LAB. MANUAL 1

MANUAL OF METHODS

OF

ANALYSIS OF FOODS

MILK AND MILK PRODUCTS

सत्यमेव जयते

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
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MANUAL FOR ANALYSIS OF MILK AND MILK PRODUCTS

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1. LIQUID MILK

Buffalo milk, cow milk, goat milk, sheep milk, mixed milk, standardised milk, full cream milk, recombined milk, toned milk, double toned milk, and skimmed milk as laid down under FSSAI Rules.

1.1. Preparation of Sample of Milk

Samples are received after few days of drawl and contain preservative (0.4% formalin). Warm the sample to 37- 40°C by transferring it to the beaker and keeping it in a water bath maintained at 40 - 45°C. Stir slowly for proper homogenisation. Mix sample thoroughly by pouring back into the bottle, mixing to dislodge any residual fat sticking to the sides and pour it back in the beaker. During mixing do not shake the bottle vigorously. Allow the sample to come to room temperature (26- 28°C) and withdraw immediately for analysis. If small clots or lumps are observed in the sample which cannot be dispersed, a few drops of liquor ammonia may be used during homogenisation. If even after homogenisation the sample shows lumps or clots or droplets of oil are visible suggestive of curdling /splitting of milk, the sample should be deemed unfit for analysis and rejected.

(*Ref:- IS 1479 (Part II) – 1961 (Reaffirmed 1997) Methods of test for Dairy Industry - Chemical Analysis of Milk. Bureau of Indian Standards, New Delhi).*

1.2. Detection of Adulterants in Milk

1.2.1. Detection and Quantification of Cane Sugar in Milk

Sucrose is absent in milk and its presence in milk indicate adulteration. Presence of sucrose in milk can be determined by the following method.

1.2.1.1. Qualitative Method: Modified Seliwanoff's Method

Fructose in cane sugar (sucrose) reacts with resorcinol in HCl to give red colour.

1.2.1.1.1. Reagent

A. Resorcinol Solution (0.5%): Weigh 0.5 g of resorcinol in about 40 ml of distilled water. Add 35 ml of concentrated HCl (12 N) to it and make up the volume to 100 ml using distilled water.

Note: The resorcinol flakes should be white in colour.

1.2.1.1.2. Procedure

Take 1 ml of milk in a test tube. Add 1 ml of Resorcinol Solution and mix. Place the tube in boiling water bath for 5 min. Withdraw the tube and observe the colour. Appearance of deep red colour indicates presence of sucrose, or a ketose sugar. In pure milk samples no such red color is developed and sample remains white in nature. The limit of detection of method is 0.1%.

(Ref :- IS 1479 (Part I) 1961 (Reaffirmed 2003) Methods of test for Dairy Industry – Rapid Examination of Milk. Bureau of Indian Standards, New Delhi).

1.2.1.2. Quantitative Determination of Cane Sugar in Milk

If the test for Cane Sugar (Sucrose) is positive, the quantitative estimation of sucrose is necessary for determination of SNF in milk sample.

The sucrose content in milk sample can be determined by volumetric method (Lane-Eynon method). In this method, the milk sample is curdled with zinc acetate and potassium ferrocyanide (Carrez Solution 1 and 2). Determine the sugar content before and after inversion. The value before inversion indicates lactose and after inversion total sugars.

1.2.1.2.1. Reagents

Same reagents those mentioned in Section 9.4. (Determination of sucrose content in condensed/evaporated milk).

1.2.1.2.2. Procedure

Proceed as in described in Section 9.4 (Determination of sucrose content in condensed/evaporated milk) and instead of condensed milk, take 40 g of milk sample. Apply the factor 0.95 for calculating the sugar content.

(Ref:- IS 1166 – 1986 (Reaffirmed 1997) Specification for Condensed milk, Partly Skimmed and Skimmed Condensed Milk(Appendix C: Determination of sucrose). Bureau of Indian Standards, New Delhi; IS 4079 – 1967(Reaffirmed 1995) Specification for Canned Rasogolla (Appendix C: Determination of sucrose). Bureau of Indian Standards, New Delhi (Appendix C)Pearson's Composition and Analysis of Foods, 9thedn, 1991 Modification of

para 1 page 297).

1.2.2. Detection and Quantification of Starch in Milk

1.2.2.1. Qualitative method

1.2.2.1.1. Reagent

A. Iodine Solution: Dissolve 2.6 g of iodine and 3 g of potassium iodide in a sufficient quantity of water and make up to 200 ml.

1.2.2.1.2. Procedure

Take about 5 ml of milk in a test tube. Bring to boiling condition and allow the test tube to cool to room temperature. Add 1-2 drops of iodine solution to the test tube. Development of blue colour indicates presence of starch which disappears when sample is boiled and reappears on cooling. The limit of detection of method is 0.02%.

(*Ref :- IS 1479 (Part I) 1961 (Reaffirmed 2003) Methods of test for Dairy Industry – Rapid Examination of Milk. Bureau of Indian Standards, New Delhi).*

1.2.2.2. Quantitative Determination of Starch in Milk

If test for starch is positive, quantitative estimation of starch is to be carried out for determination of SNF in milk sample. The sample of milk is curdled with alcohol, and made free from lactose which is naturally present in milk. The precipitated starch is washed with 50% alcohol to free it from lactose. The precipitated starch is hydrolysed to convert it into reducing sugars. Reducing sugar is determined by Lane and Eynon method (Section 9.4 (Determination of sucrose in condensed milk) and multiplied with 0.9 to get the starch content in milk.

1.2.2.2.1. Reagents

A. Ethanol (98%).

B. 10% sodium hydroxide.

C. Sodium carbonate.

1.2.2.2.2. Procedure

Weigh approximately 25 g sample in a 250 ml beaker. Add 20 ml of ethanol to curdle the milk. Filter the precipitate on a filter paper and wash the precipitate with 50% ethanol till the precipitate is free from lactose/sugar i.e. when the washings give a negative test with resorcinol. Transfer the precipitate to a 500 ml flask with about 200 ml water and add 10 ml concentrate HCl to hydrolyse the starch by refluxing in a boiling water bath for 2.5 hours. Cool and neutralise with 10% sodium hydroxide and sodium carbonate towards the end using litmus paper. Make up to 500 ml with water. Shake well and filter if necessary. Determine reducing sugar by Lane and Eynon method (Section 9.4). Calculate starch as follows:

% starch = % reducing sugar x 0.9

(Ref:- Modified Method, A.O.A.C 17thedn, 2000 Official Method 925.50, Starch in Confectionery, last para).

1.2.3. Detection of Cellulose in Milk

Cellulose in milk gives blue colour with Iodine – Zinc Chloride reagent.

1.2.3.1. Reagent

A. Iodine – Zinc Chloride reagent: Dissolve 20 g $ZnCl_2$ in 8.5 ml water and when cool, introduce the iodine solution (3 g potassium iodide and 1.5 g iodine in 60 ml water) drop by drop until iodine begins to precipitate.

1.2.3.2. Procedure

Take about 10 g of milk in a 100 ml beaker. Add 50 ml of hot water and stir thoroughly for about 2 min. Pour the mixture on a nylon cloth and wash the residue with 50 ml of hot water twice. Scrape the residue with a spatula and place it in a spotting plate. Stain a part of residue with Iodine-Zinc Chloride reagent and another part with iodine solution (see Reagent 1.2.2.2.1.). Development of blue colour in Iodine-Zinc Chloride reagent and absence of blue colour in Iodine Solution confirms presence of cellulose. The method is also applicable to milk products like curd, *rabri* and evaporated milk.

(*Ref:- Manual Methods of Analysis for Adulterants & Contaminants in Foods. I.C.M.R 1990*

page 27).

1.2.4. Detection of Added Urea in Milk

Urea is a natural constituent of milk and it forms a major part of the non-protein nitrogen of milk. Urea concentration in milk is variable within herd. 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 addition of urea to milk can be detected by using para-dimethylaminobenzaldehyde (DMAB). This method is based on the principle that urea forms a yellow complex with DMAB in a low acidic solution at room temperature.

1.2.4.1. Qualitative Method

This method is based on the principle that urea forms a yellow complex with DMAB in a low acidic solution at room temperature.

1.2.4.1.1. Reagent

A. DMAB reagent (1.6%, w/v): Dissolve 1.6 g DMAB in 100 ml ethyl alcohol and add 10 ml concentrate HCl.

1.2.4.1.2. Procedure

Mix 1 ml of milk with 1 ml of 1.6% DMAB reagent. Distinct yellow colour is observed in milk containing added urea. The control (normal milk) shows a slight yellow colour due to presence of natural urea. The limit of detection of method is 0.2%.

1.2.4.2. Quantitative Estimation of Urea in Milk

Urea is a natural constituent of milk and is present to the extent of 70 mg per 100 ml (700 ppm). The test based on the use of para-dimethylamino benzaldehyde can be used for the estimation of urea in milk after precipitation of milk proteins using trichloroacetic acid.

1.2.4.2.1. Reagents/Apparatus

A. p-Dimethyl amino benzaldehyde (DMAB) solution: Dissolve 1.6 g DMAB in 100 ml ethyl alcohol and add 10 ml concentrate HCl. The reagent is stable for 1 month. Prepare new standard curve with each new batch of reagent.

B. Phosphate Buffer pH 7.0: Dissolve 3.403 g anhydrous potassium dihydrogen orthophosphate (KH_2PO_4) and 4.355 g anhydrous dipotassium monohydrogen orthophosphate (K_2HPO_4) separately in 100 ml of distilled water. Combine solutions and dilute to 1 litre with water.

C. Trichloroacetic acid (TCA) 24%, w/v: Freshly prepared. 24.0 g TCA is dissolved in distilled water and volume made up to 100 ml.

D. Diluting Reagent: Equal volumes of 24% TCA and phosphate buffer (pH 7.0) are mixed to make the diluting reagent.

E. Urea Standard Solution: (a) Stock solution: 5 mg / ml. Dissolve 5 ± 0.001 g reagent grade urea in water and dilute to 1 litre with water. (b) Working solution:– Pipette 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 ml stock solution into 250 ml volumetric flask and dilute to volume with phosphate buffer. (c) Reference solution -Use standard solution containing 1.0 mg urea / 5 ml as reference standard. Store at less than 24°C. The reagent is stable for 1 week.

1.2.4.2.2. Apparatus

A. Spectrophotometer – Instrument with maximum band width 2.4 nm at 420 nm, with 1 cm cells

B. Whatman filter paper: Grade 42.

C. Funnels.

D. Test tubes.

1.2.4.2.3. Procedure

1.2.4.2.3.1. Preparation of standard curve

Pipette 5 ml aliquots of working standard solutions into 20 x150 mm (25 ml) test tubes and add 5 ml DMAB solution to each. Prepare reagent blank of 5 ml buffer and 5 ml DMAB solution. Shake tubes thoroughly and let stand for 10 minutes. Read A in 1 cm cell at 420 nm with reagent blank at zero A. Plot A against concentration urea Plot should be straight line

1.2.4.2.3.2. Estimation

10 ml of milk sample is mixed with 10 ml of TCA to precipitate the proteins and filtered using Whatman 42 filter paper. 5 ml of filtrate is then treated with 5 ml of DMAB reagent to develop the colour. Blank is prepared by taking 5 ml of diluting reagent and treating with 5 ml of DMAB reagent. The optical density of the yellow colour is measured at 420 nm. From standard curve the amount of urea in milk is calculated.

(Ref:- IS.1479 (Part I) – 1960 (Reaffirmed 2003) Methods of test for Dairy Industry, Part – I Rapid examination of Milk. Bureau of Indian Standards, New Delhi; Bector, B.S., Ram, M. and Singhal, O.P. (1998). Rapid platform test for the detection /determination of added urea in milk. Indian Dairyman, 50(4): 59-62).

1.2.5. Detection of Ammonium Compounds in Milk

1.2.5.1. Method 1.

1.2.5.1.1. Reagents

A. 2% Sodium hydroxide.

B. 2% Sodium hypochlorite.

C. 5% Phenol solution.

1.2.5.1.2. Procedure

Take 1.0 ml of milk add 0.5 ml of 2% sodium hydroxide, 0.5 ml of 2% sodium hypochlorite and 0.5 ml of 5% phenol solution. Heat for 20 seconds in boiling water bath, bluish colour turns deep blue in presence of ammonium sulphate. The development of pink colour shows that the sample is free from Ammonium sulphate.

(Ref:- Milk and Milk products Vol. 5 Published by N.C.E.R.T.).

1.2.5.2. Method 2.

1.2.5.2.1. Reagents

A. Nessler's reagent: Dissolve the following chemicals separately.

a. 8.0 g of mercuric chloride in 150 ml distilled water.

b. 60.0 g of sodium hydroxide in 150 ml distilled water.

c. 16.0 g of potassium iodide in 150 ml distilled water.

Add reagent 'a' to reagent 'b' and mix well. To this mixture, add reagent 'c', mix and dilute the contents to 500 ml. Leave this solution undisturbed and decant the clear upper layer of the solution and store in a stoppered glass bottle.

1.2.5.2.2. Procedure

Take 5 ml of milk sample in a test tube. Add 1 ml of Nessler's reagent. Mix the contents of the tube thoroughly. Observe and note the color. The control milk sample gives slight grayish colour. At low concentration of ammonium compounds, brownish shade appears which is distinguishable at 0.15% followed by yellowish colour and then orange colour development at higher concentration. The limit of detection of method is 0.15%.

(Ref:- Guleria, V. (1998). Detection of added ammonium salts in milk with and without the addition of formalin. M. Sc. Thesis. NDRI, Karnal, India; Sharma, R.; Rajput, Y.S. and Naik, N.L. (2012). Detection of adulterants in milk – a laboratory manual. NDRI Publication No. 88/2012, NDRI, Karnal, page 49-51).

1.2.6. Tests for Presence of Sulphates in Milk

Presence of sulfate salts, which may be added to milk to raise its SNF level in milk, can be detected by using barium chloride.

1.2.6.1. Reagents

A. Barium chloride ($BaCl_2.2H_2O$) 5% (w/v) aqueous solution: Dissolve 5.0 g barium chloride in distilled water and make the final volume to 100 ml.

B. Trichloroacetic acid (TCA), 24% (w/v, aq.): Dissolve the 24 g of TCA into distilled water and make the final volume to 100 ml obtain 24% TCA.

1.2.6.2. Procedure

Take 10 ml of milk in a 50 ml stoppered test tube. Add 10 ml of TCA solution. Filter the coagulated milk through Whatman filter paper Grade 42. Take 5 ml of clear filtrate. Add few drops of barium chloride solution. Observe for any visible precipitates in the tube. Formation of milky-white precipitates indicates the presence of

added sulfates like ammonium sulfate, sodium sulfate, zinc sulfate and magnesium sulfate etc. to milk. The limit of detection of method is 0.05%.

(Ref:- Sharma, R.; Rajput, Y.S. and Naik, N.L. (2012). Detection of adulterants in milk – a laboratory manual. NDRI Publication No. 88/2012, NDRI, Karnal, page 20-21).

1.2.7. Detection and Estimation of Added Glucose in Milk

1.2.7.1. Qualitative Method

1.2.7.1.1. Reagents

A. Modified Barford's reagent: Dissolve 24 g of Copper acetate in 450 ml of boiling distilled water. Add 25 ml of 8.5% acetic acid, shake, cool to room temperature and make up to 500 ml. After sedimentation filter the reagent and store in dark coloured bottle.

B. Phosphomolybdic acid: Take 35 g ammonium molybdate and 5 g sodium tungstate in a large beaker; add 200 ml of 10% NaOH solution and 200 ml water. Boil vigorously (20-60 min) so as to remove nearly whole of ammonia. Check removal of ammonia with the help of red litmus paper. Cool, dilute with water to about 350 ml. Add 125 ml concentrated H3PO4 (85%) and dilute further to 500 ml.

1.2.7.1.2. Procedure

Take 1 ml of milk sample in a test tube. Add 1 ml of modified Barford's reagent. Heat the mixture for exact 3 min in a boiling water bath. Rapidly cool under tap water. Add one ml of phosphomolybdic acid reagent to the turbid solution. Observe the colour. Immediate formation of deep blue color after adding phosphomolybdic acid reagent indicates the presence of added glucose in the milk sample. In case of pure milk, only faint bluish color can be observed due to the dilution of Barford's reagent. The limit of detection of method is 0.1%.

1.2.7.2. Quantitative method

The qualitative method can be further extended for the estimation of glucose content in milk. The natural level of glucose in milk is around 10 mg/100 ml.

1.2.7.2.1. Reagents

A. Modified Barford's reagent: Dissolve 24 g of Copper acetate in 450 ml of boiling distilled water. Add 25 ml of 8.5% acetic acid, shake, cool to room temperature and make up to 500 ml. After sedimentation filter the reagent and store in dark coloured bottle.

B. Phosphomolybdic acid: Take 35 g ammonium molybdate and 5 g sodium tungstate in a large beaker; add 200 ml of 10% NaOH solution and 200 ml water. Boil vigorously (20-60 min) so as to remove nearly whole of ammonia. Check removal of ammonia with the help of red litmus paper. Cool, dilute with water to about 350 ml. Add 125 ml concentrated H3PO4 (85%) and dilute further to 500 ml.

C. Acetate buffer: 1 N Sodium acetate and 1N acetic acid in equal volume having 4.75 pH.

1.2.7.2.2. Procedure

To 1 ml of milk sample or 1 ml of reconstituted milk powder in a test tube add equal volume of acetate buffer and filter. To 0.2 ml of filtrate add 2.8 ml water and 2 ml of modified Barford's reagent. Heat the tube in boiling water for 4 minutes. After cooling for 2 minutes add 3 ml of phosphomolybdic acid and mix the contents. Development of deep blue colour indicates the presence of glucose. Filter the contents of the tube through Whatman No 42 filter paper. Collect the filtrate in a colorimetric tube, after discarding first 1 ml. Measure the absorbance in a photoelectric colorimeter, using red filter or determine absorption maxima in a spectrophotometer between 620- 780 um against blank prepared identically from a pure milk sample. The concentration of glucose in the sample can be determined with the help of a standard curve prepared from milk samples containing known amounts of added glucose i.e., 0.5, 1.0, 2.0, 5.0 percent glucose in milk.

(Ref:- Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990, page 28; Roy, N.K. and Sen, D.C. (1991) Rapid analysis of milk. In: Textbook of Practical Dairy Chemistry. Vol. I. Chemical analysis of fluid milk. Kalyani Publishers, New Delhi, India).

1.2.8. Detection of Sodium Chloride in milk

The presence of extraneously added sodium chloride in milk can be detected by silver nitrate and potassium chromate reagent.

1.2.8.1. Reagents

A. Silver nitrate ($AgNO_3$) solution: 0.1 N, aqueous.

B. Potassium chromate (K_2CrO_4) solution: 10% (w/v) aqueous.

1.2.8.2. Procedure

Take 5.0 ml of milk sample and add 1.0 ml of 0.1 N silver nitrate solution (10%). Mix the content thoroughly and add 0.5 ml of 10% potassium chromate solution and observe the colour. Appearance of chocolate brown precipitate indicates the absence of dissolved chloride in milk and appearance of yellow colour indicates presence of dissolved chloride. The limit of detection of method is 0.02%.

(Ref :- Pearson's Composition and Analysis of Foods, 9thedn,1991 – Modified Mohr method, page 14).

1.2.9. Detection of Presence of Foreign Fat in Milk

In Indian context, among the various milk constituents, milk fat is the costliest. Often, unscrupulous traders, remove the milk fat from milk and admix milk with vegetable oil/refined oil with help of detergent or by adding other emulsifiers. In the following test, milk fat is isolated from given milk sample and is subject to butyro-refractometer reading (B.R.). Since, most of vegetable fat/oils have higher B.R. compared to milk fat, any increase in B.R. reading above reference value of milk fat indicate adulteration of milk with vegetable/refined oil. For isolation of milk fat from milk, modified Gerber butyrometer can be used where both ends of the butyrometer are open. Stem side opening of the butyrometer (which is generally closed) is closed with a good quality removable silicon stopper. After the milk fat test, silicon stopper is removed and milk fat is removed with the help of a syringe and same is subjected to B.R. at 40°C. Since Gerber sulfuric acid causes some hydrolysis of fatty acids/triglycerides, the B.R is multiplied by a factor to obtain corrected B.R. The fat in suspected milk sample can also be isolated by solvent extraction method in which case correction of

B.R. is not required.

1.2.9.1. Method 1. Using modified Gerber Method

1.2.9.1.1. Reagents: Gerber sulphuric acid, Iso-amyl alcohol.

1.2.9.1.2. Procedure

Isolate the fat from milk by Gerber method using specially designed milk butyrometer, which is open at both ends. Close the stem side opening with a good quality acid resistant silicon stopper. Add 10 ml of Gerber sulphuric acid, 10.75 ml milk and 1 ml iso-amyl alcohol. Close the neck side with lock stopper; mix the content and centrifuge at 1200 rpm, 5 min to get a clear fat column. Remove the silicon stopper from the stem side and take out the fat from the stem of the butyrometer using a capillary or a syringe.

For taking B.R. reading of the milk fat, clean the prism of the Butyro-Refractometer with diethyl ether. Allow the ether to evaporate to dryness. Maintain the temperature of the prism at 40°C by circulating water using a thermostatically controlled water-bath. Calibrate the Butyro-Refractometer by applying standard liquid solution of known B.R. reading. Again clean the prism with diethyl ether; apply 1-2 drops of clear, extracted fat between the prism. Wait for 2 min before taking the reading so that sample should attain temperature of 40°C. A correction of 0.55 is added to the observed B.R. reading for each degree above 40°C or subtracted for each degree below 40°C to get corrected B.R. reading of the sample.

1.2.9.1.3. Calculation

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

Corrected B.R. = Observed B.R. x 1.08

1.2.9.1.4. Interpretation

If the BR reading differs from the prescribed limit of variability (not more than 42 in case of non-cotton tract area and not more than 45 in case of cotton tract area), presence of foreign fat in the milk may be suspected.

1.2.9.2. Method 2. Other Methods for Extraction of Milk fat

A. Solvent Extraction Method

Extract fat from the milk sample by Rose-Gottlieb method (See Section 1.3.4.2). Take the B.R. reading at 40°C of the extracted fat and interpret the results as indicated in Section 1.2.11.1.4.

A. Cream Extraction Method

Separate the milk fat from the milk sample by centrifugation (5000 g, 10 min, 4°C). Carefully remove the cream plug and collect the cream in an aluminium dish. Heat the dish over burner till ghee formation takes place. Filter the ghee residues and take the B.R. reading of ghee and interpret the results as indicated in Section 1.2.11.1.4.

Note:

1. If fat is extracted using method listed in section 1.2.11.1, correction factor is required. Result can be interpreted directly from the B.R. reading, if fat is extracted using other method (Section 1.2.11.2).
2. Further check for presence of extraneous fat can be done by checking the fatty acid profile of the extracted fat by GLC (See Manual on Oils and Fats for determination of fatty acid composition of oils and fats).

(Ref:- IS.1479 (Part I) – 1960 (Reaffirmed 2003) Methods of test for Dairy Industry, Part – I Rapid examination of Milk. Bureau of Indian Standards, New Delhi; Arora, K.L.; Lal, D.; Seth, R. and Ram, J. (1996). Platform test for detection of refined mustard oil adulteration in milk. Indian J. Dairy Sci., 49 (10): 721-723.; Lal, D.; Seth, R.; Arora, K.L. and Ram, J. (1998). Detection of vegetable oils in milk. Indian Dairyman, 50 (7): 17-18).

1.2.10. Detection of Nitrates (Pond Water) in Milk

Pond water is heavier than the tap water; some unscrupulous persons for adulteration in milk usually prefer it. However, it can be easily detected by the following method. This method actually detects nitrates present in the pond water. In the pond water nitrates may come from fertilizers used in the fields.

1.2.10.1. Reagent

A. Diphenylamine (2%, w/v, in sulfuric acid): Weigh 2 g of diphenylamine and dissolve it in sulfuric acid to obtain final volume of 100 ml.

1.2.10.2. Procedure

Take 2 ml of milk in a test tube. Rinse the tube with the milk and drain the milk from the test tube. Add two-three drops of the reagent along the side of the test tube. Note the developed colour. Deep blue colour will be formed in presence of nitrate in the milk sample. Pure milk sample will not develop any colour.

(Ref:- Sharma, R.; Rajput, Y.S. and Naik, N.L. (2012). Detection of adulterants in milk – a laboratory manual. NDRI Publication No. 88/2012, NDRI, Karnal, page 47-48).

1.2.11. Detection of Neutralizers in Milk

Neutralizers (NaOH, 0.1% for Na_2CO_3 and 0.2% for $NaHCO_3$) are added to milk to neutralize the developed acidity in milk. Rosalic acid method can be used for the detection of presence of these neutralizers in milk. The other method available for detection of neutralizers in milk is through determination of alkalinity of ash.

1.2.11.1. Method 1 (Rosalic acid Method)

There are two variants of this method. Both the variants are capable of detecting neutralizers in milk.

Version 1.

1.2.11.1.1.Reagents

A. Rosalic acid solution (0.1%, w/v): Weigh 100 mg of rosalic acid powder and dissolve it in the 30 ml ethyl alcohol and make up the volume with distilled water to obtain final volume of 100 ml.

B. Ethyl alcohol (95%): Take 95 ml of ethyl alcohol in a 100 ml volumetric flask and make the volume up to the mark with distilled water and mix well.

1.2.11.1.2.Procedure

To 10 ml of milk add equal volume of 95% alcohol in a test tube. Add a few drops of 0.1% alcoholic solution (w/v) rosalic acid. If alkali is present a rose red colour appears whereas pure milk shows only a brownish colour. The limit of detection of method is 0.1% for NaOH, 0.1% for Na_2CO_3 and 0.2% for $NaHCO_3$.

Version 2.

1.2.11.1.3.Reagent

Rosalic acid solution (0.05%, w/v): First prepare 60% (v/v) ethyl alcohol solution by mixing 60 ml ethyl alcohol (95%) and 40 ml distilled water. Weigh 50 mg of rosalic acid powder and dissolve it in small quantity of 60% ethyl alcohol and make up the volume to 100 ml with 60% ethyl alcohol.

1.2.11.1.4.Procedure

Take 2 ml milk sample in a test tube and add 2 ml rosalic acid solution. Mix the contents. If alkali is present in milk, a rose red colour appears whereas pure milk shows only a brownish colour.

1.2.11.2. Method 2. (Alkalinity of ash)

1.2.11.2.1.Reagent: 0.1N HCI.

1.2.11.2.2.Procedure

Neutralisation with lime water/sodium bicarbonate/ caustic soda increases ash content and alkalinity of ash. Take 20 ml of milk in a silica dish, evaporate on a water bath and keep in muffle furnace at 550ºC to get white ash. Dissolve the ash obtained in 10 ml of water and titrate with 0.1 N HCI. The titre of more than 1.2 ml indicates the presence of neutralizers in milk.

(Ref:- IS 1479 (Part II) 1961 (Reaffirmed 1997) Methods of test for Dairy Industry – Chemical Analysis of Milk. Bureau of Indian Standards, New Delhi).

1.2.12. Detection of Hypochlorites and Chloramines in Milk

1.2.12.1. Method 1. Detection of Hypochlorite

In this test yellowish fluorescence is produced due to the presence of chlorate (potassium or sodium chlorate) in the hypochlorite solution and is proportional to the amount present. Stannous chloride in the reaction acts as reducing agent.

1.2.12.1.1.Reagent

A. Stannous chloride ($SnCl_2.2H_2O$) solution: 0.025%, (w/v) in 73.5% sulphuric acid (prepared by mixing three volumes of concentrated sulphuric acid and one volume of distilled water).

1.2.12.1.2.Apparatus

A. Centrifuge

B. Tubes for centrifuge: 12.5 ml capacity

C. Mercury vapour lamp fitted with a Wood's filter.

1.2.12.1.3.Procedure

Cool about 3 ml of milk sample 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 freezing mixture and hold for 3 min. Place the mixture in a centrifuge tube and centrifuge for 3 min at 2500 rpm. A yellow-green colour is produced in the presence of hypochlorite. Alternatively, after centrifuging, examine the tube in ultraviolet light from a mercury vapour lamp fitted with Wood's filter for the presence of any yellow fluorescence.

1.2.12.2. Method 2. Detection of Hypochlorites and Chloramines

1.2.12.2.1.Reagents

A. Potassium Iodide solution: Prepare fresh by dissolving 7 g of potassium iodide in 100 ml of water.

B. Dilute HCl: To 200 ml of water, add 100 ml of Concentrated Hydrochloric acid (sp. gr. 1.16).

C. Starch solution: - Boil 1 g starch in 100 ml water. Cool before using.

1.2.12.2.2.Procedure

A. To 5 ml of sample in a test tube add 1.5 ml of Potassium Iodide solution, mix thoroughly and observe colour.

B. If unaltered, add 4 ml of dilute HCl, mix thoroughly with a glass rod flattened at one end and note colour of curd.

C. Subsequently, place the tube in a water bath previously heated to 85°C and allow it to remain for 10 minutes. The curd will rise to the surface. Cool the tube rapidly by placing in cold water. Note the colour of the curd and the liquid.

D. Next add 0.5.to1.0 ml of starch solution to the liquid below curd and note the colour.

1.2.12.2.3.Interpretation

The proportion of available chlorine may be ascertained from Table 1.

Table.1. Reactions with various tests to detect residual chlorine in milk.

Test No*	Concentration of available chlorine					
	1:1000	1:2000	1:5000	1:10000	1:25000	1:50000
A	Yellowish brown	Deep yellow	Pale yellow	-	-	-
B	Yellowish brown	Deep yellow	Light yellow	-	-	-
C	Yellowish brown	Deep yellow	Yellow	Yellow	Pale yellow	Yellowish
D	Blue purple	Blue purple	Blue, dark purple	Dark purple	Red purple	Pale red-purple

* Indicates the step number followed in the above procedure.

Note: The method is not reliable in the presence of more than 2.5 ppm of copper.

(Ref:- IS 1479 (Part II) 1961 (Reaffirmed 1997) Methods of test for Dairy Industry – Chemical Analysis of Milk. Bureau of Indian Standards, New Delhi; A.O.A.C 17th edn, 2000 Official Method 922.08 Hypochlorites and Chloramines in milk).

1.2.13. Test for Quaternary Ammonium Compounds in Milk

Quaternary ammonium compounds (QAC) may be present in milk due to some residual detergent solution remaining after bottle washing. The following method detects about 5 mg / Kg in milk and is included in B.S 1741: Part II.

1.2.13.1. Qualitative method

1.2.13.1.1.Reagents

A. Indicator solution: Prepare a stock solution by dissolving 0.05 g eosin in 100 ml acetone. Shake 10 ml of stock solution with 90 ml of tetrachloroethane and 1 g citric

acid and filter before use.

B. Buffer: Dissolve 25 g citric acid in 100 ml water and adjust to pH 3.5 with 50% Sodium Hydroxide solution (approximately 15 ml required).

1.2.13.1.2.Procedure

To a centrifuge tube add 1 ml milk, 5 ml water, 1 ml indicator solution and 0.2 ml buffer and shake hard for 10 seconds. Centrifuge for 5 minutes at 3200 rpm. If QAC is present the bottom layer assumes a red or pink colour. Samples containing about 1 mg / kg of QAC show a faint pink colour. If the colour is deep pink or red, the amount of QAC can be approximately determined by titration with a standard anionic detergent solution.

(Ref :- B.S. 1741: Part II, Pearson's Composition and Analysis of Foods 9thedn 1991, page 548).

1.2.14. Test for Presence of Anionic Detergent in Milk

1.2.14.1. Qualitative method

Alkyl benzene sulphonic acid (ABS) or anionic detergent may be present in milk due to intentional addition of detergent in milk or due to insufficient rinsing of dairy equipments. The following method is based on the ionic interaction between the anionic detergent and cationic dye. Anionic detergents have a property to form a complex with cationic dyes. The solubility of dye and dye-detergent complex differs significantly as dye-detergent complex is relatively less polar in comparison to dye alone. Formation of dye-detergent complex between cationic dye and anionic detergents and subsequently its extraction into the hydrophobic solvent layer (lower) is the principle behind the method. The method is performed by addition of methylene blue dye solution and chloroform to milk, mixing of the content followed by centrifugation. This results in distribution of dye colour in upper layer and lower layers. Relative intensity of the colour is noticed in these layers. Appearance of relatively more blue colour in lower layer indicates the presence of detergent in milk. The developed test is sensitive to detect anionic detergent up to 0.0125% (12.5 mg/100 ml).

1.2.14.1.1.Reagents

A. Methylene blue dye: 12.5 mg is dissolved in 100 ml of distilled water. Protect the solution against direct sunlight.

B. Chloroform (Inflammable and toxic on inhalation. Mouth pipetting is not recommended).

1.2.14.1.2.Procedure

Pipette 1 ml of suspected milk sample into a 15 ml test tube. Add 1 ml of dye solution followed by addition of 2 ml chloroform. Vortex the contents for about 15 sec and centrifuge at about 1100 rpm for 3 min. Note the intensity of blue color in lower and upper layer. Relatively, more intense blue color in lower layer indicates presence of detergent in milk. Relatively more intense blue color in upper layer indicates absence of detergent in milk. The method can detect presence of 0.15% level of laboratory grade detergent (e.g. labolene) in milk.

Ref:- Rajput, Y.S.; Sharma, R. and Kaur, S. (2006) A kit for detection of detergent in milk. Indian Patent Office file no. 1970/Del/2006.

1.2.15. Test for Presence of Skimmed milk Powder in Natural milk (Cow, buffalo, goat, sheep)

As per the law, use of skimmed milk powder (SMP) is not allowed for adjustment of SNF in case of sale of cow/buffalo or mixed milk. A method has been developed for the detection of presence of SMP in liquid milk. The method is based on the fact that the coagulum obtained from reconstituted skim milk powder by addition of acetic acid, gives intense blue colour on boiling with phosphomolybdic acid due to certain reducing groups present in the proteins of milk powder which are able to cause reduction of molybdenum blue resulting in formation of blue colour.

