

14.2 Reagents

a) *Standard Sodium Hydroxide Solution* — 0·1 N. Prepare a concentrated stock solution of sodium hydroxide by dissolving equal parts of sodium hydroxide (sticks or pellets) in equal parts of water in a flask. Tightly stopper the flask with a rubber bung and allow any insoluble sodium carbonate to settle out for 3 to 4 days.

Use the clear supernatant liquid for preparing the standard 0·1 N solution. About 8 ml of stock solution is required per litre of distilled water.

b) *Phenolphthalein Indicator Solution* — Dissolve one gram of phenolphthalein in 100 ml of 95 percent ethyl alcohol. Add 0·1 N sodium hydroxide solution until one drop gives a faint pink colouration. Dilute with distilled water to 200 ml.

c) *Rosaniline Acetate Solution* (*Stock Solution*) — Dissolve 0·12 g of rosaniline acetate in approximately 50 ml of rectified spirit [*see* IS : 323-1959 Specification for Rectified Spirit (*Revised*)] containing 0·5 ml of glacial acetic acid. Make up to 100 ml with rectified spirit.

d) *Rosaniline Acetate Solution* (*Bench Solution*) — Dilute 1 ml of the stock solution to 500 ml with a mixture of rectified spirit (*see* IS : 323-1959) and distilled water in equal proportions by volume.

NOTE — The stock solution and the bench solution should be stored in dark in brown bottles securely stoppered with rubber bungs.

14.3 Procedure — Thoroughly mix the milk by pouring several times from one container to another avoiding incorporation of air bubbles. Measure accurately 10 ml of milk in two porcelain basins. Add an equal volume of freshly boiled and cooled water. Add 1·0 ml of phenolphthalein indicator solution to one of the basins and to the other basin, add 1·0 ml of bench solution of rosaniline acetate. Titrate the contents of the basin to which phenolphthalein has been added, against standard sodium hydroxide solution added drop by drop from the burette until by comparsion the colour matches the pink tint of the solution in the basin containing

the rosaniline acetate solution. Stir vigorously throughout. The time taken for complete titration shall not exceed 20 seconds. The titration shall be made in north light or under illumination from a daylight lamp.

14.4 Calculation

14.4.1 Titrable acidity (as lactic acid per 100 ml of milk) $= \frac{9V_1N}{V_2}$

where

V_1 = volume in ml of the standard sodium hydroxide required for titration,

N = normality of the standard sodium hydroxide solution, and

V_2 = volume in ml of milk taken for the test.

14.5 Interpretation — The normal range of acidity of milk varies from 0·10 to 0·17 percent lactic acid. Any value in excess of 0·17 percent can safely be reckoned as developed lactic acid.

15. DETERMINATION OF FAT

15.1 Determine fat in the sample (*see* **3.6**) according to the method prescribed in IS: 1224-1958.

16. DETERMINATION OF SOLIDS-NOT-FAT

16.1 Calculate the solids-not-fat according to the method prescribed in IS: 1183-1957* from the density reading (*see* **5**) and the percentage of fat (*see* **15**).

17. DETECTION OF ADULTERANTS

17.1 The modes of adulteration commonly encountered in market samples are:

a) removal of fat by skimming,

b) addition of separated milk or skim milk to whole milk,

c) addition of water, and

d) addition of starch and cane sugar for raising density to prevent detection of added water by lactometers.

17.2 Detection of Skimming — An indication of the removal of excess fat from milk is given by the following:

a) Lower percentage of fat,

b) Higher density reading of the sample at 27°C, and

c) Higher ratio of solids-not-fat : fat.

*Since revised.

17.3 Detection of Milk Mixed with Separated Milk or Skim Milk — When fresh separated milk or skim milk has been added to whole milk, it could be inferred from the following facts:

a) Lower percentage of fat (*see* **15**),

b) Higher density of the toned milk sample at 27°C (*see* **5**),

c) Higher percentage of solids-not-fat (*see* **16**), and

d) Higher ratio of solids-not-fat : fat.

17.4 Detection of Extraneous Water — Presence of extraneous water in milk is detected by the following facts:

a) Lower percentage of fat (*see* **15**),

b) Lower density of milk at 27°C (*see* **5**),

c) Lower percentage of solids-not-fat (*see* **16**), and

d) Depression of freezing point*.

17.5 Detection of Starch — Starch or cereal flours, may be added to make up the density of milk to prevent detection of added water. The presence of starch or cereal flours is detected by the following test:

Place in a test-tube about 3 ml of well-mixed sample. Bring it to boil by holding the tube over a flame. Allow to cool to room temperature. Add a drop of one percent iodine solution. Presence of starch is indicated by the appearance of a blue colour which disappears when the sample is boiled and re-appears on cooling.