1.2.15.1. Reagents

A. Acetic acid:4%.

B. Phosphomolybdic acid: 1% solution in water.

1.2.15.2. Procedure

Take 50 ml of milk in a 60 ml centrifuge tube. Place the tube in the centrifuge and balance it properly. Centrifuge at 5000 rpm for 15 minutes. Decant the supernatant creamy layer carefully. Add 0.5 ml of 4% acetic acid to skim milk portion for coagulation of protein. Centrifuge the tubes at 5000 rpm for 5 min. Decant the supernatant and wash the precipitate with distilled water twice. Discard the washings. Then, add 2 ml of 1% phosphomolybdic acid to the washed precipitates. Mix the contents thoroughly and heat in a water bath at boiling temperature for 15 minutes and then cool. The curd obtained from pure milk shall be greenish in colour whereas the curd of sample containing skimmed milk powder shall be bluish in colour. The intensity of bluish colour depends on the amount of the skim milk powder present in the sample

(*Ref :- Journal of Food Science and Technology, Vol 22 (1985) page 207-208).*

1.2.16. Test for Detection of Gelatine in Milk

1.2.16.1. Reagents: Mercury, Conc. HNO_3, Saturated Picric Acid solution.

1.2.16.2. Procedure

Take 10 ml of sample, add 10 ml acid $Hg(NO_3)_2$ solution (Hg dissolved in twice its weight of HNO3 and this solution diluted to 25 times its volume with water). Shake mixture, add 20 ml water, shake again, let stand 5 minutes and filter. If much gelatine is present, filtrate will be opalescent and cannot be obtained quite clear. To portion of filtrate in test tube add equal volume of saturated aqueous picric acid solution. Yellow precipitate is produced in the presence of considerable amount of gelatine, smaller amounts are indicated by cloudiness.

Note: The test is applicable to milk products also. In applying this test to sour, fermented, cultured, or very old samples of milk, cream or butter milk ; to sterilized cream or evaporated milk or to cottage cheese, use care to recognize precipitate produced by picric acid when added to the $Hg(NO_3)_2$filtrates from these materials in absence of gelatine. Such samples with or without rennet and entirely free from gelatine, give on standing distinct precipitate when treated as above. In every case, however these precipitates differ in character than those produced by picric acid with gelatine. Gelatine picric acid precipitate is finely divided, more apt to remain in

suspension, settles only slowly and adheres tenaciously to the bottom of the container, from which it is rinsed with difficulty. Precipitates produced by picric acid in the absence of gelatine are flocculent, separate readily (leaving serum practically clear) do not adhere to walls of container and are easily removed by rinsing with water. When gelatine is present in sample gelatine picric acid precipitate will remain in suspension long after flocculent precipitate has settled, but on standing overnight the characteristic sticky deposit will be found adhering tenaciously to bottom and sides of the test vessel. If gelatine is present in relatively high concentration (1%) gelatine, picric acid precipitate will be voluminous and will settle rather quickly.

(Ref :- A.O.A.C 17thedn ,2000 Official Method - 920.106. Gelatine in Milk and Milk products).

1.2.17. Test for Presence of Formalin in Milk

1.2.17.1. Method 1:Hehner's Test

1.2.17.1.1.Reagent: Concentrated sulphuric acid.

1.2.17.1.2.Procedure

Take milk sample (2 ml) in a test tube and add 2 ml of 90 percent H_2SO_4containing traces of ferric chloride from the side of the test tube slowly. Formation of purple ring at the junction indicates formaldehyde is present in milk. If sucrose is present, distil the milk sample (25 ml) and then carry out the test on the distillate by taking 2-3 ml of distillate and adding 2 ml of formaldehyde free milk. The violet coloration does not appear usually when relatively large quantities of formaldehyde are present.

Precaution: If H_2SO_4 is added from the top and not from the side of the test tube, it may burn the milk solids and affect the end result.

(Ref:- Pearson's Composition and Analysis of foods, 9th edition, 1991 page 90; IS 1479 (Part II) 1961 (Reaffirmed 1997) Methods of test for Dairy Industry – Chemical Analysis of Milk. Bureau of Indian Standards, New Delhi).

1.2.17.2. Method 2. Chromotropic Acid Test

1.2.17.2.1.Reagent

Saturated solution of 1, 8-dihydroxynaphthalene-3, 6-disulphonic acid in about 72% sulfuric acid (about 500 mg/100 ml). Light straw-colored solution should result.

1.2.17.2.2.Procedure

Take one ml of milk sample in a test tube. Add 1 ml of the chromotropic acid reagent and mix well. Appearance of yellow color confirms the presence of formalin in the sample, whereas; control sample will remain white (translucent).

(Ref :- IS 1479 (Part I) 1961 (Reaffirmed 2003) Methods of test for Dairy Industry – Rapid Examination of Milk. Bureau of Indian Standards, New Delhi).

1.2.18. Test for Presence of Hydrogen Peroxide in Milk

1.2.18.1. Method 1.Vanadium pentoxide Test

1.2.18.1.1.Reagent

A. Vanadium pentoxide solution: Dissolve 1 g of vanadium pentoxide (V_2O_5) in 100 ml dilute sulphuric acid (6 ml concentrated sulphuric acid diluted to 100 ml).

1.2.18.1.2.Procedure

Add 10 to 20 drops of vanadium pentoxide reagent in 10 ml milk sample and mix. Note the colour of the sample. Appearance of pink or red colour indicates the presence of hydrogen peroxide in milk.

(Ref:- A.O.A.C 17thedn, 2000 Official Method 957.08 Hydrogen Peroxide in milk).

1.2.18.2. Method 2. Para-phenylenediamine Test

1.2.18.2.1.Reagent

A. Para-phenylenediamine solution: Weigh 2.0 g of para-phenylenediamine and dissolve it in distilled water to obtain 100 ml solution i.e. 2% aqueous solution, w/v. Dissolution of para-phenylenediamine in water is difficult and require thorough mixing. The solution will appear pale yellow.

1.2.18.2.2.Procedure

Add about 2 ml of milk in a test tube. Add equal volume of raw milk. Add two drops of 2 % of para-phenylenediamine reagent. Mix well. Observe the color of the

solution in the tube. Blue color will developed in the presence of H_2O_2, whereas pure milk sample remain white in color.

(Ref:- IS 1479 (Part I) 1961 (Reaffirmed 2003) Methods of test for Dairy Industry – Rapid Examination of Milk. Bureau of Indian Standards, New Delhi).

1.2.18.3. Method 3. Using Potassium Iodide and Starch

1.2.18.3.1.Reagents

A. Potassium iodide solution: Weigh 20 g of potassium iodide and dissolve it in distilled water to obtain a 100 ml solution.

B. Starch solution: Take 1 g starch powder and dissolve it in distilled water by heating and make up the volume to 100 ml.

C. Potassium iodide–starch reagent: Mix equal volumes of 20% potassium iodide solution and 1% starch solution.

1.2.18.3.2.Procedure

Take 1 ml of milk sample in a test tube. Add 1 ml of the potassium iodide-starch reagent and mix well. Observe the color of the solution in the tube. Blue color will developed in the presence of H_2O_2, whereas pure milk sample remain white in color.

(Ref:- Sharma, R.; Rajput, Y.S. and Naik, N.L. (2012). Detection of adulterants in milk – a laboratory manual. NDRI Publication No. 88/2012, NDRI, Karnal, page 10).

1.2.19. Test for Presence of Boric acid and Borates

1.2.19.1. Method 1.

1.2.19.1.1.Reagents

A. Turmeric Paper: Weigh 1.5 to 2.0 g of turmeric powder in 250 ml Erlenmeyer flask and add 100 ml 80% (v/v) ethanol. Shake for 5 min and filter. Collect the filtrate in a flat bottom dish. Dip Whatman filter paper Grade 2 in the clear filtrate. Remove the paper and hang to dry in air. After 1 h, cut the paper into 6 X 1 cm strips and store in tightly stoppered bottle protected from light.

B. Conc. Hydrochloric acid (sp. gr. 1.16).

C. Ammonium hydroxide (sp. gr. 0.88).

D. Lime water or caustic soda.

1.2.19.1.2. Procedure

Take 20 ml of milk in a porcelain dish and add 1.4 ml of conc. hydrochloric acid and mix it thoroughly. Dip a strip of turmeric paper in the acidified milk. Appearance of characteristic red colour on the turmeric paper indicates the presence of boric acid or borax ($Na_2B_4O_7.10H_2O$). The red colour changes to dark blue green on adding ammonium hydroxide, but reappears on re-acidification with hydrochloric acid.

1.2.19.2. Method 2.

1.2.19.2.1. Reagents: Concentrated hydrochloric acid.

1.2.19.2.2. Procedure

Take 25 ml of milk sample in a porcelain dish. Make the sample alkaline with lime water or caustic soda and evaporate it to dryness on water bath. Ignite the dry residue in the dish by heating over low flame to complete charring of the organic matter. Cool the charred residue, re-digest with 15 ml distilled water and add concentrated hydrochloric acid, drop by drop until the ignited residue is dissolved. Then add 1 ml of concentrated hydrochloric acid in excess. Immerse a strip of turmeric paper in the solution and dry it in air. Observe the colour change. Appearance of characteristic red colour indicates the presence of boric acid or borax. A further check is made by adding a few drops of ammonium hydroxide on the red turmeric paper. It will turn into deep blue-green colour but red colour reappears on re-acidification with hydrochloric acid.

(Ref :- IS 1479 (Part I) 1961 (Reaffirmed 2003) Methods of test for Dairy Industry – Rapid Examination of Milk. Bureau of Indian Standards, New Delhi).

1.2.20. Test for Presence of Salicylic acid in Milk

1.2.20.1. Reagents: Dilute HCl, Ether, 0.5% (v/v) neutral Ferric Chloride solution**.**

1.2.20.2. Procedure

Place 50 ml of sample in a separating funnel. Add 5 ml of dilute HCl (1+ 3) and

extract with 50 ml ether, If mixture emulsifies add 10-15 ml petroleum ether and shake. If this treatment fails to break emulsion centrifuge or let stand until considerable portion of aqueous layer separates, drain latter, shake vigorously and again let separate. Wash ether layer with two 5 ml portions of water, evaporate ether in a porcelain dish and add 1 drop of 0.5 % (v/v) neutral Ferric Chloride solution. A violet colour indicates presence of Salicylic acid.

(Ref :- A.O.A.C 17th, edn Official method 975. 30 Salicylic acid in Food and Beverages / IS 1479 (Part II) 1961 (Reaffirmed 1997) Methods of test for Dairy Industry – Chemical Analysis of Milk. Bureau of Indian Standards, New Delhi).

1.3. Other Tests for Chemical Analysis of milk

1.3.1. Alkaline Phosphatase Test for Checking Efficiency of Pasteurisation in Liquid Milk

Alkaline phosphatase is an indigenous milk enzyme. The enzyme activity is destroyed at pasteurization temperature and has been adopted as an index of the efficiency of pasteurization. Since milk is a proven vector for a number of pathogenic bacteria, including *Salmonella*, *Campylobactor* and *Listeria*, the test is of very great significance to the dairy industry as a means of policing the thoroughness of heat treatments or the addition of raw milk to heated or unheated products. In the following method, a solution of disodium *p*-nitrophenyl phosphate in a buffer of pH 10.2 is used as substrate. This compound, colourless in solution, is hydrolyzed by alkaline phosphatase of milk to liberate *p*-nitrophenol, which under alkaline condition gives an intense yellow colouration to the solution. The liberated *p*-nitrophenol is measured by direct comparison with standard colour discs in a Lovibond comparator. The test does not apply to sour milk and milk preserved with chemical preservatives.

1.3.1.1. Reagents/Apparatus

All reagents should be of analytical grade.

A. Buffer solution: 1.5 g of sodium bicarbonate and 3.5 g of anhydrous sodium carbonate dissolved in water and made up to one litre. Store in a refrigerator and discard after 1 month.

B. Disodium *p*- nitrophenylphosphate. The solid substrate must be kept in the refrigerator.

C. Buffer-substrate solution - Weigh accurately 0.15 g of substrate (disodium *p*-- nitrophenyl phosphate) into a 100 ml measuring cylinder and make up to 100 ml with buffer solution. The solution should be stored in refrigerator and protected from light. The solution should give a reading of less than the standard marked 10 on comparator disc APTW or APTW 7 when viewed through a 25 mm cell (distilled water is used as a blank). The solution must be discarded after one week.

D. A Lovibond Comparator with stand for work in reflected light.

E. A lovibomd comparator disc APTW or APTW 7.

F. Two Fused glass cells of 25 mm depth.

G. A water bath or incubator capable of being maintained at 37.5±0.5°C.

H. 1 ml pipette and 5 ml pipette.

I. 1 litre graduated flask.

J. 100 ml measuring cylinder.

K. Test tubes, nominal size 150/16 mm with rubber stoppers.

1.3.1.2. Procedure

Into a test tube pipette 5 ml of buffer substrate solution, stopper and bring the temperature to 37°C. Add 1 ml of test milk to it shake and replace stopper, incubate at 37°C for 2 hrs. Incubate one blank prepared from boiled milk of the same type as that undergoing the test with each series of sample. Remove the tubes after 2 h and the content should be well mixed. Place the boiled milk blank on left hand side of the comparator stand and test sample on the right. Take reading in reflected light by revolving the disc until the test sample is matched. Record readings falling between two standards by affixing a plus or minus sign to the figure for the nearest standard.

Interpretation:- The test is considered satisfactory if it gives a reading of 10 µg or less of *p*-nitrophenyl per ml of milk. Properly pasteurized milk gives no discernible colour.

Note: Precautions

1. All glassware must be cleaned before use. Cleaning should be done by soaking in Chromic acid solution prepared by slowly adding 4 volumes of concentrated H_2SO_4 to 5 volumes of 8% potassium dichromate. After cleaning in chromic acid glassware must be rinsed in warm water and distilled water and finally dried. Glassware used for the test must not be used for any other purpose and must be kept apart from other apparatus in the laboratory.
2. A fresh pipette must be used for each sample of milk. Pipettes must not be contaminated with saliva.
3. The sample of milk should be examined as soon as possible after arrival at the laboratory. If not examined immediately it must be kept at a temperature between 3°C and 5°C until examined. The sample must be brought to room temperature immediately before being tested.

(Ref:- IS 1479 (Part II) 1961 (Reaffirmed 1997) Methods of test for Dairy Industry – Chemical Analysis of Milk. Bureau of Indian Standards, New Delhi; F.A.O Manuals of Food Quality control 14 /8 page 23; BIS (1977) IS: 8479 (Part II), Methods for determination of phosphatase activity in milk and milk products. Part I. Routine method, Bureau of Indian Standards, New Delhi).

1.3.2. Turbidity Test for Checking Efficiency of Sterilization in Liquid Milk

The turbidity test depends upon the denaturation of proteins of milk especially albumin after sterilisation. When solutions of inorganic salts or acids are added albumin separates with the casein. The sample after treatment with ammonium sulphate is filtered and heating of the filtrate shows turbidity due to presence of albumin on account of insufficient heat treatment. If milk has been sterilised properly all albumin will have been precipitated and no turbidity will be produced. The test is not suitable for UHT milk.

1.3.2.1. Reagents/Apparatus

A. Ammonium sulphate AR.

B. Conical flask, 50 ml.

C. Graduated cylinder, 25ml.

D. Test tubes 150 /16 mm.

E. Funnels, 6 cm dia.

F. Beaker, 400 ml.

G. Whatman No. 12 or equivalent, 12.5 cm folded filter paper.

H. Pipette, 20 ml.

1.3.2.2. Procedure

Pipette 20 ml of milk in a 50 ml conical flask, add 4.0±0.1 g of ammonium sulphate. Shake the flask till the ammonium sulphate is completely dissolved. Allow the mixture to settle for 5 min, filter through a folded filter paper in a test tube. Keep about 5 ml of the above filtrate in a boiling water bath for 5 min. Cool the tube in a beaker of cold water and examine the contents for turbidity by moving the tube in front of an electric light shaded from the eyes of the observer.

Interpretation:- The milk is considered sterilized when the filtrate shows no turbidity.

(Ref:- IS 1479 (Part II) 1961 (Reaffirmed 1997) Methods of test for Dairy Industry – Chemical Analysis of Milk. Bureau of Indian Standards, New Delhi; F.A.O Manual of Food Quality Control 14 / 8 page 26).

1.3.3. Determination of Total Solids (Gravimetric method)

In this procedure, a known quantity of milk is dried on a boiling water bath. Subsequently sample is dried in hot air oven at 102 ±2°C and from the weight of the residue, the total solids content in milk is determined.

1.3.3.1. Reagents/Apparatus

A. Analytical Balance having sensitivity of 0.1 mg.

B. Desiccator provided with an efficient desiccant (for example freshly dried silica gel with a hydrometric indicator.

C. Boiling water bath provided with openings of adjustable size.

D. Drying oven, ventilated capable of being maintained thermostatically at 102 ± 2°C throughout the total working space.

E. Flat bottomed dishes of height 20 – 25 mm, dia 50 - 75 mm and made of appropriate material (stainless steel, nickel or aluminium) provided with well fitted readily removable lids.

F. Water bath capable of being maintained at 35° - 40°C.

1.3.3.2. Procedure

Transfer sample to a beaker, warm slowly to 35° - 40°C on a water bath with careful mixing to incorporate any cream adhering to the sample. Cool the sample quickly to room temperature. Heat a dish with its lid alongside in the drying oven at least 1 hour. Place the lid on the dish and immediately transfer to a desiccator. Allow to cool to room temperature (at least 30 mins) and weigh to the nearest 0.1 mg. Add 5 ml of prepared sample, place the lid on the dish and weigh again. Place the dish without the lid on the vigorously boiling water bath in such a way that the bottom of the dish is directly heated by the steam. Continue heating till most of the water is removed. Remove the dish from the water bath, wipe the underside and place it in the oven alongside the lid and dry in the oven for 2 hours. Place the lid and transfer to the desiccator. Allow the dish to cool and weigh to the nearest 0.1 mg. Again heat the dish with its lid alongside in the oven for 1 hour. Place the lid on the dish and immediately transfer to the desiccator. Allow to cool and weigh again. Repeat the operation again until the difference in the two consecutive weighing does not exceed 1 mg. Record the lowest mass.

1.3.3.3. Calculation

$$\text{Total Solid Content} = \frac{M_2 - M_0}{M_1 - M_0} \times 100$$

Where

M_0 = mass in g of dish + lid

M_1 = mass in g of dish + lid and test portion

M_2 = mass in g of dish + lid and dried test portion

Round the value obtained to nearest 0.01 % (m/m)

(Ref: - IS 12333 - 1997 / ISO 6731: 1989 Milk, Cream and Evaporated milk. –

Determination of total Solids Content -reference method. Bureau of Indian Standards, New Delhi).

1.3.4. Determination of Fat in Milk

1.3.4.1. Method 1. Gerber Method

The milk is mixed with sulphuric acid and iso-amyl alcohol in a special Gerber tube, permitting dissolution of the protein and release of fat. The tubes are centrifuged and the fat rising into the calibrated part of the tube is measured as a percentage of the fat content of the milk sample. The method is suitable as a routine or screening test. It is an empirical method and reproducible results can be obtained if procedure is followed correctly.

1.3.4.1.1. Reagents /Apparatus

A. Sulphuric acid with a density of 1.807 to 1.812 g/ml at 27°C corresponding to a concentration of sulphuric acid from 90-91 percent by weight.

B. Amyl alcohol for milk testing (furfural free). It should have density between 0.808 to 0.818 g/ml at 27°C.

C. Gerber sulphuric acid: Sulphuric acid shall have a density of 1.807 to 1.812 g/ml at 27°C corresponding to a concentration of sulphuric acid from 90 to 91% by mass.

Preparation of Gerber Sulfuric acid: Take required volume of water in a pyrex flask (generally 100 ml of water is required for 900 ml of concentrated sulfuric acid) kept in a basin of ice cold water. Carefully add the commercial sulfuric acid in small quantities at a time keeping the container sufficiently cold and mix gently. Observe the following precautions while performing the above experiment.

- Sulfuric acid is very corrosive. Handle it with care.
- Add acid to water. Add small quantities of acid to water at a time and cool the mixture by stirring. Never add water to acid.
- Use heat resistant flask for dilutions.

After cooling the flaks, check the specific gravity of Gerber acid with hydrometer and if necessary adjust the Gerber acid to the correct specific gravity with addition of water

or acid taking same precautions as before till specific gravity is in the range of 1.807 to 1.812 g/ml at 27°C (or 1.815 to 1.820 g/ml at 20°C). Store the prepared acid in a glass stoppered bottle to avoid absorption of water.

D. Iso-amyl alcohol ($C_5H_{11}OH$): The iso-amyl alcohol shall have density of 0.803 to 0.805 g/ml at 27°C and should be furfural free.

E. Gerber butyrometer; 6, 8 and 10 percent (ISI marked).

F. Pipette: 10 ± 0.25 ml or automatic measure or tilt measure for sulphuric acid.

G. Pipette: 10.75 ± 0.03 ml for milk.

H. Pipette: 1 ± 0.05 ml or automatic measure or tilt measure for iso-amyl alcohol.

I. Lock stoppers for butyrometers.

J. Lock stopper key.

K. Water-bath: The water-bath shall be made of a suitable material (e.g. stainless steel). It shall be capable of being maintained at 65 ± 2°C and shall be of sufficient depth as to support the butyrometer in vertical position with their scale completely immersed. The bath shall be fitted with horizontal perforated plates to hold the butyrometers and shall also carry a suitable thermometer.

L. Gerber Centrifuge. The centrifuge may be hand-driven or electric driven. The centrifuge shall be capable of producing within 2 min when fully loaded, a relative centrifugal acceleration of 350 ± 50 g_n 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) if operated at the speed indicated against each:

Effective Radius (mm)	Revolution Per Min (± 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 (g_n) produced in a centrifuge is given by the following formula:

$$1.12 \times 10^{-6} \, r \, n^2$$

where r = effective horizontal radius in mm, and

n = speed in revolutions per min

1.3.4.1.2. Procedure

Measure 10 ml of sulphuric acid into a butyrometer tube, preferably by use of an automatic dispenser, without wetting the neck of the tube. Mix the milk sample gently but thoroughly and fill the milk pipette above the graduation line. Wipe the outside of the pipette and allow the milk level to fall so that the top of meniscus is level with the mark. Run the milk into the butyrometer tube along the side wall without wetting the neck, leave to drain for three seconds and touch the pipette's tip once against the base of the neck of the butyrometer tube. Add 1 ml of Amyl alcohol, close with a lock stopper, shake until homogeneous, inverting it for complete admixture of the acid. Keep in a water bath for 5 min. at 65±2°C taking care to have casein particles if any to dissolve fully, and centrifuge for 4 min. at 1100 rpm. The tubes should be put in centrifuge, so as to conform to radial symmetry, and as evenly spaced as possible, in order to protect bearings of the centrifuge. Allow the centrifuge to come to rest. Remove the butyrometer tubes and place in water bath for 5 min. at 65±2°C. Read the percentage of fat after adjusting the height in the tube as necessary by movements of the lock stopper with the key. Note the scale reading corresponding to the lowest point of the fat meniscus and the surface of separation of the fat and acid. When readings are being taken hold the butyrometer with the graduated portion vertical, keep the point being read in level with the eye, and then read the butyrometer to the nearest half of the smallest scale division.

Note:

1. The butyrometer must always be emptied without delay and the highly acidic waste disposed off appropriately. The tubes may be cleaned with chromic acid.
2. In homogenised milk fat separates with more difficulty and centrifuging more than once may be required. On the other hand, holding the tubes too long at 65°C or above, results in esterification of the amyl alcohol with a consequent increase in the volume of the fat layer.
3. In case of old samples, if necessary the concentration of sulphuric acid may be increased from 90-91% to 92-93% to felicitate better dissolution.

(*Ref:- F.A.O. Manual of Food Quality Control, 14/8, page 8 /IS 1479 (Part I) 1961 (Reaffirmed 2003) Methods of test for Dairy Industry – Rapid Examination of Milk. Bureau of Indian Standards, New Delhi).*

1.3.4.2. Method 2. Rose-Gottlieb Method

The milk sample is treated with ammonia and ethyl alcohol; the former to dissolve the protein and the latter to help precipitate the proteins. Fat is extracted with diethyl ether and petroleum ether. Mixed ethers are evaporated and the residue weighed. This method is considered suitable for reference purposes. Strict adherence to details is essential in order to obtain reliable results.

1.3.4.2.1. Reagents /Apparatus

A. Ammonia solution, containing approximately 25% (m/m) of NH_3, sp.gr ($\rho^{20)}$ ≈910 g/l (If ammonia of this concentration is not available, a more concentrated solution of known concentration may be used – see method).

B. Ethyl alcohol (95%).

C. Diethyl ether, peroxide-free.

D. Petroleum ether, boiling range 40-60°C.

E. Mojonnier fat extraction flask or any other suitable extraction tube (as per IS specification).

F. Cork or stopper of synthetic rubber unaffected by usual fat solvents.

G. 100 ml flat bottom flask with G/G joint or stainless steel or aluminium dishes of 5.5 cm height and 9 cm diameter or glass bowl.

1.3.4.2.2. Procedure

Weigh accurately about 10 g of sample (liquid milk), transfer to extraction tube. Add 1.25 ml of ammonia sp. gr. 0.91 (or an equivalent volume of a more concentrated ammonia solution may be used), mix and shake thoroughly. Add 10 ml ethyl alcohol and mix again. Add 25 ml of diethyl ether (peroxide free) stopper and shake vigorously for about a minute. Then add 25 ml petroleum ether (boiling range 40 – 60°C and shake again vigorously for about half a minute. Let it stand until the upper ethereal layer has separated completely and is clear. (Alternatively use low r.p.m. Mojonnier centrifuge). If there is a tendency to form emulsion, a little alcohol may be added to help separation of the layers. Decant off the clear ethereal layer into a suitable vessel (flask, glass bowl, aluminium dish, etc.). Wash the delivery end of the extraction tube with a little ether and add the washings to the flask. Repeat twice extraction of the liquid remaining in the extraction tube using 15 ml of each solvent every time. Add the ethereal extract to the same container and evaporate off completely. Dry the flask in an air oven at 102 ± 2°C for two hours, cool in a desiccator and weigh. Heat the flask again in the oven for 30 min. Cool in a desiccator and weigh. Repeat the process of heating and cooling and weighing until the difference between two successive weights does not exceed 1 mg. Wash out the fat from the flask with petroleum ether carefully leaving any insoluble residue in the flask. Dry the flask in the oven and reweigh. The difference in weights represents the weight of fat extracted from the milk. Correct weight of extracted fat by blank determination on reagents used. If reagent blank is more than 0.5 mg purify or replace reagents. Difference between duplicate determinations obtained simultaneously by the same analyst should not be more than 0.03 g fat /100g product.

1.3.4.2.3. Calculation

$$\text{Fat \% (w/w)} = \frac{\text{Weight of Extracted Fat}}{\text{Weight of milk}} \text{X100}$$

(Ref: - A.O.A.C, 17thedn, 2000 Official method 905.02 Fat in milk).

1.3.4.3. Method 3. Acid Digestion Method (Werner Schmidt Method)

In this method, milk proteins are digested with concentrated hydrochloric acid. Liberated fat is extracted with alcohol, ethyl ether and petroleum ether. Ethers are evaporated and residue left behind is weighed to calculate the fat content.

1.3.4.3.1. Reagents

A. Concentrated hydrochloric acid, Sp. gr. 1.16.

B. Ethyl alcohol, 96% by volume.

C. Diethyl ether, peroxide free.

D. Petroleum ether, boiling range 40-60°C.

1.3.4.3.2. Procedure

Weigh about 10 g of the material in a small beaker. Add 10 ml of concentrated hydrochloric acid and heat on a Bunsen burner stirring continuously with a glass rod until the contents turn dark brown, and then cool to room temperature. Transfer to the Mojonnier fat extraction flask or the Rohrig tube. Add 10 ml of ethyl alcohol first to the beaker and later transfer the contents to the Mojonnier fat extraction flask or the Rohrig tube. Mix well. Then, add 25 ml of ethyl ether to the beaker and from the beaker to the Mojonnier flask or the Rohrig tube. Stopper with cork or a stopper of synthetic rubber resistant to usual fat solvents. Shake vigorously for one minute. Add 25 ml of petroleum ether and repeat vigorous shaking for one minute. Centrifuge Mojonnier flask at about 600 rpm. If Rohrig tube is used, let it stand until upper liquid is practically clear. Decant the ether solution into a suitable flask, metal dish or glass bowl. Wash the tip and the stopper of the extraction flask or tube with a mixture of equal parts of the two solvents and add the washings to the weighing flask or tube and repeat extraction of liquid remaining in the flask or tube successively using 15 ml of each solvent. Add, if necessary, water to bring its level in tube to original mark. This may be necessary in the case of milk powder. Repeat extraction using 15 ml of each solvent. Evaporate the solvent completely on water bath at a temperature that does not cause sputtering or bumping. Dry the fat in oven at 102 ± 2°C to a constant weight. Weigh the cooled flask or

metal dish or glass bowl. Remove the fat completely from the container with warm petroleum ether and weigh as before.

Note: The Werner Schmidt Method (acid extraction procedure) is not suitable, if the milk sample contains added sugar. In such samples Rose - Gottlieb method should be adopted.

1.3.4.3.3. Calculation

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

Where,

W_1 = Weight in g of contents in the flask or metal dish or glass bowl before removal of fat.

W_2 = Weight in g of contents in the flask or metal dish or glass bowl after removal of fat and

W_3 = Weight in g of material taken for the test.

(Ref:- Pearson's Composition and analysis of foods, 9th edn, 1991 page 538).

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2. CREAM, INCLUDING STERILISED CREAM, WHIPPED CREAM AND MALAI

The fat content is the most important statutory requirement in cream. Examine samples for preservative, colouring matter and foreign fat. Whipped cream should be examined for sucrose and stabilizers.

2.1. Preparation of Sample of Cream

The preparation of cream sample depends upon its physical condition. If, at room temperature, the cream is thin to pour easily, mix by repeated inversion of the container. If the cream is too thick, stir gently, taking care that the top and the bottom layers get well mixed. It may not be possible to mix by gentle stirring if the cream is very thick and the fat is partly separated, or if on stirring, the cream becomes thick or fat separates. Under these circumstances, warm the cream sample to temperature between 30 to 40°C in water-bath and, while cooling it to room temperature, shake the container gently or stir the contents at intervals.

Keep the container covered as much as possible to avoid loss of moisture by evaporation. If the cream sample shows any abnormality, it should be recorded. If satisfactory mixing cannot be achieved, the sample should not be tested. In the laboratory, the exposure of cream sample to temperature below freezing point should be avoided. Cream samples should be protected from light, heat, contaminating odours and be kept in a cool place, at a storage temperature of 0 to 5°C.

Note: Sour cream should not be warmed but should be thoroughly stirred.

(Ref:- IS 3509(1969)Method of sampling and test for cream. Bureau of Indian Standards, New Delhi; IS 12333: 1997 / ISO 6731: 1989 – Methods for determination of total solids content in milk, cream, and evaporated milk- reference method. Bureau of Indian Standards, New Delhi).

2.2. Determination of Fat in Cream

2.2.1. Method 1.Rose Gottlieb Method

2.2.1.1. Reagents/Apparatus: See Section 1.3.4.2.1**.**

2.2.1.2. Procedure

Weigh accurately 1-2 g (depending on fat percentage) of cream into a 50 ml

beaker, add 9 ml of 0.5% sodium chloride solution to disperse and transfer to fat extraction apparatus. Add 1 ml of concentrated ammonia and mix well. Extract fat by Rose Gottleib method (Section 1.3.4.2.2).

2.2.2. Method 2. Werner Schmidt Method

2.2.2.1. Reagent: See Section 1.3.4.3.1.

2.2.2.2. Procedure

Weigh accurately 1-2 g (depending on fat percentage) of cream into a 50 ml beaker. Add 10 ml of concentrated HCl to weighed sample in a beaker, heat on a water bath till casein is dissolved. Cool and follow Werner Schmidt method (Section 1.3.4.3.2).

(Ref:- IS 3509- 1966 Method of sampling and test for cream. Bureau of Indian Standards, New Delhi).

2.3. Detection of Presence of Thickeners in Cream

2.3.1. Method 1 for Detection of Starch in Cream

Starch may be detected by addition of Iodine (See Section 1.2.2.).

2.3.2. Method 2 for Detection of Gelatine in Cream

Gelatine may be detected by Stokes Test. Mix together 10 ml cream, 20 ml water and 20 ml of Stokes reagent (Dissolve mercury in twice its weight of concentrated nitric acid and dilute to 25 times the volume with water). To the filtrate add an equal volume of saturated picric acid solution. Yellow precipitate is produced in presence of considerable amount of gelatine, smaller amounts are indicated by cloudiness.

(Ref:-A.O.A.C. Official method 920.106 Gelatine in milk and milk Products; Pearson's Composition and Analysis of foods, 9th edn,1991 page 559).

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3. CREAM POWDER

3.1. Preparation of Sample of Cream Powder

Make homogeneous either by mixing or shaking or alternately rolling and inverting container. Avoid excessive temperature and humidity when opening sample container to prevent absorption of moisture

3.2. Determination of Moisture in Cream Powder

The sample is dried to constant weight at 102°C and the loss in weight reported as moisture. The method described here has been followed from IS 11623: 1986 (Reaffirmed 1997).

3.2.1. Requirements

A. Flat-bottom moisture dishes with cover: Of stainless steel, nickel or aluminium having approximately 50 mm diameter and 25 mm depth. The dishes shall have lids which fit well and can readily be removed.

B. Hot air oven: Maintained at 102 ± 2°C.

C. Desiccator: Containing an efficient desiccant.

3.2.2. Procedure

A. Uncover a dish and place the dish and its lid in a hot air oven at 102 ± 2°C for 1 h. Place the lid on the dish, transfer the covered dish from the hot air oven to the desiccator. Allow it to cool to room temperature and weigh it.

B. Put approximately 1 g of the dried milk sample in the dish, cover the dish with the lid and weigh the covered dish accurately and quickly.

C. Uncover the dish and put it with its lid in the hot air oven maintained at 102 ± 2°C for 2 h.

D. Replace the lid, transfer the covered dish to the desiccator, allow it to cool to room temperature (for approximately 30 - 45 min) and weigh it accurately and quickly.

E. Heat the uncovered dish and lid in the hot air oven at 102 ± 2°C for further 1 h, replace the lid, allow the covered dish to cool to room temperature in the desiccator and weigh it. Repeat the process of drying, cooling and weighing, until

the successive weighing do not differ by more than 0.5 mg. It is usually found that drying is complete after the first 2 h.

3.2.3. Calculation

$$\text{Moisture \% by mass} = \frac{100\,(M_1 - M_2)}{M_1 - M}$$

Where

M = mass in g, of the empty dish;

M_1 = initial mass in g, of the dish and lid with the material taken for analysis;

M_2 = final mass in g, of the dish and lid with the material after drying.

The maximum deviation between duplicate determinations should not exceed 0.06% by mass of moisture.

(Ref:- IS 11623 (Reaffirmed 1997), Method for determination of moisture content in milk powder and similar products, Bureau of Indian Standards: India; ISO 5537|IDF 026:2004 - Dried milk - Determination of moisture content (Reference method), International Dairy Federation, Brussels).

3.3. Determination of Fat Content in Cream Powder

3.3.1. Requirement: See Section 1.3.4.2.1. (Rose-Gottlieb Method).

3.3.2. Procedure

Quickly weigh to the nearest mg about 1 g well mixed sample into a small beaker. Add 1 ml water and rub to smooth paste. Add 9 ml additional water and 1 – 1.25 ml NH_4OH and warm on steam bath. Transfer to fat extraction flask or tube. Cool and extract fat by Rose Gottlieb method after adding 10 ml of alcohol (See Section 1.3.4.2.2.- Rose-Gottlieb Method).

(Ref:- A.O.A.C 17thedn, 2000 Official method 932.06 Fat in Dried Milk).

3.4. Determination of Milk Protein in Milk Solids not Fat of Cream Powder

3.4.1. Method 1. As per AOAC Method

Total nitrogen and non-protein nitrogen content of test sample is determined separately. Subtract non protein nitrogen content from total nitrogen content, in the sample and multiply by 6.38 to get milk protein nitrogen content.

3.4.1.1. Determination of Total Nitrogen in Sample

3.4.1.1.1. Reagents: See Kjeldahl Method, Section 19.

3.4.1.1.2. Procedure

Weigh accurately about 5 g of sample and transfer to the Kjeldahl flask. Digest with sulphuric acid by using copper sulphate as catalyst and potassium sulphate as boiling point elevator to release nitrogen from protein and retain nitrogen as ammonium salt. Concentrated NaOH is added to release ammonia which is absorbed in HCl and back titrated. Multiply percent nitrogen by 6.38 to calculate total protein nitrogen.

3.4.1.2. Determination of Non Protein Nitrogen in Sample

For determining non protein nitrogen, protein is precipitated by addition of trichloroacetic acid (TCA) solution. Final concentration of TCA in the mixture is about 12 %. Precipitated milk protein is removed by filtration. Filtrate contains non protein nitrogen components of milk. Nitrogen content of filtrate is determined by Kjeldahl method.

3.4.1.2.1. Reagents

A. Trichloroacetic acid (CCl_3COOH)solution (15%; w/v): TCA is soft deliquescent crystal which should be stored in container protected from light and moisture.

B. Hydrochloric acid standard solution: 0. 01 M HCl.

3.4.1.2.2. Procedure

Reconstitute sample (about 5-10 g) with warm water at 40°C and make up to 100 ml. Pipette 10 ml into a preweighed 125 ml Erlenmeyer flask and weigh. Add 40±0.5 ml TCA in the flask. Weigh flask and contents. Swirl to mix. Let precipitate settle for 5 minutes. Filter through Whatman No1 paper or equivalent, 15 cm, Nitrogen free, and collect entire filtrate. Filtrate should be clear and free from particulate matter. If it

is not, repeat entire sample preparation. Swirl filtrate to mix. Pipette 20 ±0.2 ml into a 50 ml beaker, and weigh. Pour filtrate from beaker into Kjeldahl digestion flask that contains boiling chips, potassium sulphate and copper sulphate. Immediately reweigh empty beaker. Add H_2SO_4, and digest. Prepare a blank (16 ml of 15 % TCA and no test sample) and keep record of blank value.

Calculate percent nitrogen Protein equivalent % = Nitrogen % x 6.38 which is non-protein nitrogen expressed as protein equivalent.

3.4.1.2.3. Calculation

Subtract total non-protein nitrogen expressed as protein equivalent from total protein to determine milk protein in Milk solids not Fat.

(Ref:- A.O.A.C. 17thedn, 2000 Official Method 991.23 Protein Nitrogen content of Milk read with 991.20 Nitrogen in Milk- Kjeldahl method and 991.21Non Protein Nitrogen in Whole Milk).

3.4.2. Method 2. Determination of Protein Content Using IDF Procedure

Take 1 g of sample and refer to the method mentioned in Section 19.

A. Follow method mentioned in Section 19.1 for **Determination of Total Nitrogen/Crude Protein in Milk.**

B. Follow method mentioned in Section 19.2 for **Determination of Non-Protein Nitrogen (NPN) in Milk**

C. Determine the true protein nitrogen content by subtracting B from A.

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4. CURD OR DAHI

4.1. Preparation of Sample of Dahi

To get a representative sample particularly in case of a thick-set product it may be necessary to pour out the whole of the contents in a mortar and mix it thoroughly until the mass is homogeneous. Aliquots are weighed out from this well mixed sample.

4.2. Determination of Fat in Dahi

Refer to determination of fat in milk by Rose-Gottlieb method (See Section 1.3.4.2).

4.3. Test for the Presence of Starch in Dahi

Refer to method for the presence of starch in milk (See Section 1.2.2.)

4.4. Determination of Total Solids in Dahi

In cultured dairy products such as dahi or yoghurt, total solids are determined after neutralization of developed acidity with alkali.

4.4.1. Method 1.Sodium Hydroxide Method

In this procedure (IS 12333: 1997), NaOH is used for neutralization of developed acidity followed by determination of total solids by evaporation at 100 ± 2°C.

4.4.1.1. Reagents

A. Sodium hydroxide

B. Phenolphthalein Indicator Solution: Dissolve 1g of phenolphthalein in 100 ml of ethanol (95%, v/v). Add 0.1N NaOH solutions until one drop gives a faint pink colouration. Dilute with distilled water to 200 ml.

4.4.1.2. Apparatus

Shallow flat-bottom dishes of aluminium, nickel, stainless steel, porcelain or silica, 7-8 cm diameter, about 1.5 cm in height and provided with easily removable but closely fitting lids.

4.4.1.3. Procedure

Heat the clean dry empty dish and lid in oven maintained at 100 ± 2°C for one

hour, cool in a desiccator and weigh. Quickly weigh to the nearest 0.1 mg 4-5 g of prepared sample of curd or dahi, replace the lid and weigh again. Add 1-2 drops of phenolphthalein solution to the sample in the dish and neutralise with 0.1 N sodium hydroxide solution to a faint pink colour. Note the volume of 0.1N sodium hydroxide required to neutralise the sample. Place the dish without lid on a boiling water bath until the water is removed from the sample. Wipe the under-surface of the dish and place in the oven maintained at 100 ± 2°C, for 3 hrs. Remove the dish along with the lid and cool in a desiccator and weigh. Continue heating and re-weighing at hourly intervals until successive weighing do not vary by more than 0.5 mg. Deduct half weight of the 0.1N sodium hydroxide added to neutralize the sample from the residue after drying and calculate total solids as for whole milk.

4.4.1.4. Calculation a = $\frac{\text{N x T.V x 40}}{\text{1000 x 2}}$

Total solids percent w/w = $\frac{100\ (W_2 - \text{a})}{W_1}$

Where: N = Normality of NaOH

T. V = Titre value

W_2 = Weight in g of residue left after drying

W_1= Weight in g of the prepared sample taken

a = Half of the volume of 0.1 N Sodium hydroxide added

(Ref:-Modified IS 12333 – 1997 / I.S.O 6731-1989 Milk, Cream and Evaporated Milk - Determination of Total Solid Content – Reference method. Bureau of Indian Standards, New Delhi; Pearson's Composition and Analysis of Foods, 9thedn. Page 570).

4.4.2. Method 2.Zinc oxide Method

In the following procedure (IDF 151: 1991), the water is evaporated from a test portion of the sample in the presence of zinc oxide at a temperature of 100 ± 2°C in drying oven. Lactic acid (determined by titration method) content is determined separately in the sample and is added to the moisture determined in the drying step to compensate for the loss of water as a result of neutralizing the sample by means of zinc oxide.

4.4.2.1. Reagent: Zinc oxide

4.4.2.2. Apparatus

A. Analytical balance

B. Desiccator: provided with efficient drying agent.

C. Drying oven: ventilated, maintained thermostatically at 100 ± 2°C.

D. Flat bottom dishes: of height 20 to 25 mm, diameter 50 to 75 mm, and made of appropriate material (for example stainless steel, nickel or aluminium), provided with well-fitting, readily removable lids.

E. Boiling water-bath.

F. Spoon or spatula.

G. Homogenizer for homogenizing fruit yogurts.

4.4.2.3. Procedure

A. Bring the sample to temperature of 20-25°C. Mix the sample carefully by means of spatula using a rotatory motion which passes from the lower layers to the surface layer of the sample so as to displace and mix them well

B. Heat an open dish containing approximately 2 g of zinc oxide, together with lid and a stirring rod for 1 h in the oven maintained at 100 ± 2°C.

C. Transfer the dish along with lid and stirring rod to the desiccator, allow it to cool to room temperature (at least 45 min) and weigh the dish with the stirring rod and lid to the nearest 0.1mg.

D. Move the zinc oxide to one side of the prepared dish by tilting. Place on the clear space approximately 1.0 g of prepared test sample, replace the lid on the dish with the stirring rod on top and weigh the dish to the nearest 0.1 mg.

E. Add 5 ml of water to the test portion and thoroughly mix the diluted test portion and zinc oxide. Spread the mixture evenly over the bottom surface of the dish. Heat the dish on the boiling water bath and continue heating for approximately 30 min with frequent mixing of the contents of the dish during the early stages of the drying so as to obtain maximum evaporation of liquid.

F. Remove the dish from the water bath and wipe its base to remove any water. Leave the stirring rod in the dish and then place it together with lid by its side, in the drying oven for 3 h.

G. After drying, cover the dish with its lid and immediately transfer to the desiccator. Allow the dish and contents to cool in the desiccator to room temperature (at least 45 min) and weigh to the nearest 0.1 mg.

H. Again heat the dish and contents together with its lid for a further 1h, cover the dish and transfer to the desiccator. Allow to cool and weigh to the nearest 0.1 mg. repeat the process of reheating and weighing until the difference in mass between two successive weighing does not exceed 1 mg. Record the lowest weight.

I. In order to compensate for the loss of water as a result of neutralizing the yoghurt by means of zinc oxide, determine the titratable acidity (expressed as g of lactic acid per 100 g of product) of the sample.

4.4.2.4. Calculation:

The total solids contents, expressed as percentage by mass, is equal to:

$$= [(\frac{M_2 - M_0}{M_1 - M_0})\ x\ 100] + 0.1\ a$$

Where,

M_0= is the mass, in g of the dish (including zinc oxide), lid and stirring rod.

M_1 = is the mass, in g, of the dish (including zinc oxide), lid, stirring rod and test portion.

M_2 = is the mass, in g, of the dish, lid stirring rod and dried test portion (including zinc oxide)

a = is the mass, in g, of lactic acid as obtained in Step I. it is equal to 0.1 g per g of lactic acid content.

(Ref:- IDF (2005) IDF Standard 151, Yoghurt. Determination of total solid content, International Dairy Federation: Brussels).

4.5. Determination of Total Milk Solids in Dahi Sweetened with Cane Sugar

First determine the total solids as described in Section 4.4and then estimate the cane sugar separately by the method given for determination of cane sugar (Section 9.4.Determination of Sucrose Content in Condensed/Evaporated Milk). Deduct the amount of sugar from the total solids.

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5. CHHANNA OR PANEER

5.1. Preparation of Sample of Channa/Paneer

Pass channa sample through 8 mesh sieve three times or grate sample and mix thoroughly or grind to a uniform mass in a glass pestle and mortar. Grate the paneer sample quickly through a suitable grater. Mix the grated sample thoroughly. Transfer the grated sample to an air-tight container to await analysis, which should be carried as soon as possible after grinding. Keep sample in an airtight container until the time of analysis. If delay is unavoidable, take all precautions to ensure proper preservation of the sample, and to prevent condensation of moisture on the inside surface of the container. The storage temperature should be below 10°C.

(Ref:- IS 12758 - 1989 / I.S.O 1735-1987 Cheese and Processed Cheese products - Determination of fat content by gravimetric method - reference method. Bureau of Indian Standards, New Delhi).

5.2. Determination of Moisture in Channa/Paneer

The moisture content of channa is the loss in mass, expressed as a percentage by mass when the product is heated in an air oven at 102 ± 2°C to constant mass (IS:2785:1979; Reaffirmed 1995).

5.2.1. Apparatus

A. Flat-bottom dishes with lid: Dishes of nickel, aluminium or of other suitable metal not affected by boiling water, 70 to 80 mm in diameter and not more than 25 mm deep, provided with short glass stirring rod having a widened flat end. The dishes shall have lids which fit well and can readily be removed.

B. Hot air oven: Maintained at 102 ± 1°C.

C. Desiccator: Containing an efficient desiccant.

D. Sand: Which passes through 500 µ sieve and is retained by 180 µ sieve. It shall be prepared by digestion with concentrated HCl, followed by thorough washing with water. It shall then be dried and ignited till it is dull red.

5.2.2. Procedure

A. Heat the flat-bottomed metal dish containing 20 g of prepared sand and a stirring rod, in hot air oven for about 1 h. Allow to cool in an efficient desiccator for 30 to 40 min. Weigh accurately 3 g of the prepared sample of channa into a flat-bottomed dish (with a cover) previously dried and weighed containing about 20 g of prepared sand and a stirring rod.

B. Saturate the sand by careful addition of a few drops of distilled water, and thoroughly mix the wet sand with the channa sample by stirring with the glass rod, smoothing out lumps and spreading the mixture over the bottom of the dish.

C. Place the dish on a boiling water-bath for 20 to 30 min, then wipe the bottom of the dish. Transfer the dish containing the material, along with glass rod after uncovering in an oven maintained at 102 ± 1°C for about 4 h.

D. After 4 h replace the lid, transfer the covered dish to the desiccator, allow it to cool to room temperature and weigh it accurately and quickly to the nearest 0.1 mg.

E. Heat the uncovered dish and lid in the oven at 102 ± 1°C for further 1 h, replace the lid, allow the covered dish to cool to room temperature in the desiccator and weigh it. Repeat the process of drying, cooling and weighing, until the successive weighing do not differ by more than 0.5 mg. Record the weight.

5.2.3. Calculation

$$Moisture\ \%\ by\ mass = \frac{M_1 - M_2}{M_1 - M}\ x\ 100$$

Where,

M = mass in g, of the empty dish with containing glass rod;

M_1 = Initial mass in g of the dish, lid, glass rod along with the material taken for analysis;

M_2 = the final mass in g of the dish, lid, glass rod along with the material after drying.

Express the results to the nearest 0.01% (m/m).

(Ref:-IS 2785 -1979 (Reaffirmed 1995). Specification for Natural cheese (Hard Variety), Processed Cheese, Processed Cheese Spread and Soft Cheese. Bureau of Indian Standards, New Delhi; IS 10484 -1983 (Reaffirmed 1999). Specification for Paneer. Bureau of Indian Standards, New Delhi).

5.3. Determination of Fat (by Acid Digestion Method) in Channa/Paneer

Weigh accurately 1-2 g of prepared sample in a 100 ml beaker. Add 10 ml of conc. hydrochloric acid. Heat on a Bunsen burner, stirring continuously with a glass rod, or on a boiling water bath until all solid particles are dissolved. Cool to room temperature. Add 10 ml of ethyl alcohol first to the beaker and later transfer the contents to the Mojonnier fat extraction flask or the Rohrig tube Transfer to the Mojonnier fat extraction flask. Proceed as in determination of milk fat by acid digestion (1.3.4.3).

(Ref:- IS 2785 -1979 (Reaffirmed 1995). Specification for Natural cheese (Hard Variety), Processed Cheese, Processed Cheese Spread and Soft Cheese. Bureau of Indian Standards, New Delhi).

5.4. Detection of Starch in Channa

Weight 1 g channa sample in a porcelain dish and macerate the sample with 1 ml of distilled water. Then proceed as in Section 1.2.2. for Detection of Starch in Milk.

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6. CHEESE (ALL VARIETIES)

6.1. Preparation of Sample of Cheese

Prior to analysis, remove the rind or smear or mouldy surface layer of the cheese, in such a way as to provide a sample representative of the cheese as it is usually consumed. Grind or grate the sample by means of an appropriate device; mix the ground or grated mass quickly, and if possible grind or grate a second time, and again mix thoroughly. If the sample cannot be ground or grated, mix it thoroughly by intensive stirring and kneading. Transfer the test sample to an air-tight container to await analysis, which should be carried as soon as possible after grinding. If delay is unavoidable, take all precautions to ensure proper preservation of the sample, and to prevent condensation of moisture on the inside surface of the container. The storage should be at 10 to 12°C.

(Ref:- IS 12758 -1989 / I.S.O 1735 1987 Cheese and Processed cheese products- Determination of Fat content by gravimetric method – reference method. Bureau of Indian Standards, New Delhi).

6.2. Determination of Moisture in Cheese

Refer to determination of moisture in Channa or Paneer. See Section 5.2.

6.3. Determination of Milk Fat (by Acid Digestion Method) in Cheese

Refer to determination of fat in Channa or Paneer by acid digestion method. See Section 5.3.

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7. ICE CREAM, SOFTY ICE CREAM, CHOCOLATE ICE CREAM, KULFI, MILK ICES OR MILK LOLLIES AND FROZEN DESSERT / CONFECTION

7.1. Preparation of Sample of Ice Cream

For samples taken in small packages, remove the packaging and place the sample in a clean, dry container fitted with an airtight closure. For samples taken from bulk or from large packages, keep them in their sampling containers.

7.1.1 Plain Product

Allow the sample to soften at room temperature. It is not advisable to soften the ice-cream sample by heating on water bath or over flame because melted fat tends to separate and rises to the surface. After softening of ice-cream, mix it thoroughly by stirring with spoon or eggbeater or by pouring back and forth between beakers.

7.1.2. Fruit nut and Chocolate Ice-Cream containing Insoluble Particles

Use a mixer capable of comminuting product to fine, uniform pulp. Use 100 - 200 g of ice-cream sample to fill the cup of mixer full to about one-third. Melt the product at room temperature or in an incubator at 37°C in a closed container. Transfer entire contents to the mixer cup and mix until insoluble particles are finely divided (about 3-5 min for fruit ice-cream and up to 7 min for nut ice-cream). Alternatively, the product may be grounded in porcelain or glass pestle and mortar.

Transfer the mixed ice-cream sample to a suitable container for convenience in weighing. After weighing operation, return the remainder of the ice-cream sample to the refrigerator, preferably at a temperature not exceeding -15°C.

In case of ice cream, where the chocolate or similar covering portions forms a separate layer, it shall be removed and only the ice cream portion shall be taken for analysis.

Note: For determination of over-run in ice-cream, the entire ice-cream carton/cup should be taken as such.

(Ref:- IS 2802 – 1964 Specification for Ice cream. Bureau of Indian Standards, New Delhi / A.O.A.C 17edn,2000, Official method 969.20. Ice cream and Frozen Desserts).

7.2. Determination of Total Solids in the Ice Cream

7.2.1. Apparatus

A. Flat bottomed dishes of Aluminium, Nickel or Stainless steel not affected by boiling water, 7-8 cm in diameter and not more than 2.5 cm deep provided with short stirring rods having a widened flat end.

B. Sand which passes through 500 micron I.S sieve and is retained on 180 micron sieve. It shall be prepared by digesting with conc. HCl followed by thorough washing with water. It shall be dried and ignited to red heat.

C. Oven: Well ventilated and maintained at 100 ± 2°C.

7.2.2. Procedure

Heat the moisture dish containing 20 g of prepared sand and glass stirring rod in the oven for 1 hour. Cool and weigh. Add about 5 g of sample into the dish. Add a few drops of water to assist in spreading the sample with glass rod. Place dish on a boiling water bath for 29 - 30 minutes. Wipe bottom of dish and transfer to the air oven. Dry for about 4 hours, remove dish to an efficient desiccator, allow to cool and weigh. Replace dish in oven for a further period of 1 hour, transfer to desiccator, allow it to cool and weigh. Repeat the process of heating and cooling till consecutive weighing agree within 0.5 mg. Calculate total solids from loss in weight observed.

(Ref:- IS 2802 – 1964 – Specification for Ice cream. Bureau of Indian Standards, New Delhi.; Pearson's Composition and analysis of foods,9th edn,1991 page 604).

7.3. Determination of Weight per Unit Volume or Over-run in Ice Cream

Over-run is usually defined as the volume of ice-cream obtained in excess of the volume of the mix. It is usually expressed as a percentage. This increased volume is composed mainly of the air incorporated during the freezing process. The amount of air which is incorporated depends upon the composition of mix and the way it is processed. In this test, the volume of water and alcohol used corresponds with the volume of air originally contained in the ice-cream and the difference between the sum of these two and capacity of the flask is equivalent to the volume occupied by the sample.

7.3.1. Apparatus

A. Beaker: 400 ml.

B. Volumetric flask: 250 ml.

C. Glass funnel.

7.3.2. Reagent: *n*-Amyl alcohol (sp. gr. 0.817).

7.3.3. Procedure

A. Weigh a unit of ice-cream and from it calculate the weight of ice-cream per litre. For example, 200 ml of a full carton of ice-cream can be obtained, the ice-cream carefully removed and the empty dry carton weighed. The difference in weights between the carton when filled and when empty is, therefore, the weight of 200 ml of frozen ice-cream. Five times this weight would then equal the weight of a litre. To determine the weight of the mix, proceed as below (B):

B. Weigh and record the exact weight of a clean, dry 400 ml beaker. Into the beaker, weigh exactly 130 g of the frozen ice-cream.

C. Place the beaker in water bath warmed to 49°C and melt.

D. Weigh and record the exact weight of a 250-ml volumetric flask.

E. Using a glass funnel, transfer 130 g of melted ice-cream into the 250 ml volumetric flask.

F. Add exactly 10 g of *n*-amyl alcohol to the flask and mix to break the surface tension of the melted ice-cream and release the incorporated air. 10 g of n-amyl alcohol occupies a volume of 12.24 ml.

G. Cool the flask with contents to 15.5°C using a cold water or ice water bath.

H. Rinse the beaker containing melted mix with several small rinsing of water, adding each rinse to the 250 ml flask.

I. Again cool the flask with contents to 15.5°C and using the final rinse water, bring the volume to 250 ml mark. The bottom of the meniscus should correspond with the mark when temperature is exactly 15.5°C. Dry the outside of the flask and reweigh.

J. Calculate the weight in g of the contents. Calculate the weight in g of the water added

to the flask. Calculate the volume in ml occupied by the sample of ice-cream. Determine the sp. gr. of the mix by dividing its weight (130 g) by the volume in ml, which it occupied. Determine the weight in g per litre of mix by multiplying by the specific gravity.

(Ref:-IS:2802: 1964 (Reaffirmed 1995) Specification for ice-cream. Bureau of Indian Standards, New Delhi).

7.4. Determination of Fat in Ice-Cream (Rose-Gottlieb method)

Accurately weigh 4-5 g of the thoroughly mixed sample directly into fat extraction flask or Mojonnier tube, using free flowing pipette, dilute with water to approximately 10 ml, working sample into lower chamber and mix by shaking. Add 2 ml ammonia; mix thoroughly, heat in water bath for 20 min at 60°C with occasional shaking, cool and proceed as in Section 1.3.4.2.2 (beginning "Add 10 ml alcohol and mix well"). Identify the clear extracted fat to confirm whether it is dairy fat or not by checking refractive index at 40°C and GLC composition as per clause 1.2.11.

(Ref :- Pearson's Composition and analysis of foods 9th edn,1991 page 604).

7.5. Determination of Protein (Kjeldahl method) in Ice Cream

7.5.1. Method 1. As per DGHS Manual (2005)

The protein content is determined from the organic Nitrogen content by Kjeldahl method. The various nitrogenous compounds are converted into ammonium sulphate by boiling with concentrated sulphuric acid. The ammonium sulphate formed is decomposed with an alkali (NaOH) and the ammonia liberated is absorbed in excess of standard solution of acid and then back titrated with standard alkali.

7.5.1.1. Apparatus

A. Kjeldahl digestion flask - 500 or 800 ml

B. Kjeldahl distillation apparatus, -same digestion flask fitted with rubber stopper through which passes lower end of efficient rubber bulb or trap to prevent mechanical carry-over of NaOH during distillation.

C. Conical flask, 250 ml

D. Burette 50 ml.

7.5.1.2. Reagents

A. Concentrated Sulphuric acid – sp.gr. 1.84

B. Sodium Hydroxide solution (45%): Dissolve 450 g of Sodium Hydroxide in 1000 ml water

C. Standard Sulphuric acid solution (0.1 N)

D. Standard Sodium Hydroxide solution (0.1 N)

E. Methyl Red Indicator solution: Dissolve 0.5 g methyl red in 100 ml of alcohol

7.5.1.3. Procedure

Weigh quickly about 5-8 g of the prepared ice-cream sample and transfer to a 500 or 800 ml Kjeldahl flask taking care to see that no portion of the sample clings to the neck of the flask. Add 0.5g of copper sulphate, 15 g of potassium sulphate and 40 ml of concentrated sulphuric acid. Add two to three glass beads. Place the flask in an inclined position on the stand in the digestion chamber and digest. Heat the flask gently at low flame until the initial frothing ceases and the mixture boils steadily at a moderate rate. During heating rotate the flask several times. Continue heating for about an hour or more until the colour of the digest is pale blue. Cool the digest and add slowly 200 ml of water. Cool, add a piece of granulated zinc or anti bump granules and carefully pour down the side of the flask sufficient sodium hydroxide solution (450g/ litre) to make the contents strongly alkaline(about 110 ml) before mixing the acid and alkaline layer. Connect the flask to a distillation apparatus incorporating an efficient flash head and condenser. To the condenser fit a delivery tube which dips just below the surface of the pipetted volume of standard acid contained in a conical flask receiver. Mix the contents of the digestion flask and boil until 150 ml have distilled into the receiver. Add 5 drops of methyl red indicator and titrate with 0.1 N sodium hydroxide solution. Carry out a blank titration.

1 ml of 0.1 N H_2SO_4 = 0.0014g N.

In case of dairy ice cream / kulfi calculate milk protein as N x 6.38 In case of Frozen Dessert calculate total protein as N x 6.25

(Ref : Pearson's Composition and Analysis of Foods,9th edn,1991 page 17).

7.5.2. Method 2. Determination of Protein Content using IDF Procedure

Total nitrogen content in ice cream sample is estimated by Kjeldahl method as described for milk. The percent nitrogen obtained is multiplied by a factor to get protein content in ice cream sample.

Take 6 g of sample and refer to the method mentioned in Section 19.

A. Follow method mentioned in Section 19.1 for **Determination of Total Nitrogen/Crude Protein in Milk.**

Calculate Crude Protein Content = N x 6.38

N = Nitrogen content in sample estimated by Kjeldahl method

7.6. Determination of Added Starch in Ice Cream

The sample is made free from fat and the starch is precipitated with alcohol and made free from sugar. The precipitated starch is subjected to acid hydrolysis. The hydrolysate is freed from proteins using lead acetate and deleaded with ammonium oxalate. The reducing sugar is determined by Lane and Eynon method and multiplied with 0.9 to calculate the starch content.

7.6.1. Procedure

Weigh 20 - 50 g of ice cream sample into a 500 ml beaker depending on the approximate starch content of the sample. Defat the sample with 5-6 washings with 15-20 ml portions of petroleum ether (40-60°C). Add enough water to make 100 ml, heat to 50-60°C (avoiding any gelatinisation of starch) and let stand for 1 hour stirring frequently to ensure complete solution of sugars. Cool and add equal volume of alcohol, mix, let stand for 1 hour or more.

Centrifuge for 20 minutes at approximately 4000 rpm so that the precipitate is closely packed at the bottom of the centrifuge tubes. Filter the solution using Whatman filter paper No 1. Transfer the precipitate using 5% alcohol on the filter paper. Wash the precipitate on the filter paper with successive 50 ml portions of 50% alcohol and filter until the washings are sugar free.

Transfer the residue to a 500 ml conical flask with about 200 ml water and add 20 ml of conc. HCl of sp. gr. 1.125. Hydrolyse starch by refluxing in a boiling water bath for 2- 2 ½ hours. Cool, transfer the hydrolysate to a 250 ml volumetric flask, neutralise with Sod hydroxide and make it alkaline using litmus paper. Make up to volume. Shake thoroughly. Allow to settle for 20 minutes and filter. Determine reducing sugars by Lane and Eynon method and calculate starch content by multiplying total reducing sugars with 0.9.

(Ref :- A.O.A.C 17th edn,2000 Modified Official method- 925.50 for starch in confectionary).

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8. DRIED ICE CREAM MIX / DRIED FROZEN DESSERT / CONFECTION

8.1. Preparation of Sample of Dried Ice Cream Mix

Make homogeneous either by mixing or shaking or alternately rolling and inverting container. Avoid excessive temperature and humidity when opening sample container to prevent absorption of moisture.

8.2. Determination of Moisture Content in Dried Ice Cream Mix

Follow method described in Section 3.2.

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9. CONDENSED / EVAPORATED (SWEETENED, UNSWEETENED AND SKIMMED) MILK

9.1. Preparation of Sample of Condensed/Evaporated Milk

Shake the container thoroughly with frequent inversion. Open the container and pour the milk slowly into another container made of glass or other suitable material provided with an air tight lid, taking care to incorporate in the sample any fat or other constituents adhering to the sides of the original container. Stir vigorously and close the container. Alternatively heat the closed container in a water bath at 40°C –60°C. Remove and shake the container vigorously every 15 minutes. After 2 hrs, remove the container and cool to 20°C– 25°C. Remove the lid and mix thoroughly by stirring the milk with a spoon or spatula.

Note: If the fat separates, correct results cannot be obtained.

(Ref:- IS 12333 : 1997 / ISO 6731: 1989 – Methods for determination of total solids content in milk , cream, and evaporated milk- reference method).

9.2. Determination of Total Solids in Condensed/Evaporated Milk

9.2.1. Apparatus

A. Flat-bottomed dishes: Made of aluminum alloy, nickel, stainless steel, porcelain, 50 to 75 mm diameter, 20 to 25 mm depth, and provided with easily removable but closely fitting lids.

B. Boiling water-bath.

C. Water-bath: Maintained at 40 – 60°C. (Required for the preparation of sample, see Section 9.1)

D. Hot air oven: Maintained at 102 ± 2°C, well ventilated.

E. Desiccator with efficient desiccant (for example freshly dried silica gel with a hygroscopic indicator).

F. Short glass stirring rods: Flattened at one end and fitting in to the dish.

G. Quartz sand or sea sand: Which passes through 500 micron IS sieve and is retained on 180 micron IS sieve [IS: 460 (Part I) – 1978], and which passes the suitability test size as follows.

   a. Place approximately 20 g of sand in a dish containing a stirring rod. Heat the open dish and sand, stirring rod and lid in a hot air oven controlled at 102 ± 2°C, for at least 2 h or preferably overnight. Fit the lid, allow the dish to cool in a desiccator to room temperature and weigh to the nearest 0.1 mg.

   b. Moisten the sand with approximately 5 ml of water, mix the sand and water using the stirring rod and heat the dish and sand, stirring rod and lid in a hot air oven, controlled at 102 ± 2°C for at least 4 h. Fit the lid, allow the dish to cool in the desiccator to room temperature and weigh again to the nearest 0.1 mg. The difference between the two weighing shall not exceed 0.5 mg.

   c. If the requirements given above is not met, the sand may be made suitable for the determination as follows.

   d. Leave the sand immersed in 25% (m/m) hydrochloric acid solution for 3 days. Stir occasionally, decant off the supernatant liquid as far as possible. Then wash the sand with water until the acid reaction has reaction has disappeared. Calcine (heat) the sand at 550°C for at least 4 h using a muffle furnace. Repeat the test for the suitability of the sand as described above.

9.2.2. Procedure

A. Heat a dish containing about 20 g of the sand with its lid alongside and a stirring rod on top of the lid, in a hot air oven at 102° ± 2°C for about 1 h.

B. Place the lid (with the stirring rod on the top) on the dish, immediately transfer the dish to the desiccator, allow to cool for at least 45 min, and weigh the dish with lid and rod to the nearest 0.1 mg. Tilt the sand to one side of the prepared dish, place on the clear space 2.0 g of the prepared test sample of condensed milk, replace the lid with the stirring rod on top and weigh the dish to the nearest 0.1 mg.

C. Add 5 ml of distilled water to the test portion in the dish and mix with the stirring rod. Thoroughly mix together the diluted test portion and the sand, and spread the mixture evenly over the bottom of the dish. Leave the stirring end of the rod in the mixture with the other end resting on the rim of the dish.