17.6 Detection of Cane Sugar — Cane sugar may be added to milk to raise the density to prevent detection of extraneous water. It is detected by the following test:

To about 15 ml of milk in a test-tube, add one millilitre of concentrated hydrochloric acid and 0·1 g of resorcinol and mix. Place the tube in boiling water-bath for five minutes. In the presence of cane sugar, a red colour is produced.

18. DETECTION OF PRESERVATIVES

18.0 General — Milk and other dairy products are required by Public Health Laws to be free from preservatives. With a few exceptions, any preservative added to milk is not removed in the process of treatment or manufacture. Thus, if milk containing preservatives is accepted, the treated milk or milk product when subsequently offered for sale may contain preservatives and render the seller liable to prosecution besides constituting a hazard to health. No preservative shall, therefore, be added to milk

*The method for determining the freezing point of milk has been covered in IS: 1479 (Part IV)-1962.

except in the case of the samples which have to be preserved for chemical examination.

Some of the most common preservatives found in milk are boric acid and borax, benzoic acid, formaldehyde and salicylic acid. Hypochlorite residues may also be found in milk when chlorine sterilizers are used for sterilizing milk handling equipment unless such equipment is properly rinsed with water. Antibiotics used in the treatment of udder diseases of milch animals may also find their way in milk if proper precautions are not taken. The presence of chlorine or antibiotics in milk not only enhances the keeping quality of the milk but will reduce the plate counts and also lengthen the M.B.R. (*see* **12**) or resazurin reduction time (*see* **11**).

When testing for preservatives, it is necessary to carry out a control test with a milk sample known to be free from any preservative.

18.1 Turmeric Paper Test for Boric Acid or Borax

18.1.1 *Reagents*

a) *Turmeric paper* — dried.

b) *Concentrated hydrochloric acid* — sp gr 1·16.

c) *Ammonium hydroxide* — sp gr 0·88.

d) *Lime water or caustic soda solution.*

18.1.2 *Procedure* — Immerse a strip of the turmeric paper in a sample of milk previously acidified with hydrochloric acid in the proportion of 7 ml of concentrated hydrochloric acid to each 100 ml of milk. Allow the paper to dry spontaneously. If boric acid or borax is present, the paper will acquire a characteristic red colour. The addition of ammonium hydroxide will change the colour of the paper to a dark green, but the red colour may be restored by hydrochloric acid.

Alternatively, make about 25 ml of the sample strongly alkaline with lime water or caustic soda and evaporate to dryness on a water-bath. Ignite the residue at a low red heat to destroy organic matter. Cool, digest with about 15 ml of water, add concentrated hydrochloric acid, drop by drop, until the ignited residue is dissolved. Then add one millilitre in excess. Saturate a piece of turmeric paper with this solution, and allow the paper to dry without the aid of heat. The colour change will be the same as described above.

The latter method is more sensitive than the former.

18.2 Hehner Test for Formaldehyde

18.2.1 *Reagent*

a) *Concentrated sulphuric acid* — commercial; sp gr 1·84.

18.2.2 *Procedure* — To about 10 ml of milk in a wide mouthed test-tube add about half the volume of concentrated sulphuric acid pouring the acid carefully down the side of the tube so that it forms a layer at the bottom without mixing with the milk. A violet, or blue colour, at the junction of the two liquids indicates the presence of formaldehyde. The test is sensitive to one part in 10000.

NOTE — The test is given only in the presence of a trace of ferric chloride or other oxidizing agents. This test may be combined with the determination of fat (*see* **15**), noting whether a violet colour forms on addition of the sulphuric acid in the butyrometer.

18.3 Test for Benzoic Acid

18.3.1 *Reagents*

a) *Dilute hydrochloric acid* — 1 : 3 by volume.

b) *Ethyl ether*

c) *Ammonium hydroxide* — sp gr 0·88.

d) *Ferric chloride Solution* — 0·5 percent (*w/v*), neutral.

e) *Sodium hydroxide solution* — 10 percent (*w/v*).

f) *Potassium nitrate* — crystals.

g) *Concentrated sulphuric acid* — sp gr 1·84.

h) *Ammonium sulphide* — freshly prepared and colourless.

18.3.2 *Procedure* — Acidify 100 ml of milk with 5 ml of the hydrochloric acid. Shake until curdled. Filter and extract the filtrate with 50 to 100 ml of ether. Wash the ether extract layer with two 5-ml portions of water. Evaporate the greater portion of ether in a porcelain dish on a water-bath and allow the remainder to evaporate spontaneously. If benzoic acid is present in considerable quantity, it will crystallize from the ether in shining leaflets and give a characteristic odour on heating.