D. Heat the dish on a boiling water-bath, with as much as possible of the bottom of the dish exposed to steam, for approximately 30 min stirring the mixture frequently in the early stages of drying so that the mixture is well aerated and becomes crumbly. Lay the stirring rod flat inside the dish, dry the bottom of the dish and heat the dish, with its lid alongside, in a hot air over maintained at 102 ± 2°C for 4 h.

E. Place the lid on the dish, allow the dish to cool in the desiccator and weigh to the nearest 0.1 mg. Repeat the above operations described above (heating the dish for 1 h) until the difference in mass between two successive weighing does not exceed 0.5 mg. Record the lowest mass.

9.2.3. Calculation

$$\text{Total solids \% by mass} = \frac{(M_2 - M)}{M_1 - M} \; x \; 100$$

Where

M = mass in g, of the dish, lid and stirring rod;

M_1 = mass in g, of the dish, lid, stirring rod and test portion; and

M_2 = in g, of the dish, lid, stirring rod and dried test portion.

Note: BIS/ISO method does not recommend use of sand. However, use of sand help in even spreading of samples.

(*Ref:- IS 12333 – 1997 / ISO 6731 -1989 / Milk, Cream and Evaporated milk – Determination of Total solids content – reference method. Bureau of Indian Standards, New Delhi).*

9.3. Determination of Fat by Rose-Gottlieb's Method in Condensed/Evaporated Milk

Weigh accurately 2-2.5 g of the well mixed sample into a beaker. Add 1 ml of

water and make into a smooth paste by using a glass rod. Add 9.0 ml of additional water and 1.25 ml of ammonium hydroxide and warm on a steam bath. Transfer to Mojonnier flask. Cool and proceed as for the determination of fat by Rose Gottleib method in the whole milk (Section 1.3.4.2).

9.4. Determination of Sucrose Content in Condensed/Evaporated Milk

Sucrose content can be determined by any of the following methods: Volumetric method (Lane - Eynon) and Polarimetric method.

9.4.1. Lane-Eynon (Volumetric) Method

The principle involves clarifying the sample with the help of zinc acetate and potassium ferrocyanide, inverting a part of the sample using hydrochloric acid and determining the reducing sugars content in both inverted and non-inverted parts of the sample. The sugar content is estimated by determining the volume of the unknown sugar solution required to completely reduce a measured volume of Fehling's solution. Invert sugar reduces the copper in Fehling's solution to red insoluble cuprous oxide.

9.4.1.1. Reagents

A. Sodium hydroxide solution: Approximately 0.1N prepared from sodium hydroxide, analytical reagent grade.

B. Stock solution of invert sugar: Weigh accurately 9.5 g of pure sucrose on a watch glass and transfer it to a one litre volumetric flask with 100 ml of water. Add 5ml of concentrated hydrochloric acid. Allow this to stand for 3 days at 20 to 25°C and then make up to volume with water (This solution is stable for several months).

C. Standard solution of invert sugar: Neutralise a known aliquot of the stock solution of invert sugar (Reagent B) with sodium hydroxide solution using litmus paper and dilute with water to a known volume, so that more than 15 ml but less than 50 ml of it shall be required to reduce all the copper in the Fehling's solution taken for titration. Note the concentration of invert sugar in this solution as mg per 100 ml. Prepare this solution fresh every day.

Note: When 10 ml of Fehling's solution are taken for titration, a standard invert sugar solution containing 0.12 to 0.30 percent (W/V) of invert sugar is used. Usually 40 ml of

Stock Solution (Reagent B) is diluted to 100 ml to get titration reading of around 15 ml when titrated against Fehling solution.

D. Methylene blue indicator solution: Dissolve 0.2g of methylene blue in water and dilute to 100ml.

E. Fehling's solution (Soxhlet modification): Prepared by mixing immediately before use, equal volumes of Solution A (see 'a') and Solution B (see'b'), prepared as described below.

   a. Solution A: Dissolve 34.639 g of copper sulphate (Cu_4SO5H_2O) in water add 0.5 ml of concentrated sulphuric acid of sp. gr. 1.84 and dilute to 500ml in a volumetric flask. Filter the solution through prepared asbestos.

   b. Solution B: Dissolve 173 g of Rochelle salt (Potassium sodium tartrate – $KNaC_4H_4O_6.4H_2O$) and 50 g of sodium hydroxide analytical reagent grade in water, dilute to 500 ml in a volumetric flask and allow the solution to stand for two days, Filter this solution through prepared asbestos.

9.4.1.1.1. Standardization of Fehling's Solution

Pipette accurately 10 ml of each Fehling's Solutions (Solution A and B) into a 250 ml Erlenmeyer flask. Transfer the Standard solution of invert sugar (Reagent C). Add about 10 ml of Standard solution of invert sugar (Reagent C) to the flask and heat to boiling. Boil for about 15 seconds and add rapidly further portions of the Standard solution of invert sugar until only the faintest perceptible blue colour remains. Then add 2-5 drops of methylene blue solution, continue the heating and addition of Standard solution of invert sugar (Reagent C) drop wise until the titration is complete, which is shown by the reduction of the dye (The end point of the titration is blue to red. Do not stir the flask, mixing is achieved by continuous boiling). Note down the volume of Standard solution of invert sugar (Reagent C) required to reduce all the copper. Repeat the titration, adding before heating almost all of the Standard solution of invert sugar (Reagent C) needed to reduce all the copper so that not more than 0.5-1 ml is required later to complete the titration. Heat the mixture to boiling and boil gently for 2 minutes, lowering the flame sufficiently to prevent bumping. Without removing the flask from the flame add 2-5 drops of methylene blue indicator and complete the titration within a

total boiling time of about 3 minutes, by small additions of Standard solution of invert sugar (Reagent C) to complete decolourization of the indicator. Note down the volume of Standard solution of invert sugar (Reagent B) required to reduce all the copper.

Note 1:

- The concentration of the sugar solution should be such that the titre volume should be between 15 and 50 ml. and this can be achieved with 0.2 -0.3 g sugar per 100 ml.
- Alternatively, reagent 'L' standard solution of invent sugar can also be used for determination of factor.

F. Zinc acetate solution: Dissolve 21.9 g of crystalline zinc acetate (Zn $(CHCOO)_2$, 2HO) in water and add 3 ml of glacial acetic acid. Make up to 100 ml.

G. Potassium ferrocyanide solution: Dissolve 10.6 g of crystalline potassium ferrocyanide and make up to 100 ml with water.

H. Concentrated Hydrochloric acid solution (Sp.gr. 1.16).

I. Concentrated ammonia solution (Sp.gr. 0.88).

J. Dilute ammonia solution: 10 ml of concentrated ammonia solution diluted to 100 ml with water.

K. Dilute acetic acid solution: Approximately equivalent to the dilute ammonia solution in strength.

L. Dissolve 0.2 g of pure anhydrous dextrose in water and make up to 100 ml (previously dried at 100°C and cooled before weighing).

9.4.1.2. Apparatus

A. Volumetric flasks: 100, 250, 500 and 1000 ml capacities.

B. Burette: 50 ml, graduated to 0.1 ml.

C. Funnels.

D. Conical flasks: 250 ml capacities

E. Pipette: 1, 5 and 10 ml capacities.

F. Measuring cylinder: 25 and 50 ml capacities.

G. Whatman filter paper: Grade 1.

H. Watch glass.

I. Hot plate.

9.4.1.3. Procedure

9.4.1.3.1. Preparation of the Solution: Weigh accurately about 40 g of the well - mixed sample and transfer to 100 ml beaker. Add 50 ml of hot water at 80 to 90°C. Mix and transfer to a 250 ml measuring flask washing it with successive quantities of distilled water at 60°C, until the volume is 120 to 150 ml. Mix and cool to room temperature and add 5 ml of the dilute ammonia solution. Mix and allow to stand for 15 minutes. Add the exact equivalent of dilute acetic acid to neutralize the ammonia added. Mix and add 12.5 ml of zinc acetate solution followed by 12.5 ml of potassium ferrocyanide solution. Mix again. Make up to 250 ml mark. Allow to settle and filter. Mark this solution B-I.

9.4.1.3.1.1. Pipette 50 ml of solution B-I (obtained in Section 9.4.1.3.1) into a 100 ml volumetric flask, add 5 ml of concentrated hydrochloric acid and heat in a water bath at 65 – 68°C for 5 minutes rotating the flask for the first 3 minutes. Cool the solution and neutralize with sodium hydroxide solution. Mark this solution A- I. Make up to 100 ml.

Dilute the solutions B-I (usually 50 ml of B-I solution is required to be diluted to 100 ml to obtain titration reading of around 14 ml when titrated against Fehling solution) and A-I (usually 15 ml of A-I solution is required to be diluted to 100 ml to obtain titration reading of around 18 ml when titrated against Fehling solution)so that the volume of solution required to react with 10 ml Fehling's solution (9.4.1.3.2.)is between 15 and 50 ml. Mark them B-II and A-II, respectively.

9.4.1.3.2. Incremental Method of Titration

Pour the prepared solution (9.4.1.3.1.1.) into a 50 ml burette (see Note 6). Pipette 10 ml of mixed Fehling's solution into a 250 ml conical flask and run in from the burette 15 ml of the solution. Without further dilution, heat the contents of the flask over a wire gauze, and boil. (after the liquid has been boiling for about 15 seconds, it

will be possible to judge if the copper is almost fully reduced by the bright red colour imparted to the boiling liquid by the suspended cuprous oxide. When it is judged that nearly all the copper is reduced, add one ml of methylene blue indicator solution (see Note 2). Continue boiling the contents of the flask for one to two minutes from the commencement of ebullition, and then add the prepared solution in small quantities (one ml or less at a time), allowing the liquid to boil for about 10 seconds between successive additions, till the blue colour of the indicator just disappears (see Note 5). In case there appears to be still much unreduced copper, after the mixture of Fehling's solution with 15 ml of the prepared solution has been boiling for a quarter of a minute, add the prepared solution from the burette in larger increments (more than one ml at a time according to judgement), and allow the mixture to boil for a quarter of a minute after each addition. Repeat the addition of the prepared solution at intervals of 15 seconds until it is considered safe to add a large increment of the prepared solution. At this stage, continue the boiling for an additional one to two minutes, add one ml of methylene blue indicator solution and complete the titration by adding the prepared solution in small quantities (less than one ml at a time) (see also Note 3).

Note 2: It is advisable not to add the indicator until the neighbourhood of the end point has been reached, because the indicator retains its full colour until the end point is almost reached and thus gives no warning to the operator to go slowly.

Note 3: When the operator has had a fair amount of experience with the method a sufficiently accurate result may often be obtained by a single estimation by the incremental method of titration, but for the utmost degree of accuracy of which the method is capable second titration should be carried out by the standard method of titration (see 9.4.1.3.3).

9.4.1.3.3. Standard Method of Titration

Pipette 10 ml of Fehling solution into a 250 ml conical flask and run in from the burette almost the whole of the prepared solution B-II required to effect reduction of all the copper (determined under 9.4.1.3.2) so that, if possible, not more than 1 ml shall be required later to complete the titration. Gently boil the contents of the flasks for 2 minutes. At the end of 2 minutes of boiling add, without interrupting boiling, one ml of methylene blue indicator solution. While the contents of the flask continue to boil, begin

to add the prepared solution (one or two drops at a time), from the burette till the blue colour of the indicator just disappears (see Note 4). The titration should be completed within one minute, so that the contents of the flask boil altogether for 3 minutes without interruption.

Note 4: The indicator is so sensitive that it is possible to determine the end point within one drop of the prepared solution in many cases. The complete decolourization of the methylene blue is usually indicated by the whole reaction liquid in which the cuprous oxide is continuously generated up becoming bright red or orange in colour. In case of doubt, the flame may be removed from the wire gauze for one or two seconds and the flask held against a sheet of white paper. (A holder of paper, suitably affixed round the neck of the flask, is very convenient for this purpose as it can be left round the neck of the flask without risk of over-balancing it). The top edge of the liquid would appear bluish if the indicator is not completely decolourized. It is inadvisable to interrupt the boiling as the indicator undergoes back oxidation rather rapidly when air is allowed free access into the flask, but there is no danger of this as long as a continuous stream of steam is issuing from the mouth of the flask.

Note 5: It should be observed that with both incremental and standard methods of titration, the flask containing the reaction mixture is left on the wire gauze over the flame throughout the titration, may be removed for a few seconds to ascertain if the end point is reached.

Note 6: In adding sugar solution to the reaction mixture the burette may be held in hand over the flask. The burette may be fitted with a small outlet tube bent twice at right angles, so that the body of the burette can be kept out of the steam while adding sugar solution. Burette with glass taps are unsuitable for this work, as the taps become heated by the steam and are liable to jam.

9.4.1.3.4. Repeat the titration as given in 9.4.1.3.2and 9.4.1.3.3using solution A-11 (see 9.4.1.3.1.1.).

9.4.1.3.5. Calculation

Original Reducing sugars % (ORS) in Condensed Milk = $\frac{W_1}{V_1} \times 250 \times \frac{100}{W_2} \times \frac{1}{1000}\ x\ F_1$

Total Reducing sugars % (TRS) in Condensed Milk $= \frac{W_1}{W_2} x 25 \left[\frac{2F_2}{V_2} \right]$

Sucrose % = TRS -ORS

Note: If standardization of Fehling solution is carried out by Lactose Solution (Reagent L) instead of Standard solution of invert sugar (Reagent B) prepared under acidic conditions at room temperature, multiply the Sucrose % obtained in above formula by a factor 0.95 for calculating sucrose content.

Where,

W_1= weight in mg of sucrose corresponding to 10 ml of Fehling's solution (see 9.4.1.1.1.)

W_2 = weight in g of the material taken for the determination (see 9.4.1.3.1.)

F_2 = dilution factor for solution A - II from A - I (see 9.4.1.3.1.1.)

V_2 = volume in ml of solution A - II corresponding to 10 ml of Fehling's solution (see 9.4.1.3.4.)

F_1 = dilution factor for the solution B - II from B - I(see 9.4.1.3.1.1.)

V_1 = volume in ml of solution B - II corresponding to 10 ml of Fehling's solution (see 9.4.1.3.3.)

Derivation of above formula

1. Standardization of Fehling Solution using Standard Reducing Sugar

Let 1 ml of Standard solution of invert sugar (Reagent B) contains = M mg of Total Invert Sugar

2. Calculation of Lactose (original reducing sugar in SCM sample) estimation

Let V_1 = ml of diluted sample filtrate (**B1 prepared in Step 9.4.1.3.1.1**) required for the titration of 10 ml of Fehling's solution

Let V = ml of the Standard solution of invert sugar (**Reagent C)** required for the titration of 10 ml of Fehling's solution

Now

10 ml of Fehling's solution = V ml of standard reducing sugar solution

= V_1 ml of diluted sample filtrate

Thus

V_1 ml of diluted sample filtrate ≡ V ml of standard reducing sugar solution

≡ V x M mg of standard reducing sugar

or ≡ W_1 mg of standard reducing sugar

Therefore, 100 ml of sample filtrate contains = $\frac{W_1}{V_1} \times 100$ mg of original reducing sugar

Since 100 ml of this sample filtrate was obtained from 50 ml of B1 solution

Therefore, 250 ml of B1 sample filtrate will contain = $\frac{W_1}{V_1} \times \frac{100}{50} \times 250$ mg of original reducing sugar

Since 250 ml of sample filtrate was obtained from 40 g (say W_2 g) of SCM

Therefore, Lactose (g) present in 100 gm of SCM (say C1) = $\frac{W_1}{V_1} \times \frac{100}{50} \times 250 \times \frac{100}{W_2} \times \frac{1}{1000}$ gm

2. Calculation for Total reducing sugar (Lactose + hydrolyzed Sucrose) in given sample

Let V_2 = ml of diluted sample filtrate (**A1 prepared in Step 9.4.1.3.1.1.**) required for the titration of 10 ml of Fehling's solution

Let V = ml of the Standard solution of invert sugar (Reagent B) required for the titration of 10 ml of Fehling's solution

Now

10 ml of Fehling's solution = V ml of standard reducing sugar solution

= V_2 ml of diluted sample filtrate

Thus

V_2 ml of diluted sample filtrate ≡ V ml of standard reducing sugar solution

$\equiv$ V x M mg of standard reducing sugar

or $\equiv$ W_1 mg of standard reducing sugar

Therefore, 100 ml of sample filtrate (A-II) contains = $\frac{W_1}{V_2} \times 100$ mg of total reducing sugar

Since 100 ml of this sample filtrate was obtained from 15 ml of A1 solution

Therefore, 100 ml of A-I sample filtrate will contain = $\frac{W_1}{V_2} \times \frac{100}{15}$ mg of total reducing sugar

Since 100 ml of this sample filtrate was obtained from 50 ml of B1 solution

Therefore, 100 ml of B-I sample filtrate will contain = $\frac{W_1}{V_2} \times \frac{100}{15} x \frac{100}{50}$ mg of total reducing sugar

Therefore, 250 ml of B1 sample filtrate will contain = $\frac{W_1}{V_2} \times \frac{100}{15} x \frac{100}{50} x 250$ mg of total reducing sugar

Since 250 ml of sample filtrate was obtained from 40 g (say W_2 g) of SCM

Therefore, Total reducing sugars (g) present in 100 g of SCM (say C2) = $\left[\frac{W_1}{V_2} \times \frac{100}{15} x \frac{100}{50} \times 250 \times \frac{100}{W_2} \times \frac{1}{1000} \right]$

This calculation is on the assumption that 15 of A-1 solution (obtained in Step 9.4.1.3.1.1) was diluted to 100 ml

Total sucrose (%) in the SCM = C2 –C1 g

(Ref:- IS 4079 – 1967(Reaffirmed 1995)Specification for canned rasogolla. Bureau of Indian Standards, New Delhi).

9.4.2. Polarimetric Method for Determination of Sucrose in Condensed Milk

The method based on Clerget inversion involves treatment of milk without acid which brings about complete hydrolysis of sucrose, but almost none of lactose or other

sugars. Sugar content is obtained from change in rotating power of the solution. A clear filtrate of sample, without muta-rotation by lactose as prepared by treating the solution with ammonia followed by neutralisation and clarification with successive addition of zinc acetate and potassium ferrocyanide solution. In a part of the filtrate, the sucrose is hydrolysed by appropriate method and from the rotation of filtrate, before and after inversion, sucrose content is calculated.

9.4.2.1. Apparatus

A. Zinc Acetate solution (2 N): Dissolve 21.9 g of crystallized Zinc acetate dihydrate ($Zn(C_2H_3O_2)_2.2H_2O$)and 3 ml of acetic acid in distilled water and make up to 100 ml.

B. Potassium Ferrocyanide solution (1N): Dissolve 10.6 g of crystallized potassium ferrocyanide [$K_2Fe(CN)_63H_2O$].

C. Hydrochloric acid solution: 6.35 N ±0.2 N (20- 22%).

D. Aqueous ammonia solution: 2.0±0.2 N (3.5%).

E. Acetic acid solution: 2.0±0.2 N (12%).

9.4.2.2. Apparatus

A. Beaker: 100 ml.

B. Volumetric flasks: 200 ml and 50 ml.

C. Pipette: 40 ml.

D. Graduated cylinders: 25 ml.

E. Measuring pipettes: 10 ml.

F. Filter funnel: diameter 8 to 10 cm and provided with folded filter paper of diameter 15 cm.

G. Polarimeter tube: length 200 mm, of exactly calibrated length).

H. Polarimeter or saccharimeter

   a. Polarimeter with sodium light or mercury green light (Mercury vapour lamp with prism or the special Wraten screen No. 77A), to read accurately at least 0.0500 (angular basis).

b. Saccharimeter with international sugar scale, using white light passing through a filter of 15 mm of 6 percent solution of potassium dichromate, or using sodium light to measure accurately at least 0.1° international sugar scale.

I. Water bath: Regulated at 60 ±1°C.

9.4.2.3. Procedure

9.4.2.3.1. Preparation of Sample

A. **Samples of recently manufactured product in which no appreciable separation of components may be expected:** Open the container, transfer all material adhering to the lid into the container and thoroughly mix by an up and down movement of a spoon so that the top layers and the contents of the lower corners are intermixed. Transfer the contents of can to a jar with well-fitting lid.

B. **Samples of older products and samples in which separation of components may be expected:** Heat in a water bath at about 40°C until the sample has nearly reached this temperature, open the container and proceed as described in para above. When the product is in a can transfer the contents to a jar, scrap out all material adhering to the walls and continue the mixing until the whole mass is homogeneous. Close the jar with a well fitting lid and allow to cool.

9.4.2.3.2. Control Determination

Run a control determination as described under "Determination" in duplicate in a mixture of 100g of milk or 110 g of skim milk and of pure sucrose (a) 18 g for 40 g of condensed milk (thus containing 45 percent sucrose). Calculate the sugar contents by means of the formula given at the end of this procedure using formula (1) for m, F and P respectively, which are the quantity of milk weighed and the fat and protein content of this milk, and in formula (2) for m, the value of 40. The mean of the values found shall not differ by more than 0.1 percent from the actual 45 percent.

9.4.2.3.3. Determination

Weigh approximately 40 g of the well mixed sample to an accuracy of 10 mg, into glass beaker. Add 50 ml of hot distilled water (80-90°C) and mix well. Transfer the mixture quantitatively to 200 ml volumetric flask, rinsing the beaker with successive

quantities of distilled water at 60° C, until the total volume is between 120 – 150 ml. Mix and cool, add 5 ml of ammonia solution Mix again and then allow to stand for 15 minutes. Neutralize the ammonia by adding equivalent quantity of acetic acid having determined beforehand the exact number of milliliters by titration of the ammonia solution with bromothymol blue as indicator. Mix and add with gentle mixing by rotating the tilted flask 12.5 ml of zinc acetate solution. In the same manner add 12.5 ml of potassium ferrocyanide solution. Bring the contents of the flask to 20°C and add water up to 200 ml mark.

Note: Up to this stage, all additions of water or reagents is made so as to avoid the formation of air bubbles, and with the same object in view, all mixing should be done by rotation of the flask rather than by shaking. If air bubbles are found to be present before diluting to 200 ml, their removal can be done by temporarily connecting the flask to a vacuum pump and rotating the flask; close the flask with a dry stopper and mix thoroughly by swirling. Let stand a few minute and filter through a dry filter paper, rejecting first 25 ml of filtrate.

9.4.2.3.4. Direct Polarisation

Determine optical rotation of filtrate at 20 ± 2°C.

9.4.2.3.5. Inversion

Pipette 40 ml of the filtrate obtained above into the 50 ml volumetric flask and add 6.0 ml of hydrochloric acid. Place the flask in the water bath at 60°C for 15 minutes, the entire bulb of the flask being immersed. Mix by the rotary movement during the first 5 min; by this time the contents of the flask should have attained the temperature of the bath. Cool to 20°C and make up to the 50 ml mark with distilled water at 20°C. Mix, allow to stand for one hour at this temperature.

9.4.2.3.6. Invert Polarization

Determine the rotation of the invert solution at 20 ± 2°C. (When temperature of the liquid in the polarization tube differs by more than 0.2°C during the measurement, the temperature correction referred to under Note 2 shall be applied).

9.4.2.3.7. Expression of results

Calculation: Calculate the sucrose content S, percent by mass, by means of the following formula.

$$U = \frac{m}{100}(1.08\ F + 1.55\ P) \quad --\text{Formula 1}$$

$$S = \frac{D - {}^{5}/_{4}\ I}{Q} \times \frac{V - U}{V} \times \frac{V}{e \times m} \quad --\text{Formula 2}$$

Where,

U = Correction in ml for the volume of the precipitate formed during the clarification

m = Mass in g of the weighed sample

F = Percentage of fat in the sample

P = Percentage of protein (N x 6.38) in the sample

D = Direct polarimeter reading (polarization before inversion)

I = Polarimeter reading after inversion

Q = Inversion division factor, the values of which are given in Note1

V = Volume in ml to which the sample is diluted before filtration; and

e = Length in mm of the polarimeter tube.

Remarks:

A. When exactly 40 g of the condensed milk are weighed and a polarimeter with sodium light, angular degrees and a 2 dm polarimeter tube at 20.0 ±1°C is used the sucrose content of normal condensed milk (C=9) can be calculated from the following formula; S = (D=5/4 I) 2.833 - 0.006 I2F-0.08 78P).

B. If the invert polarisation is measured at a temperature other than 20°C, the figures should be multiplied by: I + 0.0037 (I-20) Values of the inversion division factor (Q) - The following formula give accurate values for Q, for various sources of light with corrections, where necessary for concentration and temperature:

a. Sodium light and polarimeter with angular degrees: Q=0.8825 + 0.0006 (C9) -

0.0033 (1-20).

b. Mercury green light and polarimeter with saccharimeter with international sugar scale degrees:

Q = 2.549 + 0.0017 (C-9) - 0.0095 (T-20)

In the above formula:

C = Percentage of total sugars in the invert solution as polarised; and

T = Temperature of the invert solution in the polarimetric reading.

Note 1: The percentage of total sugar C in the inverted solution may be calculated from the direct reading and the change on inversion in the usual manner, using the usual values for the specific rotations of sucrose, lactose, and invert sugar. The correction 0.0006 (C-9), etc., is only accurate when C is approximately 9. For normal condensed milk, this correction can be neglected C being close to 9.

Note 2: Variation in temperature from 20°C makes little difference in direct reading, but variation of over 0.2°C in the invert reading necessitates a correction. The correction 0.0037 (T-20) etc. is accurate only between 18 and 22°C.

9.4.2.3.8. Repeatability

The difference between the results of two determinations carried out simultaneously or in rapid succession by the same analyst in the same laboratory shall not exceed 0.3 g of sucrose per 100 g of condensed milk.

(Ref:- IS 11764 – 1986 (Reaffirmed 1997) Method for determination of sucrose content by polarimetric method in condensed milk. Bureau of Indian Standards, New Delhi; IS 4079 – 1967(Reaffirmed 1995) Specification for canned rasogolla. Bureau of Indian Standards, New Delhi).

9.5. Determination of Titratable Acidity in Condensed/Evaporated Milk

9.5.1. Reagents

A. Standard Sodium Hydroxide Solution – 0.1 N.

B. Phenolphthalein Indicator – Dissolve 1.0 g of phenolphthalein in 100 ml of 95% ethanol. Add 0.1 N NaOH solution until one drop gives a faint pink colouration. Dilute with distilled water to 200 ml.

9.5.2. Procedure

Weigh accurately about 10 g of the material in a suitable dish or basin. Add 30 ml of warm water. Add 1 ml of phenolphthalein indicator. Shake well and titrate against standard NaOH solution. Complete the titration in 20 seconds. Keep a blank by taking 10 g of material diluted with 30 ml of water in another dish for comparison of colour

9.5.3. Calculation

Titratable acidity as Lactic acid= $\frac{9\ AN}{W}$

Where,

A = Volume of standard NaOH required for titration

N = Normality of Standard NaOH solution

W = weight of the sample taken for test

(Ref:- IS 1166 – 1973 Specification for condensed milk. Bureau of Indian Standards, New Delhi).

9.6. Determination of Milk Protein in Milk Solids not Fat of Condensed/Evaporated Milk

Total nitrogen content in condensed/evaporated milk sample is estimated by Kjeldahl method as described for milk. The percent nitrogen obtained is multiplied by a factor to get protein content in condensed/evaporated milk sample.

Take 1 - 2 g of sample and refer to the method mentioned in Section 19.

A. Follow method mentioned in Section 19.1 for **Determination of Total Nitrogen/Crude Protein in Milk.**

Calculate Crude Protein Content = N x 6.38

N = Nitrogen content in sample estimated by Kjeldahl method.

10. MILK POWDER (WHOLE, SKIMMED, PARTIALLY SKIMMED) INFANT MILK FOOD, INFANT FORMULA, MILK CEREAL WEANING FOOD, PROCESSED CEREAL BASED WEANING FOOD

These products are hygroscopic in nature. Care must be taken while handling these samples to avoid moisture absorption, which can give erroneous results

10.1. Preparation of Sample of Dried Milk

Make homogeneous either by mixing or shaking or alternately rolling and inverting container. Avoid excessive temperature and humidity when opening sample container to prevent absorption of moisture.

10.2. Determination of Moisture in Dried Milk

The sample is dried to constant weight at 102 ± 2°C and the loss in weight reported as moisture. The method described here has been followed from IS 11623: 1986 (Reaffirmed 1997).

10.2.1. Requirements

A. Flat-bottom moisture dishes with cover: Of stainless steel, nickel or aluminium having approximately 50 mm diameter and 25 mm depth. The dishes shall have lids which fit well and can readily be removed.

B. Hot air oven: Maintained at 102 ± 2°C.

C. Desiccator: Containing an efficient desiccant.

10.2.2. Procedure

A. Uncover a dish and place the dish and its lid in a hot air oven at 102 ± 2°C for 1 h. Place the lid on the dish, transfer the covered dish from the hot air oven to the desiccator. Allow it to cool to room temperature and weigh it.

B. Put approximately 1 g of the dried milk sample in the dish, cover the dish with the lid and weigh the covered dish accurately and quickly.

C. Uncover the dish and put it with its lid in the hot air oven maintained at 102 ± 2°C for 2 h.

D. Replace the lid, transfer the covered dish to the desiccator, allow it to cool to room temperature (for approximately 30 - 45 min) and weigh it accurately and quickly.

E. Heat the uncovered dish and lid in the hot air oven at 102 ± 2°C for further 1 h, replace the lid, allow the covered dish to cool to room temperature in the desiccator and weigh it. Repeat the process of drying, cooling and weighing, until the successive weighing do not differ by more than 0.5 mg. It is usually found that drying is complete after the first 2 h.

10.2.3. Calculation

$$\text{Moisture \% by mass} = \frac{100\,(M_1 - M_2)}{M_1 - M}$$

Where

M = mass in g, of the empty dish;

M_1 = initial mass in g, of the dish and lid with the material taken for analysis;

M_2 = final mass in g, of the dish and lid with the material after drying.

The maximum deviation between duplicate determinations should not exceed 0.06% by mass of moisture.

Note:

1. Commercial automatic moisture determination apparatus can also be used for determination of moisture in milk powder.
2. Latest BIS (IS6623: 2008) and ISO (ISO 5537:2004) methods advocates the use of drying oven set at 87°C (for 5 hours) and use of copper tubes.

10.2.4. Interpretation

This method gives free moisture content in powder, i.e. it does not include water of crystallization of alpha lactose monohydrate. The total moisture including water of crystallization can be determined by Karl Fischer titration or toluene distillation. However, sample of commerce are expected to contain less than 5% of free moisture,

therefore oven method at 100 ± 2°C is adequate for its determination.

(Ref:- IS 11623 (Reaffirmed 1997), Method for determination of moisture content in milk powder and similar products, Bureau of Indian Standards: India; ISO 5537/IDF 026:2004 - Dried milk - Determination of moisture content (Reference method), International Dairy Federation, Brussels; F.A.O Manuals of Food quality Control 14/ 8 page 30).

10.3. Determination of Fat in Dried Milk

Weigh 1 g of the sample in a Mojonnier flask or suitable extraction flask. Add 10 ml of warm (65 ± 5°C) distilled water. Shake to dissolve the powder. Add 1.25 ml of ammonia solution. Shake well and proceed as per Rose Gottlieb method (See Section 1.3.4.2).

10.4. Determination of Titratable Acidity in Dried Milk

The method is based on the titration of the sample with sodium hydroxide to phenolphthalein end point and by comparing the colour with colour obtained by mixing rosaniline acetate or cobalt sulphate to a known volume of milk sample.

10.4.1. Reagents /Apparatus

A. 0.1N Sodium hydroxide solution.

B. Rosaniline acetate solution: Dissolve 0.12 g rosaniline acetate in 95% ethyl alcohol containing 0.5 ml of glacial acetic acid and diluted to 100 ml(Store in dark). To prepare working standard, dilute 1 ml to 500 ml with 95% ethyl alcohol and water in the ratio of 1:1.

C. Phenolphthalein solution: To 1 g of phenolphthalein dissolved in 100 ml of 95% ethyl alcohol, add 0.1 N sodium hydroxide till a faint pink colour is obtained and dilute to 200 ml with distilled water.

D. Cobalt sulphate solution: Dissolve 1.5 g of cobalt sulphate in water and dilute to 100ml.

E. Porcelain dishes 100 ml.

F. Burette 5 ml.

G. Glass rods for stirring.

10.4.2. Reconstitution of Milk Powder

Weigh l0 g of skim milk or 13 g of whole milk powder, make up to 100 ml with warm distilled water at 24°C. Stir exactly for 90 seconds. Allow the sample to stand until the foam settles. The period of standing after mixing should not exceed 15 minutes.

10.4.3. Procedure

Pipette 10 ml of reconstituted milk into each porcelain dish. To one dish, add 1 ml of working solution of rosaniline acetate or cobalt sulphate solution and stir with a glass rod. This solution will be external end point reference. To the other porcelain dish add 1 ml of phenolphthalein solution and titrate with 0.1 N sodium hydroxide, stirring to mix the sample. Continue titration until the colour is comparable to the reference solution.

10.4.4. Calculation

Calculate acidity as Lactic acid (percent m/v) if desired

1 ml 0.1 N NaOH = 0.0090 g Lactic acid.

Otherwise determine solid not fat in the sample by deducting moisture and milk fat and calculate acidity in terms of ml of 0.1N NaOH / 10 g Milk solids not Fat as per requirement of FSSAI Rule as shown below :

$$\frac{\text{Volume of 0.1 NaOH x 100 x 100 x 10}}{\text{Weight of MSNF x weight of sample}}$$

(Ref:- BS 1741-Part 10,Section 10.1, 1989 / F.A.O. Manual of Food Quality Control 14/8) page 20 / IS 11765 -1986 / ISO 6091 -1980 Method for determination of titratable acidity in milk powder and similar products. Bureau of Indian Standards, New Delhi).

10.5. Determination of Total Carbohydrates in Dried Milk

Determine total carbohydrate by difference in infant milk food. Add moisture, fat, protein and ash content and deduct the value from 100 to give carbohydrate content by difference.

Total carbohydrate including sucrose, dextrose

and dextrins, maltose or lactose percent by weight = 100 –(A+B+C+D)

Where A = Percent by mass of moisture

B = Percent by mass of total protein

C = Percent by mass of fat and

D = Percent by mass of Total ash

(Ref:- IS 1656 – 1997 Milk cereal based Weaning foods – Specification. Bureau of Indian Standards, New Delhi).

10.6. Determination of Milk Protein in Milk Solids not Fat of Dried Milk

Total nitrogen content in milk powder sample is estimated by Kjeldahl method as described for milk. The percent nitrogen obtained is multiplied by a factor to get protein content in milk powder sample.

Take 0.5 g of sample and refer to the method mentioned in Section 19.

A. Follow method mentioned in Section 19.1 for **Determination of Total Nitrogen/Crude Protein in Milk.**

Calculate Crude Protein Content = N x 6.38

N = Nitrogen content in sample estimated by Kjeldahl method

Note: For determination of true nitrogen content, determine non protein nitrogen (NPN) and subtract the same from Crude Protein Content.For determination of NPN content in milk powder, see Section 3.4.1.2 or refer Section 19.

10.7. Determination of Total Ash on Dry Basis in Dried Milk

10.7.1. Apparatus

A. Platinum or silica crucible: About 70 mm diameter and 25 to 50 mm deep.

B. Muffle furnace: Capable of being controlled at 550 ± 20°C.

C. Desiccator: Containing an efficient desiccant.

D. Safety tongs having long arms.

E. Bunsen burner or electric hot plate.

10.7.2. Procedure

Weigh accurately about 3 g of the dried milk sample in the crucible, previously dried in a hot air oven and weighed. Heat the crucible gently on a burner or hot plate at first and then strongly in a muffle furnace at 550 ± 20°C till grey ash is obtained. Cool the crucible in a desiccator and weigh it. Heat the crucible again at 550 ± 20°C for 30 min. Cool the crucible in a desiccator and weigh. Repeat this process of heating for 30 min, cooling and weighing until the difference between two successive weighing is less than 1 mg. Record the lowest mass.

10.7.3. Calculation

Total ash (on dry basis), % by mass $= \frac{M_2 - M}{(100 - M_0) \times (M_1 - M)} \; x \; 100$

Where

M_2 = mass in g, of the crucible with ash;

M = mass in g, of the empty crucible;

M_1 = mass in g , of the crucible with the material taken for the test; and

M_0 = moisture, % by mass, calculated as per the method for dried milk.

(Ref:- IS 1165 - 2005. Milk Powder – Specifications. Bureau of Indian Standards, New Delhi).

10.8. Determination of Ash Insoluble in Hydrochloric Acid (for Infant Foods and Processed Cereal based Weaning Foods)

10.8.1. Reagents

A. Dilute Hydrochloric acid: 5 N, prepared from concentrated HCl.

B. Silver Nitrate solution: 0.1 N, aq.

10.8.2. Apparatus

A. Platinum or silica crucible: About 70 mm diameter and 25 to 50 mm deep.

B. Muffle furnace: Capable of being controlled at 550 ± 20°C.

C. Desiccator: Containing an efficient desiccant.

D. Safety tongs having long arms.

E. Bunsen burner or electric hot plate.

F. Water-bath: Boling.

G. Whatman filter paper: Grade 42.

H. Watch-glass.

I. Hot air oven: Maintained at 100 ± 2°C.

10.8.3. Procedure

To the ash contained in the crucible (obtained in the previous method), add 25 ml of dilute HCl (5 N), cover with watch-glass and heat on water-bath for 10 min. Allow to cool and filter the contents of the crucible through a Whatman filter paper Grade 42. Wash the filter paper with water until the washings are free from the acid [check with $AgNO_3$ solution (0.1 N solution); till the washings do not form white precipitate with $AgNO_3$ solution] and return the washed filter paper to the crucible. Keep the filter paper in a hot air oven maintained at 100 ± 2°C for about 3 h. Ignite the crucible in a muffle furnace at 550 ± 20 C° for 1 h. Cool the crucible in a desiccator to room temperature (for approximately 30 – 45 min) and weigh it. Heat the crucible again at 550 ± 20°C for 30 min, cool in a desiccator and weigh. Repeat this process of heating for 30 min, cooling and weighing until the difference between two successive weighing is less than1 mg. Record the lowest mass.

10.8.4. Calculation

Acid insoluble ash, % by mass $= \frac{M_2 - M}{M_1 - M} \times 100$

where

M = mass in g, of the empty crucible;

M_1 = mass in g, of the crucible with the material taken for the test;

M_2 = mass in g, of the crucible with acid insoluble ash.

10.9. Determination of Crude fibre (in Processed Cereal Weaning Foods)

10.9.1. Reagents

A. Dilute Sulphuric acid -1.25 % (W/V) : Prepare accurately.

B. Sodium Hydroxide -1.25% (W/ V) : Prepare accurately.

10.9.2. Procedure

Dry to constant weight about 5 g of the material in an air oven at 105 ± 2°C. Weigh accurately about 2.5 g of the dried material into a thimble and extract with petroleum ether for 1 hour using a soxhlet flask. Transfer the fat free material to a 1 litre flask. Take 200 ml of dilutedsulphuric acid in a beaker and bring to boil. Transfer whole of the boiling acid to the flask containing the fat free material and immediately connect the flask with a reflux condenser and heat so that the contents of the flask begin to boil within 1 minute. Rotate the flask frequently taking care to keep the material from remaining on the sides of the flask out of contact with acid. Continue boiling for exactly 30 minutes Remove the flask and filter through fine linen(about 18 threads to a centimeter) held in a funnel and wash with boiling water until the washings are no longer acid to litmus.

Bring to boil 200 ml of Sodium Hydroxide solution under reflux condenser. Wash the residue on the linen into the flask with the boiling sodium hydroxide solution. Immediately connect the flask with the reflux condenser and boil for exactly 30 minutes. Remove the flask and immediately filter through the filtering cloth. Thoroughly wash the residue with boiling water and transfer to a gooch crucible prepared with a thin compact layer of ignited asbestos. Wash the residue thoroughly first with hot water and then with 15 ml of ethyl alcohol. Dry the gooch crucible with the contents in an air oven maintained at 105 ± 2°C till constant weight. Incinerate in a muffle furnace until all the carbonaceous matter is burnt. Cool in a desiccator and weigh

10.9.3. Calculation

$$\text{Crude Fibre (dry basis)} = \frac{M_1 - M_2}{M} \times 100$$

Where, M_1 = mass in g of gooch crucible and contents before ashing.

M_2 = mass of gooch crucible containing asbestos and ash

M = mass in g of dried material taken for test.

(Ref:- ISI Hand book of Food Analysis (Part XI) – 1981).

10.10. Determination of the Solubility Index in Milk Powder

The solubility index (also sometimes referred as insolubility index) of the milk powder is the content of milk powder which is unable to dissolve in water. It is expressed as the volume of sediment in ml obtained by the procedure described below.

10.10.1. Apparatus

A. Balance: Approximately 500 g capacity and 0.1 g or better sensitivity.

B. Centrifuge: Of required speed (rpm) with cups to accommodate conical centrifuge tubes. The requires speed varies with diameter of the head as follows:

Diameter, cm	Speed (rpm)
25.4	1074
30.5	980
35.5	909
40.6	848
45.7	800
50.8	750
55.9	724
61.0	695

Note: 'The diameter of head' is the distance between the inside bottoms of opposite cups measured through the center of rotation of the centrifuge head while the cups are horizontally extended.

C. Centrifuge Tubes: Conical, graduated as follows:

0 to 1.0 ml in 0.1ml divisions

1.0 to 2.0 ml in 0.2 ml divisions

2.0 to 10.0 ml in 0.5 ml divisions

10.0 to 20.0 ml in 1.0 ml divisions

And a 50.0 ml mark at least 1 to 3 cm from the top of the tube.

D. Mixing Jars: 500 ml glass stirring jar.

E. Mixer: Fitted with a motor capable of operating at 3600 rpm and a stirrer shaft. It may have control to shut off the motor at the end of 90 seconds stirring period with a timer calibrated in 5 seconds internals, and adjusted to a maximum of 5 minutes. Approximate dimension of the mixer may be height 45 cm, width 12 cm and depth 22 cm. the blades of the impeller may have a pitch of 30° and a spread of 8.7 mm between blades. (Any mixture which gives the performance specified above may be used).

F. Siphon Tube:

10.10.2. Procedure

10.10.2.1. Reconstitution of Milk Powder

Add 14 g of the sample to 100 ml distilled water at a temperature of 24°C in the mixing jar. Place the jar in the mixer and stir for exactly 90 seconds. Allow the sample to stand until the foam has separated sufficiently to permit its complete removal by a spoon. The period of standing after mixing should not exceed 15 minutes. After removal of foam, mix the sample thoroughly with a spoon for 5 seconds.

10.10.2.2. Removal of cream and fat and soluble fractions

Fill up the centrifuge tube immediately with the reconstituted milk to the 50 ml mark. Centrifuge the tube for 5 minutes at the required speed (depends on the diameter of head). Immediately siphon off the transparent liquid to within 5 ml of the surface of the sediment level, taking care not to disturb the sediment layer. Add about 25 ml distilled water at a temperature of 24°C and shake the tube to disperse the sediment, dislodging it, if necessary, with a wire. Fill the tube to the 50 ml marks with distilled water at a temperature of 24°C. Invert and shake to mix the contents thoroughly. Again centrifuge at the require speed for 5 min.

10.10.2.3. Determination of Solubility Index:

Hold the tube in a vertical position with the upper level of the sediment on a level with

the eye and read the millilitres of sediment in the tube to the nearest graduated scale division. The sediment is easily distinguished when the tube is held between the eye and a strong source of light.

10.10.3. Calculation:

Solubility index: Report the solubility index as the millilitres of sediment in the tube.

(Ref:- ISI Hand book of Food Analysis (Part XI) – 1981).

10.11. Determination of the Solubility Percent in Milk Powder

In this method, a definite quantity of powder is reconstituted in water under specified conditions. Reconstituted sample is centrifuged and the fat layer is removed. The sediments are again dissolved and total solids (TS-1) are determined in an aliquot. The sample is again centrifuged and total solids of an aliquot of supernatant layer (TS-2) is determined. Difference in total solids of two (TS -1 and TS-2) provide solubility of powder and solubility percent is then calculated by taking into account of initial weight of sample taken,

10.11.1. Procedure

10.11.1.1. Reconstitution of Milk Powder

Weigh accurately 4 g of the material into a 50 ml boiling tube. Add 32 ml of water at 50 ± 1°C. Cork the tube and shake for 10 sec. Place the tube in a water-bath maintained at 50 ± 1°C for 5 minutes and shake the tube for one minute.

10.11.1.2. Removal of Fat

Fill the reconstituted milk into a 25 ml centrifuge tube and centrifuge for 10 minutes at 2000 rpm with a radius of 17 cm (giving a force of 770 g). Cool in a refrigerator or in ice until the fat solidifies (taking care that milk does not freeze). Remove the fat layer with spoon shaped spatula. Bring the milk to room temperature (27 ± 1°C). Break up the deposit with a glass rod. Cork the tube and shake vigorously until the liquid is homogenous.

10.11.1.3. Determination of Total Solids

Transfer about 2 ml of the homogenous liquid to a previously weighed dry

tared aluminium dish (No. 1) (6 cm in diameter and 2.5 cm in height) and provided with a tight fitting lid. Weigh the dish with the lid on and place it aside.

Centrifuge the tube again for 10 min. Pipette about 2 ml of the upper layer of the supernatant liquid without disturbing the sediment, into a second aluminium dish (No. 2) of the same type as No. 1. Cover the dish and weigh. Uncover both the dishes and place them side by side on a steam-bath until apparently dry. Place the dishes in an air-oven at 100 ± 2°C for 90 min. Cover

and transfer the dishes to a desiccator. Cool and weigh the dish individually.

10.11.2. Calculation

Solubility, percent by weight = $\frac{M_4 x\, M_1 x\, 100}{M_3 x\ M_2}$

Where

M_4 = Mass in g of total solids in dish No. 2

M_1 = Mass in g of the liquid taken immediately after the removal of fat in dish No. 1

M_3 = Mass in g of total solids in dish No. 1, and

M_2 = Mass in g of the supernatant liquid taken in dish No. 2

(Ref:- ISI Hand book of Food Analysis (Part XI) – 1981).

Note :-For vitamins, minerals and microbiology of Infant foods and milk cereal based weaning foods refer to specialized publications.

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11. KHOA

11.1. Preparation of Sample of Khoa

Grind or grate the laboratory sample of khoa by means of an appropriate device; mix the ground or grated mass quickly, and if possible grind or grate a second time, and again mix thoroughly. If the sample cannot be ground or grated, mix it thoroughly by intensive stirring and kneading. Transfer the test sample to an air-tight container to await analysis, which should be carried as soon as possible after grinding. If delay is unavoidable, take all precautions to ensure proper preservation of the sample and to prevent condensation of moisture on the inside surface of the container. The storage temperature should be below 10°C.

11.2. Determination of Moisture in Khoa

Refer Section 5.2 - Determination of Moisture in Channa or Paneer).

11.3. Determination of Fat content in Khoa

11.3.1. Method 1. If Sample shows Presence of Sugar

Accurately weigh 2-3 g of the sample in a glass beaker. Add 5 ml of warm water and break the lumps with the help of a glass rod. Transfer the contents carefully to a Mojonnier flask or other extraction flask. Wash the beaker and glass rod with warm water. Add 1.25 ml of ammonia solution and proceed as per method given in Section 1.3.4.2. (Rose-Gottlieb method).

11.3.2. Method 2. If Sample shows Absence of Sugar

Accurately weigh 2-3 g of sample in a small beaker. Add a few drops of water and rub to a smooth paste by using a glass rod. Add 9 ml of water the first few drops being used to wash the tip of the glass rod. Add 10 ml of concentrated hydrochloric acid and heat on a Bunsen burner. (see Section 5.3. - Determination of Fat in Channa). Cool to room temperature. Add 10 ml of ethyl alcohol first to the beaker and later transfer the contents to the Mojonnier fat extraction flask or the Rohrig tube Transfer to the Mojonnier fat extraction flask. Proceed as in determination of milk fat by acid digestion (1.3.4.3. - Determination of Milk Fat by Acid Digestion Method (Werner Schmidt Method).

(Ref:- I.S 2785 – 1964. Specification for cheese. Bureau of Indian Standards, New Delhi).

11.4. Determination of Starch in Khoa

In this method, a definite quantity of sample is dispersed in hot water and diluted sample is treated with iodine solution. The appearance of blue colour confirms presence of starch in khoa.

11.4.1. Reagent

A. Iodine solution: Dissolve 1 g of iodine and 5 g of potassium iodide in a sufficient quantity of water and make volume up to 100 ml.

11.4.2. Procedure

A. Preparation of sample: Take 11.0 g of grated khoa sample in a beaker. Add 20 ml of hot water (80-90°C) and with the help of glass rod, make a paste. Transfer the sample quantitatively to 50 ml volumetric flask and make the volume to 50 ml. Shake well before use.

B. Take about 5 ml of prepared sample of khoa in a test tube. Add 0.5 ml of concentrated HCl followed by addition of 0.005 g of resorcinol. Mix the content and place the test tube in boiling water-bath for 5 min. Appearance of deep red colour indicates presence of sucrose in sample whereas control samples will remain light pink.

(Ref:- Kavitha, P. (2004). Studies on the validity of existing methods and their modification for the detection of adulterates in khoa and burfi. PhD. Thesis. NDRI, Karnal, India).

11.5. Detection of Sucrose in Khoa

In this method, a definite quantity of sample is dispersed in hot water and diluted sample is treated with resorcinol solution. The appearance of red colour confirms presence of sugar in khoa.

11.5.1. Reagent

A. Resorcinol flakes: Should be white in colour.

B. Concentrated HCl.

11.5.2. Procedure

A. Preparation of sample: Take 11.0 g of grated khoa sample in a beaker. Add 20 ml of hot water (80-90°C) and with the help of glass rod, make a paste. Transfer the sample quantitatively to 50 ml volumetric flask and make the volume to 50 ml. Shake well before use.

B. Take about 5 ml of prepared sample of khoa in a test tube. Add 0.2 ml of iodine solution to the test tube and mix well. Development of blue colour indicates presence of starch and control sample remains yellow. The limit of detection of method is 0.05%.

(Ref:- Kavitha, P. (2004). Studies on the validity of existing methods and their modification for the detection of adulterates in khoa and burfi. PhD. Thesis. NDRI, Karnal, India).

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12. TABLE (CREAMERY), AND DESHI BUTTER

12.1. Preparation of Sample of Butter

Warm the sample in an airtight container with the lid screwed down tightly or with the glass stopper in an oven or water bath maintained at 37 ± 2°CShake vigorously to obtain a homogeneous fluid emulsion free from unsoftened pieces. In case, the sample does not mix up properly (water separation can be seen) reject the sample. For analysis of butter fat heat a portion of emulsified butter in a beaker to a temperature of 50 – 60°C until the fat separates. Filter the fat layer through a dried filter paper into a dry vessel. Melt the filtered fat if necessary and refilter to obtain clear fat free from water.

(Ref:- IS 3507 – 1966 Method of sampling and test for Butter. Bureau of Indian Standards, New Delhi; Pearson's Composition and Analysis of Foods 9th Edition, Pg 576).

12.2. Determination of Moisture in Butter

12.2.1. Apparatus

A. Hot air oven: Maintained at 100 ± 1°C.

B. Flat bottom moisture dish: Dishes of height at least 25 mm and at least 50 mm in diameter, and made of appropriate material (for example stainless steel, nickel or aluminium) not affected by boiling water.

C. Glass rods with one end flattened and about 9 cm in length.

D. Desiccator with an efficient desiccant.

E. Boling water-bath with rings to take dishes of 50 mm diameter.

F. Clay pipe triangles.

12.2.2. Procedure

A. Clean the dish and the glass rod and dry them in the hot air oven maintained at 100 ± 1°C for at least 1 h.

B. Allow to cool to the room temperature in a desiccator and weigh the dish. Accurately weigh (to the nearest 0.1 mg) into the dish 3 to 4 g of the prepared butter sample.

C. Place the dish on a boiling water-bath supported on a clay pipe triangle for at least 20 min, stirring at frequent intervals until no moisture can be seen. Wipe the bottom of the dish and transfer it to the oven maintained at 100 ± 1°C and keep it for 90 min. Allow the dish to cool in the desiccator and weigh to the nearest 0.1 mg.

D. Heat the dish again in an oven for 30 min. Repeat the process of heating, cooling and weighing until the differences between two consecutive weights does not exceed 0.1 mg. Record the lowest mass and preserve the residue for the determination of curd.

Note: As per IDF (IDF 80-1, 2001) procedure, the weight of butter sample taken for moisture determination is 5 g and drying temperature is 102 ± 2°C for 1 h.

12.2.3. Calculation

Moisture, % by mass = $\frac{M_1 - M_2}{M_1 - M}$ x 100

Where, M_1 = mass in g, of the dish with the material before heating to constant weight;

M_2 = mass in g, of the dish with the material after heating to constant weight; and

M = mass in g, of the empty dry dish.

(Ref:- IS 3507 – 1966 Methods of sampling and test for Butter. Bureau of Indian Standards, New Delhi; A.O.A.C 17thedn, 2000, Official method 920.116 Moisture in Butter; IDF (2001) IDF Standard 80-1, Butter – Determination of moisture, non-fat solids and fat contents – Part 1: Determination of moisture content (reference method) International Dairy Federation: Brussels).

12.3. Determination of Fat and Curd (Milk solids not Fat) in Butter

Fat portion is removed with the help of petroleum ether and residue left behind is dried for determination of curd content. In case of table butter, it is curd and salt content and thus salt content has to be determined separately for calculating curd content.

12.3.1. Apparatus

A. Gooch crucible or sintered funnel - with filter flask and adapter.

B. Glass funnel with folded 12.5 cm Whatman filter paper Grade 1.

C. Flat bottom flask: 250 ml capacity

D. Desiccator with efficient desiccant.

E. Asbestos.

F. Hot air oven: Maintained at 100 ± 1°C.

G. Conical flask: 250 ml capacity.

H. Glass beads

12.3.2. Reagent

n-Hexane or, alternatively, light petroleum hydrocarbon solvent (petroleum spirit) with boiling range between 40 to 60°C. The reagent shall not leave more than 1 mg of residue after evaporation of 100 ml.

12.3.3. Procedure

A. Prepare a celite mat in a Gooch crucible or sintered funnel. Dry it in a hot air oven maintained at 100 ± 1°C, cool in the desiccator and weigh. Alternatively, dry, cool and weigh ordinary glass funnel with folded 12.5 cm filter paper.

B. Melt the residue in the moisture dish and add 25 to 50 ml of petroleum solvent and mix well.

C. Fit the crucible to the filter flask or place the funnel with filter paper on a filter stand.

D. Wet the asbestos mat or the filter paper with petroleum solvent and decant the fatty solution from the dish into the asbestos or the filter paper, leaving the sediment in the dish. Macerate the sediment twice with 20 to 25 ml of petroleum solvent and decant again the fatty solution into the asbestos or the filter paper.

E. Filter the solution and collect the filtrate in a clean, dried, tared 250 ml flat bottom flask containing 1 to 2 glass beads.

F. With the aid of a wash-bottle containing petroleum solvent, wash all the fat and sediment from the dish into the crucible or the filter paper.

G. Finally, wash the crucible or the filter paper until free from fat, collecting all the filtrate in the conical flask. Preserve the filtrate for the determination of fat. Dry the crucible or filter paper in the oven maintained at 100 ± 1°C for at least 30 min.

Note: If fat is to be determined only, transfer all the filtrate to a pre-dried and weighed fat flaks containing 2-3 glass beads. Rinse the conical flask with petroleum ether. Evaporate the ether, first on the water-bath and then in the oven at 102±2°C for 1 hour or till the time the constant weight is obtained. Calculate the fat content form the residues obtained by using following formula

$$\% \text{ fat} = \frac{(Weight\ of\ fat\ flask + fat\ residues) - Weight\ of\ empty\ flask}{Weight\ of\ sample\ taken}\ x\ 100$$

H. Cool in the desiccator and weigh. Repeat drying, cooling and weighing until the loss of weight between the consecutive weighing does not exceed 0.1 mg. Preserve the residue for the determination of salt.

12.3.4. Calculation

$$\text{Curd and salt, \% by mass (C)} = \frac{M_1 - M_2}{M} \times 100$$

Where, M_1 = mass in g, of the filter paper with residue;

M_2= mass in g, of the filter paper alone; and

M = mass in g, of the sample.

Percent Fat w/w = 100 - (M+C)

Where,

M = Moisture percent

C = Curd & salt percent

Curd percent by weight is obtained by subtracting the value of salt percent by weight from the value of C.

(Ref:- IS 3507 – 1966 Method of sampling and Test for Butter. Bureau of Indian Standards, New Delhi); Pearsons Composition and Analysis of Foods 9thedn, page 576).

12.4. Determination of Salt Content in Butter

12.4.1. Method 1. (Volhard's Method)

In this method, salt present in the butter sample is extracted with hot water

from the dried fat-free residue obtained in moisture determination. The chlorides are precipitated by adding excess of silver nitrate. The unused silver nitrate is titrated with potassium thiocyanate using ferric ammonium sulphate indicator.

Reaction

Ag^{+} (excess) + Cl^{-} ⟶ AgCl (solid)

$Ag^{+} + SCN^{-}$ ⟶ AgSCN (solid)

$Fe^{+3} + SCN^{-}$ ⟶ $[FeSCN]^{+2}$ (Reddish brown)

Reagents

A. Standard silver nitrate solution: 0.05 N, standardized against standard sodium chloride. Dissolve slightly more than theoretical quantity (8.7 g per 1 L of water) of silver nitrate (equivalent weight 169.89) in halogen-free water and dilute to volume (1 L).

B. Nitric acid: sp. gr. 1.42 approx. 70 % (m/m).

C. Nitric acid: Approximately 5 N.

D. Ferric ammonium sulphate indicator solution: Dissolve 50 g of ferric ammonium sulphate [$Fe_2(SO_4)_3.(NH_4)_2SO_4.24H_2O$] in 95 ml of water containing 5 ml of 5 N nitric acid.

E. Standard potassium thiocyanate (KCNS) solution (Approx. 0.05 N): Standardized against standard silver nitrate. Weigh approx. 5.25 g KCNS and dissolve in 1 L water. Allow to stand overnight and filter, if necessary, to get a clear solution, standardize by titration against 0.05 N $AgNO_3$ and dilute with requisite volume of water to get exactly 0.05 N KCNS solution.

12.4.1.1. Apparatus

A. Beakers: 100, 250 ml capacity.

B. Volumetric flask: 100 ml, 1 L capacity.

C. Conical flask: 250 ml capacity.

D. Water-bath: Maintained at 60 to 70°C.

12.4.1.2. Procedure

A. Extract the salt from the residue of curd and salt by repeated washing of the Gooch crucible or filter paper with hot water, or by placing the crucible or filter paper in a beaker of hot water.

B. Collect the rinsing in a 100 ml volumetric flask passing the solution through a filter paper. Allow to cool to room temperature and make up to volume.

C. Take 25 ml water extract into a 250 ml conical flask, and add an excess (normally 25 to 30 ml) of 0.05 N silver nitrate solution.

D. Acidify with nitric acid; add 2 ml of the indicator solution and 1 ml nitrobenzene. Mix and determine the excess of silver nitrate by titration with the potassium thiocyanate solution until the appearance of an orange tint, which persist for 15 s.

E. In the same manner determine the equivalent of 25 ml or the added amount of silver nitrate as thiocyanate using the same volumes of reagents and water.

12.4.1.3. Calculation

$$\text{NaCl, \% by mass} = \frac{23.38 \times N \times (A-B)}{M}$$

where

N = normality of potassium thiocyanate solution (0.005 N);

A = volume in ml, of potassium thiocyanate in blank titration;

B = volume in ml, of potassium thiocyanate in the sample titration; and

M = mass in g, of the butter sample.

(Ref:- IS 3507 – 1966 Methods of sampling and test for butter. Bureau of Indian Standards, New Delhi).

12.4.2. Method 2. (Mohr's Method)

In this method, the butter sample is melted in hot water, and the chlorides present in the mixture are titrated with a solution of silver nitrate using potassium chromate as indicator.

Reaction

$$AgNO_3 + NaCl \longrightarrow AgCl + NaNO_3$$

$$2AgNO_3 + K_2CrO_4 \longrightarrow Ag_2CrO_4 + 2KNO_3$$
(Brick–red ppt)

12.4.2.1. Reagents

A. Standard silver nitrate solution (0.1 N): Standardized against standard sodium chloride. Dissolve slightly more than theoretical quantity of silver nitrate (equivalent weight 169.89) in halogen-free water and dilute to volume. Dissolve between 17 g and 19 g of silver nitrate in 1 L of water which is practically free from carbon dioxide. Standardize the silver nitrate solution against standard sodium chloride solution. Store the solution away from direct sunlight.

B. Potassium chromate indicator (5%, w/v): Dissolve 50 g of potassium chromate (K_2CrO_4) in 1 L of water.

C. Calcium carbonate: Analytical Grade, free from chloride.

12.4.2.2. Apparatus

A. Conical flask: 250 ml capacity.

B. Burette: 50 ml capacity, graduated to 0.1 ml.

C. Pipette: capable of delivering 2.0 ml

D. Measuring cylinder: 100 ml capacity, graduated.

12.4.2.3. Procedure

A. Weigh accurately 5 g of butter sample into the 250 ml conical flask. Carefully add 100 ml of boiling distilled water. Mix the contents of the conical flask. Allow to stand with occasional swirling for 5 to 10 min.

B. After cooling to 50 to 55°C (titration temperature), add 2 ml of potassium chromate solution. Mix by swirling. Add about 0.25 g of calcium carbonate and mix by swirling.

C. Titrate at 50 to 55°C with standard silver nitrate solution while swirling continuously, until the brownish colour persists for half a minute.

D. Carry out a blank test with all the reagents in the same quantity except the butter sample. The maximum deviation between duplicate determinations should not exceed 0.02% of sodium chloride.

12.4.2.4. Calculation

NaCl, % by mass = = $\frac{5.844 \text{ x N } (V_1 - V_2)}{M}$

where

N = normality of silver nitrate solution (0.1N);

V_1 = volume in ml, of silver nitrate used in the sample titration;

V_2 = volume in ml, of silver nitrate used in the blank titration; and

M = mass in g, of the butter sample.

(Ref:- IS 3507 – 1966 Methods of sampling and test for butter. Bureau of Indian Standards, New Delhi).

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13. GHEE / BUTTER FAT /BUTTER OIL AND ANHYDROUS MILK FAT / ANHYDROUS BUTTER OIL

13.1. Preparation of Sample of Ghee

Mix the ghee sample in the container in which it is received until homogenous. Carry out this operation in a cool place, away from direct sunlight, and complete it in shortest possible time. In the event of any separation taking place in between, that is, mixing and commencement of the chemical analysis for moisture determination, remix the sample. Use this for determining of moisture. After moisture determination, place the container or glass bottle containing ghee in a water-bath at a temperature not higher than 50°C till completely melted. Filter through a dried, fluted open-texture 15 cm Whatman filter paper Grade 4 with the help of a hot water funnel, directly into receiving bottle. The filtered ghee should be bright and clear.

(Ref:- IS 3508 – 1966 (Reaffirmed 1997) Methods of sampling and test for Ghee (Butterfat). Bureau of Indian Standards, New Delhi).

13.2 Determination of Moisture in Ghee

The moisture content of ghee is the loss in mass, expressed as a percentage by mass when the product is heated in a hot air oven at 105 ± 1°C to constant mass.

13.2.1 Apparatus

A. Moisture Dish: Aluminium, Nickel or Stainless steel, 7-8 cm in diameter, 2-2.5cm deep provided with tight fitting sip on covers.

B. Desiccator: Containing an efficient desiccant

C. Air oven: Electrically heated with thermostatic control.

13.2.2 Procedure

Weigh accurately about 10 g of the sample into a moisture dish which has been dried previously and weighed. Place in air oven for 1 hour at 105 ± 1°C.Remove the dish from the oven, cool in a desiccator and weigh. Repeat the process by keeping the dish in the oven for half hour each time, cool and weigh till two successive weighing do not exceed 1 mg.

13.2.3 Calculation

$$\text{Moisture and volatile matter \%/w} = \frac{(M_1 - M_2)}{(M_1 - M)} \times 100$$

Where,

M_1 = Mass in g of dish with ghee before drying

M_2 = Mass in g of dish with ghee after drying

M = Mass in g of empty dish

(Ref:- IS 3508 – 1966 (Reaffirmed 1997) Methods of sampling and test for Ghee (Butterfat). Bureau of Indian Standards, New Delhi).

13.3 Determination of Butyro Refractometer Reading in Ghee

Refractive index is the ratio of the velocity of light in vacuum to the velocity of light in the sample medium; more generally, it is expressed as the ratio between the sine of angle of incidence to the sine of the angle of refraction, when a ray of light of a definite known wavelength (usually 589.3 mμ the mean of the D-line of sodium) passes from air into ghee. The refractive index of ghee may be read on an Abbe refractometer which gives the true refractive index or on a butyro-refractometer, which reads on an arbitrary scale (B.R. reading) at constant temperature. Since ghee is often solid or semi-solid at room temperature, the B.R. reading is usually taken at 40°C, at which the ghee sample is clear and transparent (BIS, 1966). The general values for B.R. reading of milk fat (40 to 43) and vegetable oils and fats (above 50) are so wide apart that this property can be employed as an indicator for milk fat adulteration with vegetable oils and fats, except coconut oil (38 to 39) and palm oil (39 to 40). The B.R. reading of animal body fats are in the range of 44 to 51 (Kumar *et al*., 2002). An increase in B.R. reading in ghee is also caused by a decrease in the content of lower chain fatty acids, or by an increase either in higher saturated or unsaturated fatty acids, particularly the latter.

13.3.1 Apparatus/ Reagents

A. Precision Butyro-refractometer fitted with an accurate thermometer reading from 40 to 50°C. Check the calibration of the instrument as per the manufacturer's instructions.

B. Hot water circulating device to maintain the temperature of prism constant at 40 ± 1°C.

C. Sodium lamp: Daylight can also be used if the refractometer has an achromatic compensator.

D. Standard fluid for checking the accuracy of the instrument. Usually this fluid is provided by the manufacturer for calibration of the refractometer.

13.3.2 Procedure

A. The sample should be rendered optically clear and free from water and suspended impurities. This can be done by using following procedure.

B. After moisture determination, place the container or glass bottle containing ghee in a water-bath at a temperature not higher than 50°C till completely melted. Filter through a dried, fluted open-texture 15 cm Whatman filter paper Grade 4 with the help of a hot water funnel, directly into receiving bottle. The filtered ghee should be bright and clear. The correctness of the butyro-refractometer shall be tested before carrying out the test with liquid of known refractive index (at 40°C). Open the double prism of the instrument and place a few drops of the ghee sample on prism. Ghee shall completely fill the space between the two prisms, and shall show no air bubble. Close prisms firmly. Allow the instrument to stand for few min before reading is taken so that temperature of ghee sample and instrument are same. The reading shall be taken after ghee has been kept in the prism for 2 to 5 min and after it has been ensured that it has attained constant temperature (40°C) by taking two or more readings. B.R. reading of ghee decreases with the rise in temperature and vice versa. A correction is needed whenever; temperature of the butyro-refractometer is either below or above 40°C.