Dissolve the residue in hot water, divide into two portions and test as follows:

a) Make one portion alkaline with a few drops of ammonium hydroxide, expel the excess of ammonia by evaporation, dissolve the residue in a few millilitres of hot water, filter if necessary. Then add a few drops of the neutral ferric chloride solution. A salmon coloured precipitate of ferric benzoate indicates the presence of benzoic acid.

b) To the other portion, add one or two drops of sodium hydroxide solution and evaporate to dryness. To the residue, add five to ten drops of concentrated sulphuric acid and a small crystal of potassium nitrate. Heat for 10 minutes in a glycerol bath at

120 to 130°C, or for 20 minutes in a boiling water-bath. The temperature of glycerol bath shall not exceed 130°C. After cooling, add one millilitre of water, make distinctly ammoniacal and boil the solution to decompose any ammonium nitrate that may have been formed. Cool, pour into a test-tube, and add a drop of fresh colourless ammonium sulphide, without allowing layers to mix.

A red-brown ring indicates benzoic acid. On mixing, the colour diffuses through the whole liquid, and on heating finally, changes to greenish yellow. This differentiates benzoic acid from salicylic acid. The latter forms coloured compounds, which are not destroyed by heating.

18.4 Ferric Chloride Test for Salicylic Acid

18.4.1 *Reagents*

a) *Dilute hydrochloric acid* — 1 : 3 by volume.

b) *Ethyl ether*

c) *Ferric chloride* — 0·5 percent, neutral.

18.4.2 *Procedure* — Acidify 100 ml of milk with 5 ml of dilute hydrochloric acid. Shake until curdled and filter. Extract with 50 to 100 ml of ether. Wash the ether layer with two 5-ml portions of water. Evaporate the greater portion of ether in a porcelain dish on a steam-bath, allow the remainder to evaporate off. Add one drop of the ferric chloride solution. A violet colour indicates the presence of salicylic acid.

18.5 Test for Hydrogen Peroxide

18.5.1 *Reagent*

a) *Paraphenylenediamine solution* — 2 percent (w/v).

18.5.2 *Procedure* — Add to about 5 ml of milk in a test-tube, an equal volume of raw milk, followed by five drops of a two percent solution of paraphenylenediamine. A blue colour is developed in presence of hydrogen peroxide.

NOTE — Hydrogen peroxide is destroyed when milk is heated or stored for a long interval.

18.6 Detection of Hypochlorite

18.6.1 *Apparatus*

a) *Centrifuge*

b) *Tubes for centrifuge* — each of capacity 12·5 ml.

c) *Mercury vapour lamp* — fitted with a Wood's filter.

18.6.2 *Reagent*

a) *Stannous chloride solution* — 0·025 percent (*w/v*) in 73·5 percent sulphuric acid (prepared by mixing three volumes of concentrated sulphuric acid and one volume of water).

18.6.3 *Procedure* — Cool about 3 ml of milk taken in a test-tube in a freezing mixture of ice and salt to 2 to 5°C. In another test-tube, take an equal volume of the stannous chloride solution and similarly cool and add to milk. Shake the tube whilst in the freezing mixture and hold for 3 minutes. Place the mixture in a 12·5-ml centrifuge tube and centrifuge for 3 minutes at 2 500 rpm. A yellow-green colour is produced in the presence of hypochlorite. Alternatively, after centrifuging, examine the tube in ultra-violet light from a mercury vapour lamp fitted with a Wood's filter, for the presence of any yellow fluorescence.

19. DETECTION OF NEUTRALIZERS

19.0 General — Neutralizers in the form of lime water, or sodium bicarbonate may be added to neutralize developed acidity before milk is processed. Such a practice is not permissible.

19.1 Rosalic Acid Test for Carbonates — To about 5 ml of milk in a test-tube, add 5 ml of alcohol, a few drops of a one percent (*w/v*) alcoholic solution of rosalic acid, and mix. If a carbonate is present, a rose-red colour appears, whereas pure milk shows only a brownish colouration.

19.2 Test for Alkalinity of Ash — Neutralization of milk, whether with lime, soda ash or caustic soda, invariably increases the ash content, and total alkalinity of the ash from a fixed quantity of milk. This is detected by ashing accurately measured 20 ml of milk and titrating the ash after dispersing in 10 ml of water. The amount of standard 0·1 N hydrochloric acid required to neutralize the alkalinity shall not exceed 1·20 ml.