C. Method of measurement is based upon observation of position of border line on the scale of the instrument. Hold sector firmly and move backward or forward until field of vision is divided into light and dark portion. Line dividing these portions may not be sharp but a band of colours. The colours are eliminated by rotating screw head of compensator until sharp, colourless line is obtained. Read the BR of the sample directly on the scale.

Notes

1. It should be born in mind that presence of free fatty acids considerably lowers the refractive index.
2. In the homologues series of saturated fatty acids from butyric to stearic, the refractive index rises steeply among the lower members, and flattens out at higher chain lengths. A double bond elevates the refractive index: stearic acid has a lower refractive index than oleic, which in turn has a lower value than linoleic.
3. For conversion of refractive index values into butyro-refractometer reading and vice versa use the following formula (Rangappa and Achaya, 1974) or table (BIS, 1966) as follows:

Inter-conversion of Refractive index and Butyrometer Degrees

A. Refractometer index to Butyrometer degrees

Butyrometer reading in degrees = 42.0 + Factor (Observed refractive index - 1.4538)

The factor to be used varies with the range of refractive index, as shown below:

Observed refractive index range	Factor
1.4500 - 1.4515	1400
1.4515 - 1.4530	1410
1.4530 - 1.4545	1420
1.4545 - 1.4560	1430
1.4560 - 1.4575	1440

B. Butyrometer degrees to Refractometer index

Refractometer index = 1.4538 + Factor (Observed BR - 42.0)

The factor to be used varies inversely with temperature

Observed BR reading range	Factor
37.5 - 40.0	0.00072
40.0 - 42.5	0.00071

42.5 - 45.0	0.00070
45.0 - 47.5	0.00069

4. The refractive index decreases with a rise, and increases with a fall in temperature. If the temperature is not exactly at 40°C, X is added to the observed reading for each degree above or subtracted for each degree below 40°C *pro rata,* where

X for butyro-refractometer = 0.55

X for Abbe refractometer = 0.000365

Normally the temperature of observation shall not deviate by more than ± 2°C.

Table 2: Butyro-refractometer Readings and Indices of Refraction

B.R. Reading	Refractive Index	B.R. Reading	Refractive Index	B.R. Reading	Refractive Index
35.0	1.4488	40.5	1.4527	46.0	1.4565
35.5	1.4491	41.0	1.4531	46.5	1.4569
36.0	1.4495	41.5	1.4534	47.0	1.4572
36.5	1.4499	42.0	1.4538	47.5	1.4576
37.0	1.4502	42.5	1.4541	48.0	1.4579
37.5	1.4506	43.0	1.4545	48.5	1.4583
38.0	1.4509	43.5	1.4548	49.0	1.4586
38.5	1.4513	44.0	1.4552	49.5	1.4590
39.0	1.4517	44.5	1.4555	50.0	1.4593
39.5	1.4520	45.0	1.4558		
40.0	1.4524	45.5	1.4562		

The refractive index decreases with a rise, and increases with a fall in temperature. If the temperature is not exactly at 40°C, *X* is added to the observed reading for each degree above or subtracted for each degree below 40°C *prorata,* where

X for butyro-refractometer = 0.55

X for Abbe refractometer = 0.000365

Normally the temperature of observation shall not deviate by more than $\pm$ 2°C.

13.3.3 Accuracy of the Method

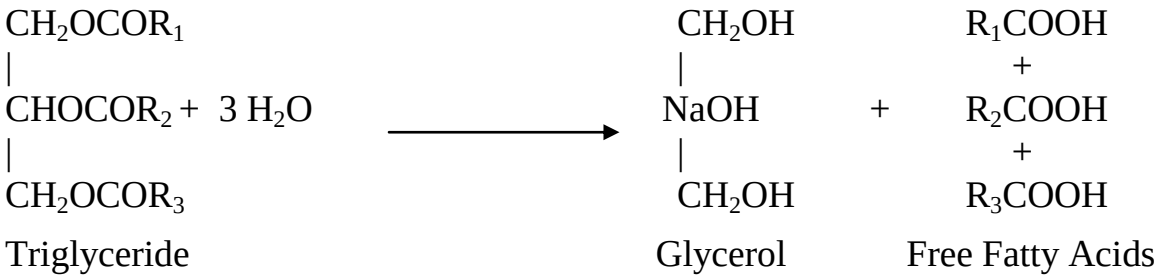
The maximum difference between duplicate determinations shall not exceed 0.0002units for the refractive index and 0.1 for the butyro-refractometer reading.

(Ref : IS 3508 – 1966 (Reaffirmed 1997) Methods of sampling and test for Ghee (Butterfat). Bureau of Indian Standards, New Delhi; Kumar, A.; Lal, D.; Seth, R. and Sharma, R.(2002) Recent trends in detection of adulteration in milk fat: a review: Indian J. Dairy Sci., 55 (6): 319-330. Rangappa, K.S. and Achaya, K.T. 1974. In: Indian Dairy Products. Asia Pub. House, Bombay).

13.4 Determination of Free Fatty Acids in Ghee

The acidity (free fatty acid content) of a fat is normally a measure of the extent to which hydrolysis has liberated the fatty acids from there ester linkage with the parent glyceride molecule. Partly for this reason, acidity of ghee is extensively quoted as a free fatty acid content percent (% FFA). The FFA content of fresh ghee varies from 0.09 to 0.28% with an average of 0.16%. The sensory quality of ghee deteriorates with increase in FFA content. As per FSSAI Rules (2011), ghee should not contain FFA more than 3%.

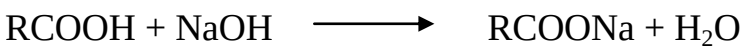
Reaction 1



Level of acidity shows the proneness of fat to oxidation. Highly acidic ghee shows faster oxidation, and thus has poor keeping quality. With the increase in acidity, there is decrease in consumer acceptability.

The FFA present in ghee can be estimated by acid-base titration with alkali (NaOH) using phenolphthalein as an indicator and the end point comes at around pH 8.3 (BIS, 1966).

Reaction2



Apparatus/ Reagents

A. Conical flasks: 250 ml capacity.

B. Burette: 50 ml, graduated to 0.1 ml.

C. Ethanol or rectified spirit: 95% (v/v), sp. gr. 0.816, neutral to phenolphthalein.

D. Sodium hydroxide or Potassium hydroxide: 0.1 N aqueous solution accurately standardized against oxalic acid (AR grade) or potassium phthalate.

E. Phenolphthalein indicator: 1.0% solution in 95 % (v/v) ethanol or rectified spirit.

13.4.1 Procedure

Weigh 10 g of the ghee sample in a 250 ml conical flask. In an another flask bring 50 ml of ethanol to the boiling point and while still above 70°C, neutralize it to phenolphthalein (using 0.5 ml) with 0.1 N NaOH. Add the neutralized alcohol to flask containing ghee sample and mix the contents of the flask. Bring the mixture to boil and while it is still hot, titrate with 0.1 N NaOH, shaking vigorously during the titration. The end point of the titration is reached when the addition of single drop produces a slight, but a definite colour change persisting for at least 15 sec.

13.4.2 Calculation

The acidity of ghee can be expressed in different ways:

13.4.2.1 Acid value: The number of mg of KOH required to neutralize the free fatty acids present in 1 g of the ghee sample using following formula:

Acid value = $\frac{T}{M}$ x 5.61

Where,

T = volume of 0.1 N alkali required for titration in ml;

M = mass in g, of sample taken.

13.4.2.2 Free fatty acids: The acidity of ghee is frequently expressed as the percentage of free fatty acids in the sample, calculated as oleic acid, using following formula:

Free fatty acids (as Oleic acid) = $\frac{T}{M}$ x 2.82

Where

T = volume of 0.1 N alkali required for titration in ml;

M = mass in g, of ghee sample taken.

13.4.2.3 Degree of Acidity

It is the total titratable acidity present in the ghee sample expressed as percentage:

Degree of acidity = $\frac{N}{M}$ x 100

Where,

N = ml of 1 N alkali used for titration;

M = mass in g, of ghee sample taken.

The maximum deviation between duplicate determination shall not exceed 0.2 degree of the acidity or equivalent.

Derivation

1 ml of N NaOH = 1 ml of N oleic acid = 0.282 g of oleic acid

1 ml of 0.1N NaOH = 0.0282 g of oleic acid

Now, if T ml of 0.1N NaOH is used for W gram of the fat sample.

$$\%\text{FFA} = \frac{2.82\ x\ V}{W}$$

(Ref:- IS 3508 – 1966 (Reaffirmed 1997) Methods of sampling and test for Ghee (Butterfat). Bureau of Indian Standards, New Delhi).

13.5 Determination of Reichert-Meissel and Polenske Value in Ghee

The Reichert-Meissl (R.M.) value is the number of ml of 0.1 N aqueous alkali solution required to neutralize the water-soluble, steam volatile fatty acids distilled from 5 g of ghee under the precise conditions specified in the method.

The Polenske value (P.V.) is the number of ml of 0.1 N aqueous alkali solution required to neutralize the water-insoluble, steam volatile fatty acids distilled from 5 g of ghee under the precise conditions specified in the method.

The R.M. value is substantially a measure of the lower fatty acids of ghee like butyric ($C_{4:0}$), and caproic ($C_{6:0}$). Butyric acid contributes about three fourth and caproic acid one-fourth to the R.M. value, while the P.V. is made up of one-fourth caprylic ($C_{8:0}$) and three fourth capric acid ($C_{10:0}$). Since the presence of lower fatty acids is peculiar to milk fat, the R.M. and P.V. are important characteristics of ghee. In general, R.M. value and P.V. for cow and buffalo ghee is about 28 and 1.5 respectively. Of the common

vegetable oils, only coconut and palm kernel contains steam volatile acids, and both exhibit R.M. of 7 and P.V. of 13. In general, ghee is required to have a R.M. value not less than 28. It is however of interest that ghee from milk of animals fed cotton seeds has much lower R.M. value of about 20. Polenske value of cow ghee is higher (2 to 3) than buffalo ghee (1 to 1.5). No significant seasonal variations have, however, been recorded for their fat constants.

In the following method (BIS, 1966), ghee (5 g) is saponified using glycerol-potash diluted with water and acidified, and thereafter steam distilled in a glass apparatus (Polenske distillation apparatus) at a controlled rate. The condensed and cooled distillate is filtered; the water-soluble acids which pass through are estimated by titration with alkali to give the R.M. value, while the water-insoluble acids collected on the filter paper are dissolved out in alcohol and titrated to give the P.V.

Reaction

A. Saponification Step

$$\begin{array}{l} CH_2OCOR_1 \\ | \\ CHOCOR_2 + \ 3NaOH \\ | \\ CH_2OCOR_3 \end{array} \longrightarrow \begin{array}{l} CH_2OH \quad + \ R_1COONa \\ | \\ CHOH \quad + \ R_2COONa \\ | \\ CH_2OH \quad + \ R_3COONa \end{array}$$

Triglyceride — Glycerol — Sodium salt of Fatty acids (soap)

B. Addition of H_2SO_4 after saponification

$$RCOONa + H_2SO_4 \longrightarrow Na_2SO_4 + RCOOH$$

C. Titration of distilled fatty acids for determination of R.M. and P.V.

$$RCOOH + NaOH \longrightarrow RCOONa + H_2O$$

Fatty acids — Sodium salt of fatty acid

13.5.1 Apparatus

A. Graduated Cylinders: 25 ml and 100 ml capacities.

B. Pipette: 50 ml capacity.

C. The assembly of the apparatus for the distillation:

a. Flat-bottom boiling flask (Polenske): The flask shall be made of heat-resistance glass and shall conform to the following details:

Volume contained to bottom of neck	310 ± 10 ml
Length of neck	75 ± 5 mm
Internal diameter of neck	21 ± 1 mm
Overall height	160 ± 5 mm
Diameter of base	45 ± 5mm

Still-head: The still-head shall be made of glass tubing of wall thickness 1.25 ± 025 mm, and shall conform to the shape shown in Figure 5.3, and with the following dimensions:

A	180 ± 5 mm
B	107.5 ± 2.5 mm
C	80 ± 5 mm
D	70 ±5 mm
E	20 ± 2 mm
F	4 ± 1 mm
G (external diameter of bulb)	37.5 ± 2.5 mm
Internal diameter of tubing	8.0 ± 0.5 mm
Acute angle between slopping part of still-head and vertical	60 ± 2°

A rubber stopper, fitted below the bulb of the longer arm of the still-head, and used for connecting it to the flask shall have its lower surface 10 mm above the centre of the side hole of the still-head.

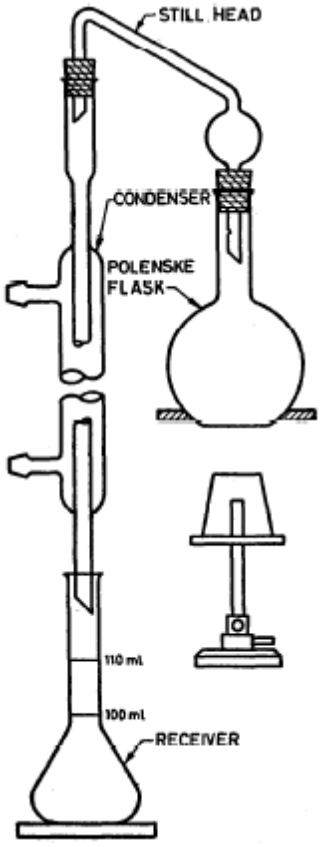
b. Condenser: The condenser shall be made of glass and conform to the following

dimensions:

Overall length	520 ± 5 mm
Length of water jacket	300 ± 5 mm
Length of widened part above water jacket	70 ± 10 mm
Wall thickness of widened part	1.25 ± 0.25 mm
Internal diameter of widened part	20 ± 1 mm
External diameter of inner tube within water jacket	12 ± 0.5 mm
Wall thickness of inner tube	1.0 ± 0.2 mm
Wall thickness of outer jacket	1.25 ± 0.25 mm
External diameter of water jacket	30 ± 2 mm

c. Receiver: The receiver shall be a flask with two graduation marks on the neck, one at 100 ml and the other at 110 ml.

d. Asbestos-Board: An asbestos-board of 120 mm diameter and 6 mm in thickness, with a circular hole of about 65 mm in diameter shall be used to support the flask over the burner. During distillation the Polenske flask shall fit snugly into the hole in the board to prevent the flame from impinging on the surface of the flask above the hole. A new asbestos-board may conveniently be prepared by bevelling the edge of the hole, soaking in water, moulding the edge with a flame, and drying.



e. Gas Burner: The burner should be sufficiently large to allow the distillation to be completed in the time specified in the procedure.

The apparatus shall be supported on a retort stand.

D. Glass Funnel: Of approximate diameter 6 cm.

13.5.2 Reagents

Figure. Apparatus for determination of R.M. Value of Ghee

A. Glycerol: 98% (m/m) conforming to AR grade.

B. Sodium hydroxide solution (50% (m/m)): Dissolve sodium hydroxide in an equal weight of water and store the solution in a bottle protected from carbon dioxide. Use the clear portion free of the solution from deposit.

C. Dilute sulfuric acid: Dilute 25 ml of concentrated sulfuric acid to 1:1 and its concentration is adjusted with water until 40 ml of diluted sulfuric acid neutralize 2 ml of the 50% NaOH solution.

D. Ethanol: 95 % (v/v), neutralized to phenolphthalein immediately before use, or neutralized denatured spirit.

E. Glass Beads: Approximately 1.5 to 2.0 mm in diameter.

F. Phenolphthalein indicator: 0.5% solution in 95% (v/v) ethanol or rectified spirit.

G. NaOH solution: Approximately 0.1 N aqueous solution of NaOH of accurately determined strength.

H. Barium hydroxide solution: 0.1 N.

I. Silver sulfate: Powdered AR grade.

J. Whatman filter paper: Grade 4.

13.5.3 Procedure

A. Weigh 5.00 ± 0.01 g of ghee sample into a Polenske flask. Add 20 g of glycerol and 2 ml of 50% NaOH solution. Heat the flask over a direct flame using a Bunsen burner, with continuous mixing, until ghee, including any drops adhering to the upper parts of the flask, is saponified, and the liquid becomes perfectly clear; avoid overheating during this saponification. Cover the flask with a watch-glass.

B. Make a blank test without ghee, but using the same quantities of reagents and following the same procedure, again avoiding overheating; such overheating would be indicated by darkening of the solution. Measure 93 ml of boiling distilled water, which has been vigorously boiled for 15 min, into a 100 ml graduated cylinder.

C. When the soap is sufficiently cool to permit addition of the water without loss, but before the soap has solidified, add water, draining the cylinder for 5 sec, and dissolve the soap. If the solution is not clear (indicating incomplete saponification), or is darker than yellow (indicating overheating), repeat the saponification with a fresh sample of ghee. Add two glass beads, followed by 50 ml of the dilute sulfuric acid, and connect the flask at once with the distillation apparatus.

D. Heat the flask without boiling its contents, until the insoluble acids are completely melted, then increase the flame and distil 110 ml in between 19 and 21 min. Keep the water flowing in the condenser at a sufficient speed to maintain the temperature of the issuing distillate between 18 and 21°C.

E. When the distillate reaches the 110 ml mark, remove the flame and replace the 110-ml flask by a cylinder of about 25 ml capacity, to collect drainings. Close 110 ml flask with its stopper and without mixing the contents; place it in water at 15°C for 10 min so as to immerse the 110 ml mark. Remove the flask from the water, dry from outside, and invert the flask carefully avoiding wetting the stopper with insoluble acids. Mix the distillate by four or five double inversions, without violent shaking.

F. Filter through a dry 9 cm open-texture filter paper (Whatman filter paper Grade 4) which fits snugly into the funnel. Reject the first runnings and collect 100 ml in a dry volumetric flask; cork the flask and retain the filtrate for titration.

G. Detach the still-head and wash the condenser with three successive 15 ml portions of cold distilled water, passing each washing separately through the cylinder, the 110 ml flask, the filter and the funnel, nearly filling the paper each time and draining each washing before filtering the next. Discard the washings.

H. Dissolve the insoluble acids by three similar washings of the condenser, the cylinder, and the filter, with 15 ml of neutralized ethanol, collecting the solution in the 110-ml flask and draining the ethanol after each washing. Cork the flask, and retain the solution for titration (for Polenske value).

I. Reichert-Meissl or soluble volatile acid value: Pour 100 ml of the filtrate (obtained in step F) containing the soluble volatile fatty acids into a titration flask, add 0.1 ml of phenolphthalein indicator and titrate with 0.1 N NaOH solution until the liquid

becomes pink.

J. Polenske or insoluble volatile acid value: Titrate the alcoholic solution of the insoluble volatile acids after addition of 0.25 ml of phenolphthalein indicator, with the 0.1 N barium or sodium hydroxide solution until the solution becomes pink.

13.5.4 Calculations

Reichert-Meissl Value = 1.10 (T_1– T_2)

Polenske value = T_3– T_4

Where

T_1 = volume in ml of 0.1 N barium or sodium hydroxide solution used for sample under step I;

T_2 = volume in ml of 0.1 N barium or sodium hydroxide solution used for blank under step I;

T_3 =volume in ml of 0.1 N barium or sodium hydroxide solution used for sample under step J,

T_4 = volume in ml of 0.1 N barium or sodium hydroxide solution used for blank under step J;

Polenske values, and to a much slighter extent Reichert – Meissl values, have been found to be low when determined at low barometric pressures, such as may occur at high altitudes. The following factors may be applied to values determined at a barometric pressure to convert them to the values determined at normal pressure.

$$\text{Correct Reichert Value} = \frac{(\text{Observed value} - 10)\log 760}{\log P} + 10$$

$$\text{Corrected Polenske Value} = \text{Observed value} \times \frac{760 - 45}{P - 45}$$

Where,

P = barometric pressure in mm of mercury at the place and time of determination.

13.5.5 Accuracy of the Method

Reichert-Meissl Value: The maximum deviation between duplicate determinations shall not exceed 0.5 units.

Polenske Value: The maximum deviations between duplicate determinations shall not exceed 0.3 units.

(Ref:- IS 3508 – 1966 (Reaffirmed 1997) Methods of sampling and test for Ghee (Butterfat). Bureau of Indian Standards, New Delhi).

13.6 Test for Detection of Vanaspati in Ghee (Boudouins Test)

The development of a permanent pink colour in a sample of ghee with furfural solution in the presence of hydrochloric acid, indicates the presence of sesame oil and the test is known as Baudouin test. According to the Govt. of India, Ministry of Agriculture Notification (1962), the edible hardened oil (vanaspati) shall contain raw or refined sesame (til) oil not less than 5% by weight i.e. addition of sesame oil in other oils is a must for the manufacture of hydrogenated edible fat. This has been made compulsory because the specific chromogenic constituents of sesame oil can be easily detected, and hence adulteration of ghee with hydrogenated fats can be easily established. The unsaponifiable matter of sesame oil contains two chromogenic constituents namely sesamolin and sesamol which are not found in other fats. The sesamol on condensation with furfural produces the pink colour in the Baudouin test.

13.6.1 Reagents

A. Hydrochloric acid (fuming): Sp. gr. 1.19.

B. Furfural solution: 2 percent solution of furfural, in ethanol, distilled not earlier than 24 h prior to the test from rectified spirit.

13.6.2 Procedure

A. Take 5 ml of the melted ghee in 25 ml measuring cylinder provided with a glass stopper, and add 5 ml of hydrochloric acid and 0.4 ml of furfural solution. Insert the glass stopper and shake vigorously for 2 min. Allow the mixture to separate.

B. The development of a pink or red colour in the acid layer indicates presence of sesame oil. Confirm by adding 5 ml of water and shaking again. If the colour in acid

layer persists, sesame oil is present. If the colour disappears, it is absent.

(Ref:- IS 3508 – 1966 (Reaffirmed 1997) Methods of sampling and test for Ghee (Butterfat). Bureau of Indian Standards, New Delhi).

13.7 Detection of Vegetable Fat in Ghee

Please refer to Methods of Analysis of Oils and Fats for above tests

13.8 Determination of Peroxide Value in Ghee

The most common cause of milk fat deterioration is rancidity which is due to oxidation, thereby affecting its flavour and quality. The acceptability of ghee largely depends on the extent to which the oxidative deterioration has occurred. It is generally considered that the first product formed by oxidation of an oil or fat is a hydroperoxide. The peroxides further decompose to secondary oxidation products i.e. aldehydes and ketones which impart off flavour in ghee. The usual method of assessment of rancidity in ghee is by determination of peroxide value (PV) which is reported in units of milliequivalents of peroxide oxygen per kg of fat or ml of 0.002 N sodium thiosulphate per g of sample. The most common method for PV determination is based on iodometric titration which measures the iodine produced from potassium iodide by the peroxides present in a fat or oil. PV is an indicator of products of primary oxidation and thus measures the rancidity or degree of oxidation but not the stability or shelf-life of a fat. Fresh ghee has a PV equal to zero. According to its PV, ghee is graded as follows:

Peroxide value	Grade
Below 1.5	very good
1.6 to 2.0	good
1.1 to 2.5	fair
1.6 to 3.5	poor
3.6 to 4.0	not acceptable

However, peroxide value varies considerably at the organoleptic threshold of the rancidity. As per BIS (BIS, 1966), two methods are recommended for the determination

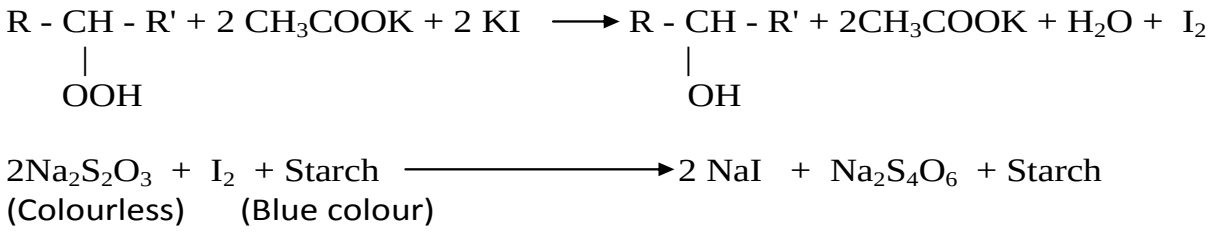
of PV of ghee i.e. iodometric method and oxygen absorption method. Here iodometric method has been described.

13.8.1 Iodometric method

Hydroperoxides are the first detectable products of autooxidation and are sufficiently stable to keep accumulating for some time. Hydroperoxides are oxidizing agent and they liberate iodine from KI and the liberated iodine can be estimated by titrating against standard sodium thiosulphate ($Na_2S_2O_3$) using starch as indicator. The liberated iodine is directly proportional to PV of the ghee sample.

The method described here is very simple and inexpensive, requiring only conventional laboratory glassware. However, this method is not satisfactory as considerable flavour deterioration occurs at peroxide value below the limit that can be accurately determined by this method. On storage of ghee at 37°C for two months, no peroxides could be detected. At the end of three and four months storage period, the average peroxide value of ghee has been reported to 1.80 and 2.70 respectively. Buffalo ghee samples showed higher rate of development of peroxides as compared to cow ghee.

Reaction



13.8.1.1 Reagents

A. Acetic acid – Chloroform solution - Mix 3 volumes of acetic acid with 2 volumes of chloroform.

B. Potassium iodide solution, saturated – Dissolve excess KI in freshly boiled water. Excess solid must remain. Store in dark. Test daily by adding 0.5 ml to 30 ml CH_3COOH-$CHCl_3$ (solution a), then add 2 drops of 1% starch solution. If solution turns blue requiring more than 1 drop of 0.1 N Na_2S_2O3 to discharge colour, prepare

fresh solution.

C. Sodium Thiosulphate standard solution – 0.1 N and 0.01N. For 0.01 N dilute 0.1 N with freshly boiled and cooled water.

13.8.1.2 Procedure

Weigh 5±0.5 g sample in a 250 ml glass stoppered Erlenmeyer flask. Add 30 ml of acetic acid – chloroform solution and swirl to dissolve. Add 0.5 ml of saturated KI solution from Mohr pipette, let stand with occasional shaking 1 minute and add 30 ml water. Slowly titrate with 0.1 N sodium thiosulphate solution with vigorous shaking until yellow is almost gone. Add about 0.5 ml of starch solution and continue titration shaking vigorously to release all iodine from chloroform layer until blue just disappears. If less than 0.5 ml 0.1 N $Na_2S_2O_3$ is used, repeat determination with 0.01 N $Na_2S_2O_3$. Conduct blank determination (must be less than 0.1 ml 0.1N $Na_2S_2O_3$). Subtract from sample titration.

Peroxide Value (millieqvt oxygen / kg oil) = $\frac{S \times N \times 1000}{\text{Weight of Sample}}$

Where

S = ml NaS_2O_3 (blank corrected) and

N = Normality of NaS_2O_3

(Ref :- A.O.A.C 17thedn, 2000 Official method 965.33 Peroxide Value of Oils and Fats).

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14.1. Preparation of Sample – Refer to Section 4.1 on Dahi.

14.2. Determination of Milk Solids – Refer to Section 4.4 on Dahi.

14.3. Determination of Fat – Refer to Section 5.3 on Channa.

14.4. Determination of Protein – Refer to Section 7.5 on Ice Cream.

14.5. Determination of Titratable Acidity -Refer to Section 9.5 on Condensed milk.

14.6. Determination of Total Ash – Refer to section 10.7 on Milk Powder.

14.7. Determination of Sucrose Content – Refer to section 9.4 on Condensed Milk.

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15. YOGHURT

15.1. Preparation of Sample of Yoghurt

For plain, skimmed, low fat, flavoured and sweetened yoghurt: Bring the yoghurt sample to the room temperature (preferably 25°C). Mix the sample carefully by means of spatula or spoon using a rotary motion which passes from the lower layers to the surface layers of the sample so as to displace and mix them well.

For Fruit yoghurt: Bring the fruit yogurt sample to the room temperature (preferably 25°C). Homogenize it using an appropriate device, in order to facilitate the grinding and dispersion of fruits etc.

15.2. Determination of Milk Solids not Fat in Yoghurt

Determine Total solids as in curd Section 4.4(Either by method described in Section 4.4.1 or 4.4.2) and sugar as described in Section 9.4.1- Lane – Eynon Method for Determination of Sucrose in Condensed Milk).

Total milk solids = Total solids – Added sugar

15.3. Determination of Milk Fat in Yoghurt

Refer to determination of fat in milk using Rose Gottleib Method (See Section 1.3.4.2).

15.4. Determination of Protein Content in Yoghurt

Total nitrogen content in yoghurt sample is estimated by Kjeldahl method as described for milk. The percent nitrogen obtained is multiplied by a factor to get protein content in yoghurt sample.

Take 4 -5 g of sample and refer to the method mentioned in Section 19.

A. Follow method mentioned in Section 19.1 for **Determination of Total Nitrogen/Crude Protein in Milk.**

Calculate Protein Content as follows

Plain Yoghurt = N x 6.38

Fruit Yoghurt = N x 6.25

N = Nitrogen content in sample estimated by Kjeldahl method

15.5. Determination of Titratable Acidity in Yoghurt

Refer to Section 9.5 (Determination of Titratable acidity in Condensed Milk).

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16. WHEY POWDER

16.1. Preparation of Sample of Whey Powder

Make homogeneous either by mixing or shaking or alternately rolling and inverting container. Avoid excessive temperature and humidity when opening sample container to prevent absorption of moisture.

16.2. Determination of Moisture in Whey Powder

Follow method given in Section 10.2 for Milk Powder.

16.3. Determination of Fat in Whey Powder

Follow method given in Section 10.3. for Milk Powder.

16.4. Determination of Milk Protein in Whey Powder

Total nitrogen content in whey powder sample is estimated by Kjeldahl method as described for milk. The percent nitrogen obtained is multiplied by a factor to get protein content in whey powder sample.

Take 1 – 1.3 g of sample and refer to the method mentioned in Section 19.

A. Follow method mentioned in Section 19.1 for **Determination of Total Nitrogen/Crude Protein in Milk.**

Calculate Protein Content as follows = N x 6.38

N = Nitrogen content in sample estimated by Kjeldahl method

16.5. Determination of Total Ash in Whey Powder

Follow method given in Section 10.7 for Milk Powder.

16.6. Determination of pH in Whey Powder

Prepare 10% solution of sample by dissolving 10 g of whey powder sample in distilled water and making final volume to 100 ml. Determine electrometrically with a pH meter calibrated against standard buffer solutions.

16.7. Determination of Lactose in Whey Powder

Determine reducing sugars as per Section 9.4 (for Condensed Milk) and calculate

as anhydrous lactose.

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17. EDIBLE CASEIN PRODUCTS

17.1. Preparation of Sample of Casein/Caseinates

Thoroughly mix the sample by repeatedly shaking and inverting the container. Transfer about 50 g of the thoroughly mixed laboratory sample to the test sieve. Transfer about 50 g of the thoroughly mixed sample to the test sieve (500 μm). If the 50 g portion directly passes or almost completely passes through the sieve, use it for the determination. Otherwise, grind the 50 g portion, using the grinding device until it passes the sieve. Immediately transfer the entire sieved sample to an air-tight container of sufficient capacity and mix thoroughly by repeatedly shaking and inverting. During these operations take precautions to avoid any change in the water content of the product. After the test sample has been prepared, the determination should be proceeded with as soon as possible.

17.2. Determination of Moisture in Casein/Caseinates

Follow method given in Section 10.2 for Milk Powder.

17.3. Determination of Fat in Casein/Caseinates

In the following procedure, a test portion is digested with hydrochloric acid followed by addition of ethanol. The acid-ethanolic solution is subsequently extracted with diethyl ether and light petroleum followed by removal of solvents by distillation or evaporation and determination of mass of the substances extracted which are soluble in light petroleum. The following method is as per IDF guidelines.

17.3.1. Reagents

A. Hydrochloric acid solution: ρ_{20} approximately 1.125 g/ml. Dilute 675 ml of concentrated hydrochloric acid , ρ_{20} 1.18 g/ml, to 1000 ml with water.

B. Ethanol or ethanol denatured by methanol : at least 94% (w/v).

C. Congo red solution: Dissolve 1 g of Congo red in water and dilute to 100 ml

D. Diethyl ether: free from peroxides containing no, or not more than 2 g/kg of antioxidants.

E. Light Petroleum: having any boiling range between 30 and 60°C.

F. Mixed solvents: prepared shortly before use by mixing equal volumes of diethyl ether and the light petroleum.

17.3.2. Apparatus

A. Analytical balance.

B. Centrifuge: For the centrifugation of fat-extraction flasks or tubes at a 500 to 600 rpm to produce a gravitational field of 80 to 90 g at the outer end of the flasks or tubes (the use of the centrifuge is optional but recommended).

C. Distillation or evaporation apparatus: To enable the solvents and ethanol to be distilled from the fat-collecting flasks or to be evaporated from beakers and dishes at a temperature not exceeding 100°C.

D. Drying oven capable of being controlled at 102 ± 2°C.

E. Boiling water bath or hot plate.

F. Mojonnier type fat-extraction tube: the flask shall be provided with good quality bark corks or stoppers of other material for e.g. silicon rubber or polytetrafluoroethylene (PTFE) unaffected by the reagents used.

G. Fat-collecting vessels: for e.g. flat bottom flask of capacity 125 to 250 ml.

H. Measuring cylinder: of capacities 5 and 25 ml.

I. Pipettes: graduated of capacity 10 ml.

17.3.3. Procedure

A. Mix the test sample and weigh immediately to the nearest 1 mg, directly or by difference, into a fat-extraction flask, or into 100 ml beaker or flask, 2 to 3 g of the test sample. Add 7.5 to 10 ml of HCl solution so as to wash the test portion into the small bulb of the extraction flask. (as per BIS method, take 5 g of the sample and 10 ml of HCl solution).

B. Carry out a blank test simultaneously with the determination, using the same procedure and same reagents, but omitting the test portion.

C. Heat by gently moving the vessel in a boiling water-bath or on a hot plate, until all the particles are entirely dissolved. Allow the vessel to stand for 20 to 60 min in the

boiling water bath, shaking occasionally during the initial 15 min or keep it gently boiling over the flame or on to the hotplate for 10 min. Cool in running water.