20. DETECTION OF MASTITIS

20.0 General — Mastitis or inflammation of the udder is caused by certain species of streptococci, principally *Streptococcus agalactiæ*, staphylococci and a few other types of organisms. Though in most cases mastitis is caused by the non-pathogenic types of bacteria, their occurrence interferes with the efficient secretion of milk and is invariably a source of great economic loss. It is, therefore, necessary to regularly examine all the milking animals in a herd to ensure that none is suffering from mastitis, and whenever positive cases are detected, to take prompt measures to check and eliminate the disease.

It is essential that the test for the detection of mastitis be made on single quarter samples since milk from normal quarters may obscure

detection of the abnormal ones if composite samples are taken. Any quarter giving an abnormal reaction should then be followed up. The first 2 or 3 streams of milk shall always be used for diagnostic tests as these will show the greatest change if the quarter is abnormal.

20.1 Bromothymol Blue Test — This test is based on the fact that milk from infected udders is usually alkaline and shows *p*H as high as 7·4.

The indicator solution may be prepared by triturating one gram of bromothymol blue powder with 160 ml of exactly 0·01 N sodium hydroxide solution, and diluting with 590 ml of distilled water and the test carried out as follows:

Place one millilitre of bromothymol blue solution in a test-tube and add 5 ml of milk. Mix and observe the colour (*see* 7).

21. HANSA TEST

21.0 Hansa test is used to detect the adulteration of cow milk with buffalo milk.

21.1 Instructions for Preservation of Sample

21.1.1 As soon as the serum is received, transfer it to a refrigerator or ice box. It should be preserved at a temperature of 4°C or as near to this temperature as possible. But it should not be frozen. Remember that it is a living tissue and its life depends on the care and gentleness with which it is handled. It should always be thoroughly mixed before use, but not shaken in such a way that foam appears.

21.2 Cleanliness

21.2.1 Keep all glassware and polytheneware thoroughly clean. The slides and pipettes should be thoroughly washed in distilled water and spirit, and dried before and after use each time. Be most careful about the pipettes introduced into the serum, which should be absolutely clean and dry. With care it is possible to keep the equipment clean even under the most primitive village conditions, but without cleanliness it is not possible to get good results.

21.3 Procedure

21.3.1 Place 2 to 3 ml (accurately measured) of the milk sample to be tested in a test-tube. Label it according to a convenient code. Add about 19 times the quantity of water to the milk, so that the sample is diluted 1/20 with water. Take a pipette fitted with a rubber bulb. Put a drop of the mixture on the centre of a glass slide. With another pipette, take out a drop of serum after proper mixing. Place a drop of the serum on the drop of milk (without touching the milk with the pipette). Mix the two-drops together thoroughly with a clean tooth pick or glass rod.

Pick up the slide and gently rock it in your hand so as to give the whole material a swirling motion. Curdy particles develop in the milk containing buffalo milk within half minute. If such particles do not develop for half a minute, the test is negative and there is no buffalo milk present in the sample (provided the serum is in good condition and the test was carried out properly).

21.3.2 *Control* — Before testing unknown samples, test several known samples of pure cow milk, buffalo milk, and cow milk containing buffalo milk in the minimum possible proportion (say 10 percent). Also, it would be a good idea to take another drop of diluted milk and test it with a drop of water in place of serum. If flocculations or particles are observed, the milk sample is spoilt (possibly due to excessive acidity) and is unsuitable for the test by the standard technique.

21.4 Preserved Milk Sample — Milk preserved with any of the standard preservatives and kept in the refrigerator can be used for Hansa test for as long as two weeks. Most satisfactory results have been obtained with 0·4 percent formalin (mix drops of commercial formalin in 28·4 ml). Boiling, addition of sugar and other common additives and stabilizers have no effect on the test.

21.5 Life of the Serum — If all the precautions are observed, and the serum is always handled with care, it should be possible to use it up to three months. In old sera, precipitates may sometimes be found, and these may cause errors in reading the test. The particles can be seen when sera are taken in a pipette. They can be easily removed by centrifugation.

BUREAU OF INDIAN STANDARDS

Headquarters:
Manak Bhavan, 9 Bahadur Shah Zafar Marg, NEW DELHI 110002
Telephones: 23230131, 23233375, 23239402 **Fax**: 91+011 23239399, 23239382
E - Mail : info@bis.org.in website : http://www.bis.org.in

Central Laboratory:	***Telephone***
Plot No. 20/9, Site IV, Sahibabad Industrial Area, SAHIBABAD 201010	277 0032
Regional Offices:	
Central : Manak Bhavan, 9 Bahadur Shah Zafar Marg, NEW DELHI 110002	2323 7617
*Eastern : 1/14 CIT Scheme VII M, V.I.P. Road, Kankurgachi, KOLKATA 700054	2337 8662
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