D. Add 10 ml of the ethanol and mix gently but thoroughly by allowing the contents of the flask to flow backward and forward between the two bulbs; avoid bringing the liquid too near to the neck of the flask.

E. Add 25 ml of the diethyl ether, close the flask with a cork saturated with water or with a stopper wetted with water, and shake the flask vigorously but not excessively (to avoid the formation of persistent emulsions) for 1 min with the flask in a horizontal position and the small bulb extending upwards, periodically allowing the liquid in the large bulb to run into the small bulb.

F. Carefully remove the cork and add 25 ml of the light petroleum. Close the flask with the rewetted cork (by dipping in water) and shake the flask gently for 30 sec.

G. Centrifuge the closed flask for 1 to 5 min at a 500 to 600 rpm. If a centrifuge is not available, allow the closed flask to stand in the rack for at least 30 min until the supernatant layer is clear and distinctly separated from the aqueous layer. If necessary, cool the flask in running water.

H. Carefully remove the cork and rinse it and the inside of the neck of the flask with a little of the mixed solvent so that the rinsings run into the flask or into the fat-collecting vessel. If the interface is below the bottom of the stem of the flask, raise it slightly this level by gently adding water down the side of the flask to facilitate the decantation of solvent.

I. Carefully decant as much as possible of the supernatant layer into the fat-collecting vessel containing a few boiling aids avoiding decantation of the aqueous layer. Rinse the outside of the neck of the Majonnier flask with a little of the mixed solvent, collecting the rinsings on the fat collecting vessel.

J. Carry out a second extraction by repeating the operations described above but using only 15 ml of the diethyl ether and 15 ml of the light petroleum; use the ether to rinse the inside of the neck of the Mojonnier flask. If necessary, raise the interface to slightly above the middle of the stem of the flask to enable the final decantation of solvent to be as complete as possible.

K. Remove the solvents (including ethanol) as completely as possible from the flask by distillation, or from fat-collecting vessel by evaporation, rinsing the inside of the neck of the flask with a little of the mixed solvent before commencing the distillation.

L. Heat the fat-collecting vessel (flask placed on its side to allow solvent vapour to escape) for 1 h in the drying oven, controlled at 102 ± 2°C. Remove the fat-collecting vessel from the oven, allow to cool (not in a desiccator, but protected from the dust) to room temperature (for at least 1 h) and weigh to the nearest 0.1 mg. Do not wipe the vessel immediately before weighing. Place the vessel on the balance using tongs (to avoid in particular, temperature variations).

M. Repeat the operations of heating and weighing until the mass of the fat-collecting vessel decreases by 0.5 mg or less, between 2 successive weighing. Record the minimum mass as the mass of the fat-collecting vessel and extracted matter.

17.3.4. Calculation

The fat content, expressed as a percentage by mass, is equal to

$$= \frac{(M_1 - M_2) - (M_3 - M_4)}{M_0} \, x \, 100$$

Where,

M_0 is the mass in g of the test portion.

M_1 is the mass in g of the fat-collecting vessel along with the extracted matter.

M_2 is the mass in g of the empty fat-collecting vessel.

M_3 is the mass in g of the fat-collecting vessel used in the blank test and any extracted matter determined.

M_4 is the mass in g of the empty fat-collecting vessel used in blank test.

Calculate result to the nearest 0.01 % (m/m).

17.4. Determination of Milk Protein in Casein/Caseinates

Total nitrogen content in casein/caseinates sample is estimated by Kjeldahl method as described for milk. The percent nitrogen obtained is multiplied by a factor to get protein content in casein/caseinates sample.

Take 1 – 1.3 g of sample and refer to the method mentioned in Section 19.

A. Follow method mentioned in Section 19.1 for **Determination of Total Nitrogen/Crude Protein in Milk.**

Calculate Protein Content as follows = N x 6.38

N = Nitrogen content in sample estimated by Kjeldahl method

17.5. Determination of Casein in Protein in Casein/Caseinates

Casein is precipitated from milk at pH 4.6 using acetic acid and sodium acetate solutions in kjeldahl flask. The acidified solution which contains the non-casein nitrogen components is separated from casein precipitate by filtration. Nitrogen content of the casein precipitate is determined by kjeldahl method and multiplied by 6.38 to obtain casein in protein

17.5.1. Reagents

A. Sodium acetate solution – 1 M / litre using AR sodium acetate or sodium acetate trihydrate – Transfer 4.10±0.1 g sodium acetate or 6.80±0.1 g sodium acetate trihydrate into 50 ml volumetric flask and dilute to volume with water. Prepare fresh weekly.

B. Acetic acid solution – 10% using A.R grade glacial acetic acid

C. Buffer solution – Dilute 1± 0.1 ml sodium acetate and 1± 0.1 ml 10 5 acetic acid to 100 ml with water. Prepare fresh weekly.

17.5.2. Procedure

Weigh 1- 2 g sample into Kjeldahl flask, add 50 ml water. Add 0 .75 ml 10% acetic acid to flask and swirl gently. Leave mixture for 10 minutes. Add 0.75 ml of sodium acetate and swirl gently. Pour mixture from kjeldahl flask on to pleated filter paper (Whatman No1, 15 cm) and collect filtrate. Let drain completely before next pour.

Add 30 ml of buffer solution to kjeldahl flask, swirl to mix. Pour mixture on to filter paper after first filtration is complete and combine filtrates. Add another 30 ml of buffer solution and add filtrate to the previous 2 filtrates. Filtrate should be clear and free of particulate matter. If particulates appear recycle filtrate through the same filter paper or repeat test. Remove filter paper. Ensure no precipitate is lost on filter paper. Drop filter paper into kjeldahl flask, add pot sulphate and copper sulphate and Sulphuric acid and digest Determine nitrogen content and multiply by 6.38 to obtain casein Protein.

(Ref:- A.O.A.C 17th edn, 2000 Official Method 998.06 Casein Nitrogen Content of Milk).

17.6. Determination of Lactose Content in Caseins/caseinates (by Photometric Method)

The first step in this procedure (IS: 11963-1987) is the dissolution of a test portion of the sample by any of the following procedure:

- in hot water in the case of caseinates.
- in hot water with the addition of sodium hydrogen carbonate in the case of acid caseins.
- In hot water with the addition of pent sodium triphosphate in the case of rennet casein.

The casein is then precipitated by addition of acetic acid and sodium acetate solution at pH 4.6, followed by filtration to obtain a protein-free solution of the carbohydrates. The colour is developed in an aliquot portion of filtrate (by the addition of phenol and concentrated sulphuric acid) which is proportional to the amount of lactose present. The developed colour is read at 490 nm is a spectrophotometer.

17.6.1. Reagents

A. Sodium hydrogen carbonate ($NaHCO_3$): For analysis of acid casein.

B. Pent sodium triphosphate ($Na_5P_3O_{10}$): For analysis of rennet casein.

C. Hydrochloric acid or sulphuric acid: 0.1N.

D. Acetic acid: 100 g/L solution.

E. Sodium acetate (CH_3COONa) solution: 1 N.

F. Phenol: 80% (m/m) solution.

G. Concentrated sulphuric acid : ρ_{20} = 1.84 g/ml.

H. Lactose (20g/L) solution: Weigh 2.105 ± 0.001 g of lactose monohydrate, corresponding to 2.00 g of the anhydrous lactose, into a 100 ml volumetric flask. Dissolve lactose in hot water, make up to volume and mix well. Store the solution at 0°C.

17.6.2. Apparatus

A. Conical flasks: 100 ml capacity.

B. Pipettes: 1, 2 and 10 ml capacities.

C. Graduated pipettes: 25 ml capacity.

D. Volumetric flasks: 100 ml capacity.

E. Test tubes: 40 ml capacity ground neck and fitted with ground glass stoppers.

F. Automatic dispenser: Capable of dispensing 5 ml of concentrated sulphuric acid within 1sec.

G. Micropipettes: 0.2 ml capacity with 0.001 ml divisions

H. Filter paper: Whatman filter paper Grade 42.

I. Water-baths: Maintained at (a) 60 to 70°C, (b) at 20°C.

J. Spectrophotometer: Suitable for making measurements at a wavelength 490 mm, provided with cuvettes of optical path length of 1 cm.

17.6.3. Procedure

17.6.3.1. Determination

A. Weigh to the nearest 1 mg about 1 g of the test sample into a 100 ml conical flask. Add the reagents as the case may be:

a) in the case of acid casein, add 0.1 ± 0.001 g of the sodium hydrogen carbonate.

b) In the case of rennet casein, add 0.1 ± 0.001 g of the pent sodium Add 25 ml of water, place the flask in the water-bath controlled at 60 - 70°C, and mix occasionally by shaking.

B. When the test portion is completely dissolved (after 10 to 15 min), cool and add successively:

- 15 ml of water,
- 8 ml of hydrochloric acid or sulfuric acid solution.
- 1 ml of the acetic acid solution.
- Stopper and mix the contents by shaking after each addition.

C. Leave for 5 min and then add 1 ml of the sodium acetate solution. Mix by shaking.

D. Allow the casein precipitate to settle, then filter through a dry Whatman filter paper Grade 42. Discard the first few ml of the filtrate.

E. Simultaneously carry out a blank by taking 0.1 ± 0.001 g of sodium hydrogen carbonate or 0.1 ± 0.001 g of pent sodium triphosphate, as appropriate, (the reagent shall be same as used in case of test sample), using the same apparatus and reagents in the same amount in above 3 steps.

F. Pipette 2 ml of the filtrate into a test tube, add 0.2 ml of the phenol solution by means of a micropipette, and mix by shaking. Then add from the automatic dispenser, in less than 1 sec, 5 ml of concentrated sulfuric acid, directing the stream of acid against the liquid surface rather than against the side of the test tube in order to obtain good mixing. Immediately mix, using the mixer, and allow to stand for 15 min. Cool for 5 min in the water bath at 20°C.

G. Wipe the tube and precede immediately measure the absorbance of the solution at 490 nm using the blank solution as the reference. If the absorbance is above the upper limit of the calibration graph, repeat step 7 using 2 ml of a suitable dilution of the filtrate instead of 2 ml of the filtrate itself.

Note: If such a dilution is made, the formula given for calculation has to be modified accordingly.

17.6.3.2. Preparation of the Calibration Graph

A. Pipette 10 ml of the lactose solution (20 g/L solution) into 100 ml volumetric flask and dilute to the mark with water (solution A); 1 ml of solution A corresponds to 2 mg of anhydrous lactose.

B. Prepare three standard solutions by pipetting 1, 2 and 3 ml of solution A into three 100 ml volumetric flasks and making up the volumes with water. The anhydrous lactose concentrations of the standard solution obtained are respectively 20, 40 and 60 µg/ml.

C. Using four test tubes, proceed in accordance with step 7, but replace 2 ml of filtrate respectively by 2 ml of each of the three standard solutions and by 2 ml of water.

D. Measure the absorbances of the three standard matching solutions using the solution obtained by treatment of the 2 ml of water as the reference liquid.

E. Construct a calibration curve by plotting the absorbances of the standard matching solutions against their anhydrous lactose concentrations in µg/ml. Draw the best-fitting line through the calibration points.

17.6.3.3. Calculation

The lactose content of the sample, expressed as anhydrous lactose as a percentage by mass, is calculated using the following formula.

$$= \left(\frac{\frac{C}{106} \times 50}{M}\right) \times 50$$

Where,

c =concentration, in µg/ml, of anhydrous lactose in the test solution, read from the calibration curve.

m = mass in g, of the test portion.

(Ref:- IS 11963 – 1987 ((Reaffirmed 1997). Method for determination of lactose content by photometric method in caseins and caseinates. Bureau of Indian Standards, New Delhi).

17.7. Determination of Ash Content in Casein/Caseinates

In this method (IS: 11962-1987) a test portion of the sample is incinerated at 825 ± 25°C and the weight of the residue obtained is recorded. The following method is as per IDF guidelines.

17.7.1. Apparatus

A. Silica or platinum dish: about 70 mm diameter and 25 to 50 mm deep.

B. Electrical furnace with air circulation, capable of being controlled at 825 ± 25°C.

C. Desiccator: Containing an effective desiccant.

17.7.2. Procedure

A. Heat the dish in the electrical furnace, controlled at 825 ± 25°C, for 30 min. allow the dish to cool in the desiccator to the room temperature and weigh to the nearest to 0.1 mg.

B. Weigh, to the nearest 0.1 mg directly in or by difference into the prepared dish, approximately 3 g of the test sample.

C. Heat the dish with its content on a low flame until the test portion is completely charred, taking care that it does not burst into flame.

D. Transfer the dish to the electrical furnace, controlled at 825 ± 2°C, and heat for at least 1 h until all carbon has disappeared from the dish. Allow the dish to cool in the desiccator to the room temperature and weigh to the nearest 0.1 mg.

E. Repeat the operations of heating in the electrical furnace, cooling and weighing, until the mass remains constant to within 1 mg or begins to increase. Record the minimum mass.

17.7.3. Calculation

The ash of the sample, as a percentage by mass, is calculated using the following formula.

$$= \frac{M_1 - M_2}{M_0}\ x\ 100$$

Where

M_0 = mass in g, of the test portion.

M_1 = mass in g, of the dish and residue.

M_2 = mass in g, of the prepared dish.

Calculate the ash to the nearest 0.01% and report the final result to the nearest 0.1%.

Calculate the ash of the sample on the dry basis

To calculate the ash of the sample on the dry basis, as a percentage by mass, multiply the result obtained as above by

$$= \frac{100}{100 - M}$$

where, M is the water content of the sample.

(Ref:- IS 11962 – 1987 (Reaffirmed 1997). Method for determination of ash in rennet caseins and caseinates (Reference Method). Bureau of Indian Standards, New Delhi).

17.8. Determination of Fixed Ash (ash including P_2O_5) Content in in Casein/Caseinates

The designation "fixed ash" is used to indicate that the organic phosphorus is retained in the ash. In the following procedure (IS: 11919-1987) a test portion of the sample is incinerated at 825 ± 25°C in the presence of magnesium acetate (added to bind all phosphorus of organic origin). The resulting ash is weighed and mass of the ash originating due to magnesium acetate is subtracted.

17.8.1. Apparatus

A. Pipette: 5 ml capacity.

B. Silica or platinum dishes: About 70 mm diameter and 25 to 50 mm deep.

C. Hot air oven: Maintained at 102 ± 2°C.

D. Electrical furnace with air circulation, capable of being controlled at 825 ± 25°C.

E. Boiling water-bath.

F. Desiccator: containing an effective desiccant.

17.8.2. Reagent

Magnesium acetate tetra hydrate [$Mg(CH_3CO_2)_2.4H_2O$]: 120 g/L solution.

17.8.3. Procedure

A. Heat two dishes in the electrical furnace controlled at 825 ± 2±°C for 30 min. Allow the dishes to cool in the desiccator to the room temperature and weigh to the nearest 0.1 mg.

B. Weigh, to the nearest accurately 0.1 mg directly in or by difference into one of the prepared dish (A), approximately 3 g of the test sample.

C. Using the pipette, add to the dish (A) exactly 5 ml of the magnesium acetate solution so as to wet this entire portion and allow standing for 20 min. To the other prepared dish (B), add 5 ml of magnesium acetate solution.

D. Evaporate the contents of both dishes (A and B) to dryness on the boiling water-bath.

E. Place both the dishes in the oven controlled at 102 ± 2°C for 30 min. Heat dish A with its content on a low flame until the test portion is completely charred, taking care that it does not burst into flame.

F. Transfer both the dishes (A and B) to the electrical furnace controlled at 825 ± 25°C, and heat for at least 1 h until all carbon has disappeared from dish A. Allow both dishes to cool in the desiccator to the room temperature and weigh to the nearest 0.1 mg.

G. Repeat the operations of heating in the electrical furnace, cooling and weighing, until the mass remains constant to within 1 mg. Record the minimum mass.

17.8.4. Calculation

The "fixed ash" of the sample, including phosphorus, as a percentage by mass is calculated by the following formula

$$= \frac{(M_1 - M_2) - (M_1 - M_4)}{M_0}\ x\ 100$$

Where

M_0 = mass in g, of the test portion;

M_1 = mass in g, of dish A and residue;

M_2 = mass in g, of the prepared dish A;

M_3 = mass in g, of the dish B and residue;.

M_4 = mass in g, of the prepared dish B.

Calculate the "fixed ash" to the nearest 0.01% and report the final result to the nearest 0.1%.

Calculation of the "fixed ash" of the sample on the dry basis

To calculate the "fixed ash" of the sample on the dry basis, as a percentage by mass, multiply the result obtained as above by

$$= \frac{100}{100 - M}$$

where, M is the water content of the sample.

(Ref:- IS 11919 – 1987 (Reaffirmed 1997). Method for determination of fixed ash in rennet caseins and caseinates (Reference Method). Bureau of Indian Standards, New Delhi).

17.9. Determination of Free Acidity in Caseins/Caseinates

Free acidity in caseins is the volume in ml, of a 0.1 N standard sodium hydroxide solution required to titrate an aqueous extract of 1 g of casein sample using phenolphthalein as indicator. This is determined by (IS: 11964-1987) aqueous extraction of test portion of the sample at 60°C followed by, filtration and then titration of the filtrate with a standard NaOH solution to the end point of phenolphthalein.

17.9.1. Reagents

A. Standard sodium hydroxide solution: 0.1 N.

B. Phenolphthalein indicator: Dissolve 1g of phenolphthalein in 100 ml of 95% ethanol (v/v).

17.9.2. Apparatus

A. Conical flask: 500 ml capacity, with ground neck and fitted with a ground glass stopper and 200 ml capacity.

B. Pipette: 100 ml capacity.

C. Burette; 50 ml capacity, graduated in 0.1 ml.

D. Measuring cylinder: 250 ml capacity.

E. Water-bath Maintained at a temperature of 60 ±2°C.

F. Appropriate filter.

17.9.3. Procedure

A. Weigh about 10 g of the test sample to the nearest 10 mg and transfer it to the conical flask (500 ml capacity).

B. Using the 250 ml measuring cylinder, add 200 ml of water (at 60°C and already boiled). Stopper the flask, mix by swirling and place in the water-bath at 60°C for 30 min. Shake the flask at intervals of about 10 min.

C. Filter the content and cool the filtrate to about 20°C. The filtrate must be clear.

D. Transfer 100 ml of the cooled filtrate into the conical flask (200 ml capacity) using the pipette. Add 0.5 ml of the ethanolic phenolphthalein solution using the pipette.

E. Titrate with the standard NaOH solution until the appearance of faint pink colour, persisting for at least 30 sec. Record the volume used to 0.01 ml.

17.9.4. Calculation

The free acidity of the casein is calculated by suing following formula.

$$= \frac{20 \times V \times T}{M}$$

where,

V = volume, in ml, of the standard NaOH solution used;

T = normality of the standard NaOH solution;

M = mass in g, of the test portion.

Calculate the free acidity to the nearest 0.01.

Calculation of the free acidity of the sample on the dry basis

To calculate the free acidity of the sample on the dry basis, multiply the result obtained as above by

$$= \frac{100}{100 - M}$$

Where, M is the water content of the sample.

(Ref:- IS 11962 – 1987 (Reaffirmed 1997). Method for determination of free acidity in rennet caseins (Reference Method). Bureau of Indian Standards, New Delhi).

17.10. Determination of pH in Casein/Caseinates

In this method (IS: 11978-1987), pH of an aqueous extract of casein or an aqueous solution of caseinate is determined at 20°C, using a pH meter.

17.10.1. Reagents

Buffer solutions, for calibration of the pH meter: Two standard buffer solutions with pH values at 200°C which are known to the second decimal place and will bracket the pH value of the sample under test, for example phthalate buffer solution of pH approximately 4 and a borax buffer solution of pH approximately 9. In addition, a phosphate buffer solution of pH approximately 7 may be used.

17.10.2. Apparatus

A. pH meter, minimum sensitivity 0,05 pH unit, with a suitable glass electrode and a calomel or other reference electrode.

B. Thermometer: Accuracy 0.5°C.

C. Conical flask: 100 ml capacity, fitted with a ground glass stopper.

D. Beaker: 50 ml capacity.

E. Mixer.

F. Beaker: For the mixer, of at least 250 ml capacity.

17.10.3. Procedure

17.10.3.1. Calibration of pH meter

Adjust the temperature of the buffer solutions to 20°C and calibrate the pH meter in accordance with the manufacturer's instructions.

Notes

1. The calibration should be carried out while the flasks are standing for 20 min (see Step B-1 and B-2).
2. If a series of samples is being tested, check the calibration of the pH meter with one or more of the standard buffer solutions at least every 30 min.

17.10.3.2. Preparation of test solution

A. Caseins: Weigh to the nearest 0.1 g, into the 100 ml conical flask, 5.0 g of the test sample, add 30 ml of water previously adjusted to 20°C and stopper the flask. (If desired, 7.0 g of the test sample and 42 ml of water can be taken.) Shake the flask by hand for 10 sec and allow it to stand for 20 min at about 20°C.

B. Caseinates: Transfer to the 250 ml beaker 95 ml of water, add 5.0 g of the test sample, and mix using the mixer for 30 s. Allow to stand for 20 min at about 20°C.

17.10.3.3. Measurement of pH

A. Caseins: Decant the supernatant liquid into the 50 ml beaker and immediately determine the pH of this liquid, using the pH meter , after having rinsed the glass electrode carefully with water.

B. Caseinates: Pour about 20 ml of the solution into the 50 ml beaker and immediately determine the pH of this liquid, using the pH meter, after having rinsed the glass electrode carefully with water.

17.10.3.4. Calculation

Recording of pH

Caseins: Record, as the pH of the aqueous extract of casein, the value read from the dial of the pH meter to at least one decimal place.

Caseinates: Record, as the pH of the aqueous solution of caseinate, the value read from

the dial of the pH meter to at least two decimal places.

(Ref:- IS 11918 – 1987 (Reaffirmed 1997) Method for determination of pH in caseins and caseinates (Reference Method). Bureau of Indian Standards, New Delhi).

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18. DETERMINATION OF LACTOSE BY COLORIMETRIC METHOD IN MILK BASED SWEETS

18.1. Determination of Lactose by Colorimetric Method

This method is useful in distinguishing milk sweets prepared from Khoa and Paneer or Channa. The above method is based upon the reaction of lactose with methylamine in hot alkaline solution to form a red complex which absorbs at 540 nm. The method is useful for differentiating Khoa based and Chhanna based sweets.

18.1.1. Apparatus

A. Volumetric flasks, 10 ml

B. Whatman No. 1 filter paper

C. Test tubes 25 ml

D. Constant temperature water bath

E. Spectrophotometer or colorimeter

F. Pipettes to deliver 5 ml, 10 ml and 25 ml.

18.1.2. Reagents

A. Zinc Acetate - Phosphotungstic acid (ZAPT) Dissolve 25.0 g of zinc acetate and 12.5 g of Phosphotungstic acid in water. 20 ml of glacial acetic acid is added and made up to 100 ml.

B. Glycine-NaOH buffer Mix 150 ml of glycine solution containing 2.4768 g of glycine and 1.9359 g NaCl with 850 ml of 0.385 N NaOH to give pH of 12.8.

C. Methylamine solution Dissolve 5.0 g of methylamine HCl in distilled water and dilute to 100 ml and store in refrigerator.

D. Sodium Sulphite solution 1% (W/V) Dissolve 1.0 g of sodium sulphite in distilled water and dilute to 100 ml prepare fresh.

E. Standard Lactose solution

F. Stock solution Dissolve 2.5315 g of lactose monohydrate (USP grade) in 200 ml of 0.1 percent (W/V) benzoic acid and store in refrigerator.

G. Working solution Dilute 10, 15, 20, 25 and 30 ml of stock solution to 250 ml separately to get 0.5 to 1.5 mg of lactose per ml.

H. 1N NaOH solution: Dissolve 40 g of NaOH in distilled water and make the volume to one litre. one litre of carbon dioxide free water.

18.1.3. Procedure

18.1.3.1. Preparation of Sample

To 8.0 g of well mixed sample add 1 ml of ZAPT reagent, dilute to 10 ml, and after 10 minutes filter using Whatman No. 1 filter paper. ii) To 0.5 ml of the filtrate add 0.5 ml of NaOH solution, dilute to 10 ml and filter using Whatman No. 1 filter paper. Dilute 5 ml of the filtrate to 10 ml.

18.1.3.2. Preparation of Standard Cure and Measurement in Sample

Pipette 5 ml each of working standard lactose and unknown solution into 25 ml test tubes. Add 5 ml of glycine NaOH buffer, 0.5 ml of methylamine solution and 0.5 ml of sodium sulphite solution in each tube, mix thoroughly. Heat tubes in a thermostatically controlled water bath at 65°C for 25 min. and cool immediately in an ice water bath for 2 min. to stop the reaction. Read absorbance against blank at 540 nm in a spectrophotometer or a suitable spectrophotometer. Draw a standard curve by plotting absorbance against concentration of lactose and determine the concentration of lactose from it.

(Ref:- Nickerson, et al. (1976) Colorimetric estimation of Lactose and its hydrolytic products. Journal Dairy Science 59, No 3, page 386).

18.2. Determination of Lactose by high-performance liquid chromatography (HPLC)

In this method (ISO 22662: 2007), an internal standard [D(+)-melezitose] is added to a weighed volume of milk and to lactose standards. A chemical reagent (Biggs-Szijarto solution) is added to precipitate out the fat and the protein component fractions of milk. The sample is filtered twice prior to injection, first through paper filter and then through a 0.45 µm nylon filter. The lactose and the internal standard are separated by a cation exchange column in the lead form and detected by a differential refractometer

detector or other suitable detector. As mobile phase, HPLC grade water is used.

18.2.1. Apparatus

A. HPLC ion exchange resin column: Length 300 mm, of internal diameter 7.8 mm, with 8% cross-linked copolymer, based on polystyrene-divinylbenzene cation exchange resin and packed in the lead form.

B. Guard column: In order to prolong ion exchange resin column life, replace the guard column after about 200 injections.

C. Micro-guard holder.

D. Column heater: Capable of maintaining a constant temperature of 85°C±1°C.

E. HPLC pump: Capable of maintaining a flow rate of between 0 ml/min and 10 ml/min.

F. HPLC autosampler.

Note: Manual injection can also be used.

G. Differential refractometer detector, highly sensitive.

Note: Other detectors, e.g. an evaporative light scattering detector, can also be used.

H. Software, capable of: automating injections, performing data acquisition, processing, and managing chromatographic information.

I. Water purification unit, capable of providing water complying with grade 1 requirements of ISO 3696, with a resistivity of between 10 MΩ·cm and 18 MΩ·cm.

J. Solvent filtration unit, including a vacuum source, with a membrane filter of 0.45 µm pore size and of diameter 47 mm.

K. Analytical balance, capable of weighing to the nearest 1 mg, with a readability of 0.1 mg.

L. Water bath, capable of maintaining a temperature of between 38 - 40°C.

M. Accurate dispenser, accurate automatic pipette, or one-mark pipettes: Class A, of capacity 2 ml.

N. Filter funnel: Diameter 75 mm.

O. Filter paper: Diameter 110 mm, Whatman1) No 1 or equivalent.

P. Nylon syringe filter: Porosity 0.45 µm.

Note: An in-line filter of the same porosity may also be used.

Q. Syringe: With Luer-lock, of capacity 5 ml.

R. HPLC vials, with caps.

S. One-mark volumetric flasks: Capacity 10 ml ± 0.02 ml.

Note: Flasks with a capacity of more than 10 ml can also be used by taking into account the concentration factor.

18.2.2. Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

A. Degassed HPLC grade water: Filter the water, conforming to the requirements of ISO 3696, Grade 1, obtained from the water purification unit using the solvent filtration unit. To improve the pump performance and to obtain a stable baseline, degas the mobile phase daily by selecting one of the available techniques such as sparging with helium, sonication, vacuum or in-line degassing system.

B. D(+)-Melezitose hydrate solution ($C_{18}H_{32}O_{16}\cdot H_2O$) (50 mg/ml): Dissolve an amount of D(+)-melezitose hydrate in water to give a final concentration equivalent to 50 mg/ml of the anhydrous form. The D(+)-melezitose solution can be stored at 4°C for no longer than 1 week.

C. α-Lactose monohydrate ($C_{12}H_{22}O_{11}\cdot H_2O$): Before use, dry the α-lactose monohydrate at 70°C for 4 h. Cool it to room temperature in a desiccator.

Note: After drying, the lactose remains in the monohydrate form.

D. Biggs-Szijarto solution: Dissolve 25 g of zinc acetate dihydrate, $Zn(CH_3COO)_2\cdot 2H_2O$ and 12.5 g of phosphotungstic acid monohydrate ($W_{12}O_{36}\cdot H_3PO_4\cdot H_2O$) in about 100 ml of HPLC grade water in a 200 ml one-mark volumetric flask. Add 20 ml of glacial acetic acid (CH_3COOH). Dilute to the 200 ml mark with HPLC grade water and mix. After use, the solution may be stored at 4°C for no longer than 1 week.

18.2.3. Procedure

18.2.3.1. Preparation of test sample

For fluid milk and cream, warm the test sample in the water bath to between 38°C and 40°C. Gently mix the test sample by repeatedly inverting the bottle. Cool the sample quickly to 20 ±1°C while gently mixing the sample immediately prior to weighing the test portion. Prepare milk powder and other samples as the case.

18.2.3.2. Preparation of standard solution

A. In a 10 ml one-mark volumetric flask, weigh, to the nearest 1 mg, the appropriate amount of α-lactose monohydrate to give the equivalent of a 20 mg/ml anhydrous α-lactose solution.
B. Dissolve the α-lactose monohydrate in about 5 ml of HPLC grade water. Add 2 ml of D(+)-melezitose solution, used as internal standard, to the flask. Make up to the mark with HPLC grade water and mix by inverting the flask. Express the final α-lactose concentration in milligrams of the anhydrous form per millilitre.
C. Filter the standard solution through a pleated filter paper using a filter funnel. Aspirate the filtrate into a syringe. Screw the nylon syringe filter to the syringe and then transfer each filtrate into an HPLC vial. Inject each standard solution at least twice. The standard solution thus prepared can provide three sets of calibration solutions. Use each set once only to calibrate the HPLC column. Store non-used sets of lactose standard solution at 4°C for no longer than 1 week. Before use, bring all refrigerated standard solutions to approximately 20°C. In order to monitor the calibration, inject the standard solution as unknown sample at the beginning and at the end of the set of the test portions.

18.2.3.3. Preparation of test portion

Depending on the type of sample, treat the sample accordingly as per following

Fluid milk test sample: Weigh, to the nearest 1 mg, about 3 ml of prepared test sample (see Clause 8) into a 10 ml one-mark volumetric flask. Proceed as in Section 18.2.3.4.

Milk powder test sample: Weigh, to the nearest 1 mg, about 0.300 g of test sample into a 10 ml one-mark volumetric flask. Add about 5 ml of HPLC grade water pre-warmed to between 50°C and 60°C. Mix thoroughly until the solution becomes homogenous. Allow the test solution thus obtained to cool to 20 ± 1°C. Proceed as in Section 18.2.3.4.

Cream test sample: Weigh, to the nearest 1 mg, about 1 g of prepared test sample into a 10 ml one-mark volumetric flask. Proceed as in Section 18.2.3.4.

18.2.3.4. Preparation of filtrate

Add 2 ml of D(+)-melezitose internal standard solution and 1.2 ml of Biggs-Szijarto solution to the content of the flask obtained above (Section 18.2.3.3), as appropriate. Dilute to the mark with HPLC grade water. Gently mix the contents by inverting the flask five times. Allow to stand at room temperature for 10 min. Repeat the mixing and standing process two more times. Filter the contents of the flask through a pleated filter paper using a filter funnel. Collect the filtrate with a syringe. Screw the nylon syringe filter to the syringe and then transfer the filtrate into a HPLC vial. Inject the test solution at least twice.

Note: The filtration step through the filter paper can be replaced by centrifugation of the test sample.

18.2.3.5. HPLC determination

A. Preliminary preparation of HPLC: In order to get a stable baseline, turn on the detector at least 24 h before starting the analysis. Set the internal temperature at 35°C. Set the HPLC pump to deliver a flow rate of 0.2 ml/min for at least 20 min while the column heater is set to room temperature.

   Increase the column heater temperature to 85°C. When that temperature is reached, gradually increase the flow rate from 0.2 ml/min to 0.6 ml/min. Allow the system to equilibrate at a flow rate of 0.6 ml/min and at 85°C for 2 h or until a stable baseline is obtained.

Note: Checking and recording the pressure of the system from day to day can help to detect whether abnormal pressure changes occur.

B. Chromatographic conditions: The chromatographic conditions are as follows:

Conditions	Details
Mobile phase	Degassed HPLC grade water
Internal detector temperature	35°C
Guard column temperature	Ambient temperature
Column temperature	85°C
Flow rate	0.6 ml/min
Volume to be injected	20 µl
Run time	15 min
Retention time of D(+)-melezitose	9 min ± 1 min
Retention time of lactose	11 min ± 1 min

Carefully choose the acquisition and integration parameters such as sensitivity, scale factor, time constant, peak width and threshold. See Figure 1 for an example of a chromatogram.

Measure the column efficiency, also called theoretical plate count, N, at least once per week. A decrease in N is related to the band spreading of the peak which is often due to a loss in column performance. Calculate N by using the following equation:

$$N = 5.54\ x\ \left[\frac{t_R}{w}\right]^2$$

Where,

t_R is the retention time, in minutes, of the lactose peak;

w is the width of the lactose peak, equivalent to time difference in minutes, at 50 % of its height.

When the theoretical plate count decreases by more than 25% compared to the original measurement, a replacement of the column is recommended.

Note: In most cases, a used column performing with low efficiency can be restored to its original form by back washing with an appropriate regenerating solvent described in the manufacturer's documentation.

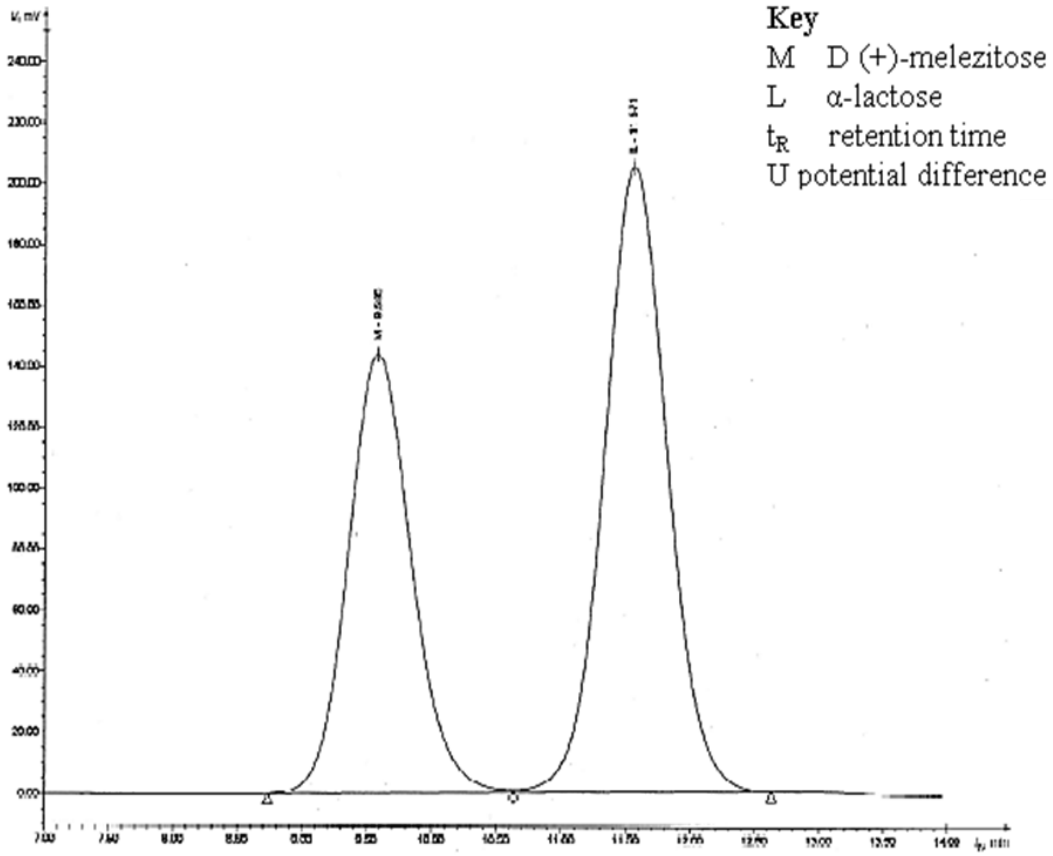

Figure 1 - Example of a chromatogram from a raw milk sample containing the internal standard

18.2.3.6. Calculation and expression of results

A computer performs the calculations as follows:

First, the software generates a curve by plotting the response ratio of the lactose standard peak area, A_s, to that of the internal standard, A_{is}, multiplied by the internal standard concentration, C_{is} i.e. $(A_s/A_{is}) \times C_{is}$, against lactose concentration, C_l. The curve fit is linear through the origin.

To quantify an unknown test sample, the software divides the concentration derived from the calibration curve by the mass of the test sample to calculate the anhydrous lactose mass fraction expressed as a percentage.

Express the test results to three decimal places.

(Ref:- ISO22662 – 2007Milk and milk products - Determination of lactose content by high-

performance liquid chromatography (reference method), International Organization for Standardization, Geneva; ISO3696 – 1987 Water for analytical laboratory use - Specification and test methods, International Organization for Standardization, Geneva;).

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19. DETERMIANIOTN OF TOTAL NITROGEN CONTENT IN MILK/Milk PRODUCT BY KJELDAHL METHOD

The most widely used method for determining protein content is by Kjeldahl method for nitrogen determination. Since nitrogen is a characteristic element in protein, by its accurate determination, protein concentration can be calculated. In 1883, Johann Kjeldahl developed the basic procedure to analyze organic nitrogen. The method involves two major steps. In the first step, the protein is digested using concentrated sulphuric acid in presence of a catalyst. In this step all the organic material is oxidized except nitrogen, the reduced form of which is retained in digest as ammonium sulphate. Neutral salts such as potassium sulphate are used in the digestion step to raise the boiling point of the reaction mixture and thereby effectively increase the digestion rate. Metallic catalyst such as copper sulfate is used to hasten the digestion and clearing the reaction mixture. The digest is neutralized with alkali to liberate ammonia.

In the second step, ammonia is distilled off, collected in boric acid and titrated with standard acid. Boric acid provides the most convenient absorbent for ammonia in that, the need for a standard alkali in titration is eliminated, and neither the amount nor the concentration of boric acid needs to be precise, since the boric acid itself is not involved in the titration, but simply reacts with the ammonia to form an ammonium borate complex. The strongly basic ammonium-borate that is formed is titrated directly with acid in the presence of a methyl red-bromocresol green indicator until the green distillate changes through colourless to pink (methyl red – methylene blue indicator can also be used; See Micro Kjeldahl method).

Reaction

$$\text{Protein} \xrightarrow[\text{Heat}]{K_2SO_4, CuSO_4, H_2SO_4,} (NH_4)_2SO_4$$

$$(NH_4)_2SO_4 + 2NaOH \longrightarrow 2NH_3 + Na_2SO_4 + 2H_2O$$

$$NH_3 + H_3BO_3 \longrightarrow NH_4^+.H_2BO_4^-$$

$NH_4^+.H_2BO^-_4 + HCl \longrightarrow NH_4Cl + H_3BO_3$

(Green) (Pink at pH < 4.8)

The quantity of acid required for titration is equivalent to the concentration of ammonia in the distillate and to the nitrogen content of the original protein containing sample. A reagent blank should be run to subtract reagent nitrogen from sample nitrogen. The result of the analysis represents the crude protein content of the sample since some nitrogen also comes from non-protein components of milk.

On average, milk proteins contain 15.65% nitrogen. Thus, for milk sample of unknown composition, the total amount of protein may be calculated using the conversion factor of 6.38 (100/15.65).

The Kjeldahl method has traditionally served as the basis for comparison of results obtained by all other quantitative methods. Repeatability and reproducibility of Kjeldahl method has led to its adoption as the standard method for determination of total protein in variety of food products.

19.1. Determination of Total Nitrogen/Crude Protein in Milk

19.1.1. Method 1. Macro Kjeldahl Method (IDF 20B: 1993)

This method employs the use of traditional Kjeldahl apparatus using Kjeldahl flask.

19.1.1.1. Reagents

Use all the reagents of AR Grade quality, unless otherwise specified, and use distilled or demineralized water only.

A. Potassium sulfate (K_2SO_4): Nitrogen free or low in nitrogen content.
B. Copper (II) sulfate solution: Dissolve 5.0 g of copper (II) sulfate pentahydrate ($CuSO_4.5H_2O$) in water and make up the final volume to 100 ml in a 100 ml volumetric flask.
C. Concentrated sulphuric acid: At least 95 - 98% (m/m), nitrogen free, ρ_{20} approximately = 1.84 g/ml.

D. Sodium hydroxide solution, 50%, m/m (low in nitrogen): Dissolve 50 g NaOH pellets in water and finally make to 100 g.

E. Indicator solution: Dissolve 0.1 g of methyl red in 95% (v/v) ethanol and dilute to 50 ml with ethanol. Dissolve 0.5 g of bromocresol green in 95% (v/v) ethanol and dilute to 250 ml with ethanol. Mix 1 part of methyl red solution with 5 parts of bromocresol green solution or combine all of both solutions.

F. Boric acid solution (H_3BO_3): Dissolve 40 g of boric acid in hot water, allow the solution to cool and dilute to 1 L. Add 3 ml of methyl red - bromocresol indicator solution, mix and store the solution in borosilicate glass bottle. The solution will be light orange in colour. Protect the solution from light and sources of ammonia fume during storage.

G. Standard Hydrochloric acid solution: 0.1 ± 0.0005 N.

H. Ammonium sulfate [$(NH_4)_2SO_4$]: Minimum assay 99.9% on dried material. Immediately before use dry the ammonium sulfate at 102 ± 2°C for not less than 2 h. Cool to room temperature in a desiccator.

I. Tryptophan ($C_{11}H_{12}N_2O_2$) or Lysine hydrochloride ($C_6H_{15}ClN_2O_2$): Minimum assay 99%, do not dry these reagents in an oven before use.

J. Sucrose with a nitrogen content of not more than 0.002% (m/m). Do not dry in an oven before use.

19.1.1.2. Apparatus

A. Kjeldahl flasks: Kjeldahl, hard, moderately thick, well-annealed glass, 500 or 800 ml capacity.

B. Distillation flask: Same Kjeldahl flask as in 1, fitted with rubber stopper through which passes lower end of sufficient rubber bulb or trap to prevent mechanical carryover of NaOH during distillation. Connect upper end of the bulb to condenser tube by rubber tubing. Use graduated 500 ml Erlenmeyer titration flask to collect distillate. Trap outlet of condenser in manner to ensure complete absorption of ammonia distilled into boric acid solution.

C. Digestion apparatus: To hold the Kjeldahl flasks in an inclined position approximately 45°C with electric heater or gas burners that do not heat the flasks above the level of their contents, and with a fume extraction system. The heater

source should be adjustable to determine the maximum heater setting to be used during digestion. Preheat the heat source at the heater setting for evaluation. In the case of a gas heater, the preheat period shall be 10 min and for an electric heater the preheat period shall be 30 min. Determine the heater setting that brings 250 ml of water including 5 – 10 boiling aids with an initial temperature of 25°C to a rolling boil in 5 – 6 min for each type of heaters. This is the maximum heater setting to be used during digestion.

D. Conical or Erlenmeyer flask: 500 ml capacity, graduated at every 200 ml.

E. Burette: 50 ml capacity, graduated at least at every 0.1 ml.

F. Boiling aid: Mesh size 10 high purity amphoteric alundum granules, plain. Do not reuse the aids. Glass beads of approximately 5 mm diameter are also used, but they do not promote as efficient boiling as the alundum granules and more foaming problems will be encountered during digestion with glass beads.

G. Measuring cylinders: 50, 100 and 500 ml capacities, graduated.

19.1.1.3. Procedure

19.1.1.3.1. Test Portion and Pre-treatment

Add to the clean and dry Kjeldahl flask, 5 – 10 boiling aids, 15 g K_2SO_4, 1.0 ml of the copper sulfate solution, approximately 5 ± 0.1 g of prepared milk sample (or milk product sample containing equivalent amount of protein), weighed to the nearest 0.1 mg, and add 25 ml of concentrated sulfuric acid. Use the 25 ml acid also to wash down any copper sulfate solution, K_2SO_4 or milk left on the neck of the flask. Gently mix the contents of the Kjeldahl flask.

19.1.1.3.2. Determination

19.1.1.3.2.1. Digestion

A. Turn on the fume extraction system of the digestion apparatus prior to beginning the digestion. Heat the Kjeldahl flask and its contents on the digestion apparatus using a heater setting low enough such that charred digest does not foam up the neck of the Kjeldahl flask. Digest at this heat-setting for at least 20 min or until white fumes appear in the flask. Increase the heater setting to half way to the

maximum setting as determined previously (See Digestion apparatus) and continue the heating period for 15 min. At the end of 15 min period, increase the heat to maximum setting.

B. After the digest clears (clear with light blue-green colour), continue boiling for 1 h to 1.5 h at maximum setting. The total digestion time will be between 1.8 – 2.25 h.

Note: To determine the specific boiling time required for analysis conditions in a particular laboratory using a particular set of apparatus, for milk analysis, select a high-protein, high-fat milk sample and determine its protein content using different boil times (1 h – 1.5 h) after clearing. The mean protein result increases with increasing boil time, becomes consistent and then decreases when boil time is too long. Select the boil time that yields the maximum protein result.

C. At the end of digestion, the digest shall be clear and free of undigested material. Allow the acid digest to cool to room temperature over a period of approximately 25 min. If the flasks are left on hot burners to cool, it will take longer to reach room temperature. The cooled digest should be liquid or liquid with a few small crystals at the bottom of the flask at the end of 25 min cooling period. Do not leave the undiluted digest in the flask overnight. The undiluted digest may crystallize during this period and it will be very difficult to get that back into the solution to avoid this situation.

Note: Excessive crystallization after 25 min is the result of undue acid loss during digestion and can result in low test values. Undue acid loss is caused by excessive fume aspiration or an excessively long digestion time caused by an incorrect maximum burner setting.

D. After the digest is cooled to room temperature, add 300 ml of water to 500 ml Kjeldahl flask or 400 ml of water when using 800 ml Kjeldahl flask. Use the water to wash down the neck of the flask too. Mix the contents thoroughly ensuring that any crystals which separate out are dissolved. Add 5 - 10 boiling aids. Allow the mixture to cool again to room temperature prior to the distillation. Diluted digests may be stoppered and held for distillation at a later time.

19.1.1.3.2.2. Distillation

A. Turn on the condenser water for the distillation apparatus. Add 75 ml of 50% (m/m) sodium hydroxide solution to the diluted digest by carefully pouring the solution down the inclined neck of the Kjeldahl flask, so as to form a clear layer at the bottom of the bulb of the flask. There should be a clean interface between the two solutions.

B. Immediately after the addition of sodium hydroxide solution to the Kjeldahl flask, connect it to the distillation apparatus, the tip of whose condenser outlet tube is immersed in 50 ml of boric acid solution with indicator contained in a 500 ml Erlenmeyer flask. Vigorously swirl the Kjeldahl flask to mix its contents thoroughly until no separate layers of solution are visible in the flask any more. Set the flask down on the burner. Turn on the burner to a setting high enough to boil the mixture. Continue distillation until irregular boiling (bumping) starts and then immediately disconnect the Kjeldahl flask and turn off the burner. Turn off the condenser water.

C. The distillation rate shall be such that approximately 150 ml distillate is collected when irregular boiling (bumping) starts and the volume of the contents of the conical flask will be approximately 200 ml. If the volume of distillate collected is less than 150 ml, then it is likely that less than 300 ml of water is added to dilute the digest. The efficiency of the condenser shall be such that the temperature of the contents of conical flask does not exceed 35°C during distillation.

19.1.1.3.2.3. Titration

Titrate the boric acid receiving solution with standard hydrochloric acid solution (0.1 N) to the first trace of pink colour. Take the burette reading to at least the nearest 0.05 ml. A lighted stir plate may aid visualization of the end point.

19.1.1.3.3. Blank Test: Simultaneously carry out a blank test by following the procedure as described above taking all the reagents and replacing the milk sample with 5 ml water and about 0.85 g of sucrose.

Note:

1. The purpose of sucrose in a blank or a recovery standard is to act as organic material to consume an amount of sulfuric acid during digestion that is roughly equivalent to a test portion. If the amount of residual free sulfuric acid at the end of digestion is

too low, the recovery of nitrogen by both recovery tests (See Section 19.1.1.3.4. i.e. Nitrogen recovery test) will be low. If the amount of residual acid present at the end of the digestion is sufficient to retain all the nitrogen, but the temperature and time conditions during digestion were not sufficient to release all the nitrogen from a sample, then the nitrogen recovery will be acceptable as per Section 19.1.1.3.4.-A and the nitrogen recovery will be low as per section 19.1.1.3.4.-B.

2. The amount of titrant used in the blank should always be greater than 0.00 ml. Blanks within the same laboratory should be consistent across time. If the blank is already pink before the beginning of titration, something is wrong. Usually, in such cases, the conical flasks are not clean or water from the air that may condense on the outside of the condenser apparatus has dripped down into the collection flask to cause the contamination.

19.1.1.3.4. Nitrogen Recovery Test

A. The accuracy of the procedure should be checked regularly by means of following recovery tests, carried out in accordance with procedure as in the preceding Steps (See Steps 19.1.1.3.1, 19.1.1.3.1 and 19.1.1.3.1).

B. Check that no loss of nitrogen occurs by using a test portion of 0.12 g of ammonium sulfate along with 0.85 g of sucrose. Add all other reagents (except milk sample) as stated in Step A. Digest and distill under same conditions as for a milk sample (See Steps 19.1.1.3.1 and 19.1.1.3.1).

C. The percentage of nitrogen recovered shall be between 99.0 and 100.0% for the given apparatus. In the case recoveries of nitrogen exceed 100%, ammonium sulfate is only useful to determine whether nitrogen loss has occurred or the normality of titrant is lower than the stated value. For recoveries less than 99%, the loss could be in the digestion or distillation step. It is possible to use a mixture of ammonium sulfate and small amount of sulfuric acid (the amount of residual remaining at the end of digestion) in a Kjeldahl flask. Dilute it with the normal value of water, add the normal amount of NaOH solution and distill. If the nitrogen recovery is still low by the same amount, the loss of nitrogen is in the distillation apparatus and not in that of the digestion. The probable cause might be a leaky tubing in a traditional system or the tips of the condensers not submerged under the surface of boric acid solution

early in the distillation. The apparatus should pass this test before going on to check recoveries by the procedure described below.

a. Check the efficiency of digestion procedure by using 0.16 g of lysine hydrochloride or 0.18 g of tryptophan along with 0.67 g of sucrose. Add all other reagents (except milk sample) as stated in Steps 19.1.1.3.1. Digest and distill under same conditions as for a milk sample. At least 98% of the nitrogen shall be recovered. If the recovery is lower than 98% after having a 99 - 100% recovery on ammonium sulfate, then the temperature or time of digestion is insufficient or there is undigested sample material (i.e., char) on the inside of the Kjeldahl flask.
b. The final evaluation of performance is best done by participation in a proficiency testing system, where within and between laboratories statistical parameters are computed based on analysis of milk samples.
c. Lower results in either of the recovery tests (or higher than 100% in case of ammonium sulfate) will indicate failures in the procedure and/or inaccurate concentration of the standard hydrochloric acid solution.

19.1.1.3.5. Calculations

Calculate the nitrogen content, expressed as a percentage by mass, by following formula

$$W_n = \frac{1.4007 \text{ x } (V_s - V_B) \text{x } N}{W}$$

W_n = nitrogen content of sample, expressed as a percentage by mass;

V_S = volume in ml of the standard hydrochloric acid used for sample;

V_B = volume in ml of the standard hydrochloric acid used for blank test;

N = Normality of the standard hydrochloric acid expressed to four decimal places;

W = mass of test portion in g, expressed to nearest 0.1 mg.

Express the nitrogen content to four decimal places.

19.1.1.3.5.1. Calculation of Crude Protein Content

The crude protein content, expressed as a percentage by mass, is obtained by multiplying the nitrogen content by 6.38. Express the crude protein results to three decimal places.

19.1.2. Method 2. Block Digestion/ Steam Distillation method (IDF 20B: 1993)

In this method, test portion of the milk sample is digested using a block digestion apparatus with a mixture of concentrated sulfuric acid and potassium sulfate using of copper (II) sulfate as a catalyst, thereby converting organic nitrogen present in the sample to ammonium sulfate. Excess of sodium hydroxide is added to the cooled digest to liberate ammonia. The steam distillation of ammonia from the digest and trapping the ammonia into an excess of boric acid solution is carried using either a manual or semiautomatic steam distillation unit followed by titration of boric acid with standard hydrochloric acid.

19.1.2.1. Reagents

See Reagents as already described in Macro Kjeldahl method for the determination of total nitrogen content in milk (Method I – Section 19.1.1.1).

Note: 40% (m/m) sodium hydroxide solution may be used instead of 50% (m/m), if plugging of the flow system in an automatic distillation unit is a problem.

19.1.2.2. Apparatus

A. Digestion block: Aluminium alloy block or equivalent apparatus, fitted with an adjustable temperature control and device for measuring block temperature.
B. Digestion tubes: 250 ml capacity, suitable for use with digestion flask.
C. Exhaust manifold: Suitable for use with the digestion tubes.
D. Centrifugal scrubber apparatus or filter pump or aspirator: Constructed of acid resistant material and for use with main water supply.
E. Measuring cylinder: 25, 50 and 100 ml capacities, graduated.
F. Distillation unit: Manual or semi-automatic, for steam distillation, suited to accept the 250 ml digestion tubes and 500 ml conical flasks.
G. Conical or Erlenmeyer flask: 500 ml capacity, graduated at 200 ml.
H. Burette: 50 ml capacity, graduated at least at every 0.1 ml.

19.1.2.3. Procedure

19.1.2.3.1. Test Portion and Pre-treatment

Add to the clean and dry digestion tube, 12 g K_2SO_4, 1.0 ml of the copper sulfate solution, approximately 5 ± 0.1 g of prepared milk sample, weighed to the nearest 0.1 mg, and add 20 ml of concentrated sulfuric acid. Use the sulfuric acid also to wash down and copper sulfate solution, K_2SO_4 or milk left on the upper walls of the digestion tube. Gently mix the contents of the tube.

Note: The amount of acid used in the block digestors is less than that used in the Macro Kjeldahl method because the volumes of acid greater than 20 ml in the block digestion systems gives excessive foaming problem during digestion and variable results. Users of block digestors must note that maintaining sufficient residual sulfuric acid at the end of digestion needs more attention by the analyst in the block digestors than in Macro Kjeldahl method. Excessive acid loss due to over aspiration of fumes is more of concern in block digestors than Macro Kjeldahl method.

19.1.2.3.2.Determination

19.1.2.3.2.1. Digestion

A. Set the digestion block at a low initial temperature so as to control foaming (approximately a temperature between 180 – 230°C). Transfer the tube to the digestion block and place the exhaust manifold which is itself connected to a centrifugal scrubber of similar device in the top of the tube. The suction rate of the centrifugal scrubber or similar device shall be just sufficient to remove fumes. The complete apparatus may need to be kept inside a fume hood.

B. Digest the test portion for 30 min or until white fumes develop. Then increase the temperature of digestion block to a temperature between 410 – 430°C and continue digestion of the test portion until the digest is clear.

Note: It may be necessary to increase the temperature gradually over a period of approximately 20 min to control foaming. In any event, do not let foam rise higher than 4 – 5 cm below the surface of exhaust manifold inserted into the top of the digestion tube.

C. After the digest clears (clear with light blue-green colour) continue digestion at a temperature of between 410 – 430°C for at least 1 h. During this period the sulfuric acid must be boiling. If visible boiling of the clear liquid is not apparent as bubbles forming at the surface of the hot liquid around the perimeter of the tube, then the temperature of the block may be too low. The total digestion time will be between 1.75 – 2.5 h.

D. To determine the specific boiling time required for analysis conditions in a particular laboratory using a particular set of apparatus, select a high-protein, high-fat milk sample and determine its protein content using different boil times (1 – 1.5 h) after clearing. The mean protein content increases with increase in boil time, becomes consistent and then decreases when boil time is too long. Select the boil time that yields the maximum protein results. At the end of digestion, the digest should be clear and free of undigested material. Remove the tube from the block with the exhaust manifold in place. Allow to cool to room temperature over a period of approximately 25 min. The cooled digest should be liquid or liquid with a few small crystals at the bottom of the tube. Do not leave the undiluted digest in the tube overnight. The undiluted digest may solidify and it will be very difficult to get that back into the solution with water.

Note: Excessive crystallization after 25 min is the result of undue acid loss during digestion and can result in low test values. Undue acid loss is caused by excessive fume aspiration or an excessively long digestion time caused by digestion for too long a period at a temperature below the maximum temperature of the analysis. To reduce acid loss, reduce the rate of fume aspiration.

E. After the digest is cooled to room temperature, remove the exhaust manifold and carefully add 85 ml of water to each tube. Swirl to mix while ensuring that any crystals which separate out are dissolved. Allow the contents of the tube to cool again to room temperature.

19.1.2.3.2.2. Distillation

A. Turn on the condenser water for the distillation apparatus. Attach the digestion tube containing the diluted digest to the distillation unit. Place a conical flask containing

50 ml of the boric acid solution under the outlet of the condenser, in such a way that the delivery tube is below the surface of the boric acid solution. Adjust the distillation unit to dispense 55 ml of sodium hydroxide solution.

Note: Where 40% (m/m) sodium hydroxide solution is used, the dispensed volume should be adjusted to 65 ml. If the automatic delivery of sodium hydroxide solution is extremely variable due to the partial plugging of the delivery tubing for the sodium hydroxide, then large variability in duplicate results will occur.

B. As per the manufacturer's instructions, operate the distillation unit in such a way as to steam distill the ammonia liberated by addition of sodium hydroxide solution, collecting the distillate in the boric acid solution. Continue with the distillation process until at least 150 ml of distillate has been collected. Remove the conical flask from the distillation unit.

19.1.2.3.2.3. Titration

Titrate the boric acid receiving solution with standard hydrochloric acid solution (0.1 N) to the first trace of pink colour. Take the burette reading to at least the nearest 0.05 ml. A lighted stir plate may aid visualization of the end point.

19.1.2.3.3. Blank Test

Simultaneously carry out a blank test by following the procedure as described above taking all the reagents and replacing the milk sample with 5 ml water and about 0.85 g of sucrose.

Note:

1. The purpose of sucrose in a blank or a recovery standard is to act as organic material to consume an amount of sulfuric acid during digestion that is roughly equivalent to a test portion. If the amount of residual free sulfuric acid at the end of digestion is too low, the recovery of nitrogen by both recovery tests (See Section 19.1.2.3.4. i.e. Nitrogen recovery test) will be low. If the amount of residual acid present at the end of the digestion is sufficient to retain all the nitrogen, but the temperature and time conditions during digestion were not sufficient to release all the nitrogen from a sample, then the nitrogen recovery will be acceptable as per

Section 19.1.2.3.4.-A and the nitrogen recovery will be low as per Section 19.1.2.3.4.-B.

2. The amount of titrant used in the blank should always be greater than 0.00 ml. Blanks within the same laboratory should be consistent across time. If the blank is already pink before the beginning of titration, something is wrong. Usually, in such cases, the conical flasks are not clean or water from the air that may condense on the outside of the condenser apparatus has dripped down into the collection flask to cause the contamination.

19.1.2.3.4.Nitrogen Recovery Test

A. The accuracy of the procedure should be checked regularly by means of following recovery tests, carried out in accordance with procedure as described in the preceding Steps (See Steps 19.1.2.3.1, 19.1.2.3.2 and 19.1.2.3.3).

B. Check that no loss of nitrogen occurs by using a test portion of 0.12 g of ammonium sulfate along with 0.85 g of sucrose. Add all other reagents (except milk sample) as stated in Step A. Digest and distill under same conditions as for a milk sample (See Steps B and C).

C. The percentage of nitrogen recovered shall be between 99.0 and 100.0%. In the case recoveries of nitrogen exceed 100%, ammonium sulfate is only useful to determine whether nitrogen loss has occurred or the normality of titrant is lower than the stated value. For recoveries less than 99%, the loss could be in the digestion or distillation step. It is possible to use a mixture of ammonium sulfate and small amount of sulfuric acid (the amount of residual remaining at the end of digestion) in a digestion tube. Dilute it with the normal value of water, add the normal amount of NaOH solution and distill. If the nitrogen recovery is still low by the same amount, the loss of nitrogen is in the distillation apparatus and not in that of the digestion. The probable cause might be a leaky tubing in the distillation system or the tips of the condensers not submerged under the surface of boric acid solution early in the distillation. The apparatus should pass this test before going on to check recoveries by the procedure described below.

a. Check the efficiency of digestion procedure by using 0.16 g of lysine hydrochloride or 0.18 g of tryptophan along with 0.67 g of sucrose. Add all other reagents (except

milk sample) as stated in Step 19.1.2.3.1. Digest and distill under same conditions as for a milk sample. At least 98% nitrogen shall be recovered. If the recovery is lower than 98% after having a 99 - 100% recovery on ammonium sulfate, then the temperature or time of digestion is insufficient or there is undigested sample material (i.e., char) inside the digestion tube.

b. The final evaluation of performance is best done by participation in a proficiency testing system, where within and between laboratories statistical parameters are computed based on analysis of milk samples.

c. Lower results in either of the recovery tests (or higher than 100% in case of ammonium sulfate) will indicate failures in the procedure and/or inaccurate concentration of the standard hydrochloric acid solution.

19.1.2.3.5.Calculations

Calculate the nitrogen content, expressed as a percentage by mass, by following formula.

$$W_n = \frac{1.4007 \text{ x } (V_s - V_B) \text{x } N}{W}$$

Where

W_n = nitrogen content of sample, expressed as a percentage by mass;

V_S = volume in ml of the standard hydrochloric acid used for sample;

V_B = volume in ml of the standard hydrochloric acid used for blank test;

N = Normality of the standard hydrochloric acid expressed to four decimal places;

W = mass of test portion in g, expressed to nearest 0.1 mg.

Express the nitrogen content to four decimal places.

19.1.2.3.6.Calculation of Crude Protein Content

The crude protein content, expressed as a percentage by mass, is obtained by multiplying the nitrogen content by 6.38. Express the crude protein results to three decimal places.

19.1.3. Method 3. Micro Kjeldahl Method

A routine method (Micro Kjeldahl method) for the determination of nitrogen content in milk being followed in most of dairy laboratories is also described below. This method also gives satisfactory results and is quite agreeable with Macro Kjeldahl Method for determination of nitrogen in milk.

The nitrogen percentage in a milk sample by Micro Kjeldahl method can be calculated as:

$$N\% = \frac{14.007 \text{ X (Sample titre} - \text{ Blank titre) x Normality of HCl x Volume made up of digest}}{\text{Aliquot of the digest taken x Weight of sample taken x } 1000} \; x \; 100$$

19.1.4. Reagents

A. Concentrated sulphuric acid: 95 - 98%, nitrogen free.

B. Copper (II) sulfate ($CuSO_4.5H_2O$): Nitrogen free.

C. Anhydrous potassium sulfate (K_2SO_4): Nitrogen free.

D. Sodium hydroxide solution (Nitrate free): 50%, w/w, aq.

E. Mixed indicator: Mix equal volume of 0.2 g per 100 ml solution of methyl red in 95% (v/v) ethanol and 0.1 g per 100 ml solution of methylene blue in 95% (v/v) ethanol.

F. Boric acid solution with mixed indicator: Dissolve 40 g of boric acid in hot water (80 – 85°C) and dilute to 1 L and add 3 ml of mixed indicator prepared as above. This solution will be purple in colour.

G. Standard hydrochloric acid: 0.02 N.

H. Ammonium sulfate: 99.99% pure.

I. Sucrose: AR grade, nitrogen free.

19.1.5. Apparatus

A. Digestion flask: Kjeldahl, hard, moderately thick, well-annealed glass, capacity 300 ml.

B. Distillation (micro distillation) system: Traditional apparatus as shown in the Figure.

C. Titration burette: 50 ml capacity.

D. Volumetric flasks: 100 ml capacity.

E. Pipette: 10 ml capacity.

F. Measuring cylinder: 10 ml capacity, graduated.

G. Conical flask: 150 ml capacity.

H. Beakers: 100, 250 ml capacities.

I. Glass funnel: 65 mm diameter.

J. Glass beads.

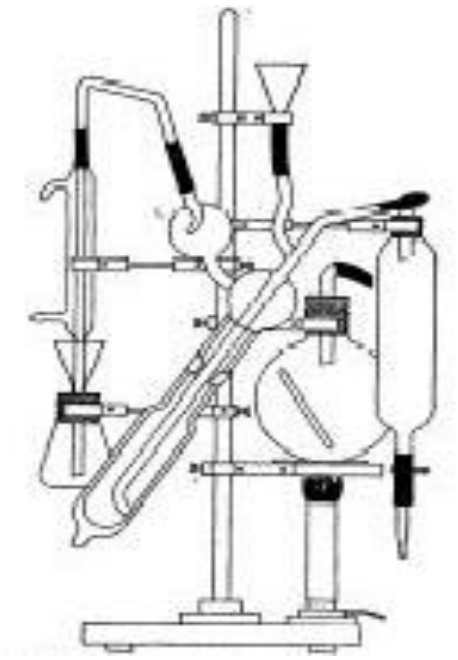

Figure. Traditional Kjeldahl distillation apparatus

19.1.6. Procedure

19.1.6.1. Digestion

A. Weigh accurately about 5 g of well-mixed sample of milk in a 300 ml Kjeldahl flask. Add 1.0 g of potassium sulfate and 0.2 g of copper sulfate to the Kjeldahl flask. Add 4-5 glass beads in the flask.

B. Add 25 ml concentrated sulphuric acid, rinsing any milk on the neck of flask down into bulb.

C. Place the flask on burner (or coil heater) so that the neck is inclined at an angle of 45° to the horizontal and flame does not touch the flask above the level of the liquid in bulb. Start heating by setting low heat so that sample does not foam up neck of Kjeldahl flask. Digest for at least 20 min or until white fumes appear in the flask. Increase flame setting half way to maximum burner setting. When the digest clears (clears with light blue-green colour), continue to heat the contents of the flask for 1 – 1.5 h at maximum heating. Remove the flask from the flame and allow the contents of flask to cool at room temperature for 15 - 20 min.

D. Wash down the sides of the Kjeldahl flask with a fine jet of distilled water (approximately 20 ml) and swirl to mix. Again place the flask on the flame and heat the flask for 1 h. At the end of digestion, the digest should be clear and free from

undigested material (no black particle should be visible). Let the flask cool at room temperature and cooled digest should be liquid or liquid with a few small crystals. Large amount of crystallization indicates too little residual sulphuric acid at the end of digestion and can result in low test values. Under such conditions repeat the experiment from the beginning.

E. Transfer the digest to a 100 ml volumetric flask using funnel. Add distilled water (15 ml) to the digestion flask and rinse it properly and transfer the washings to the volumetric flask. Repeat this step twice to ensure complete washing of the residual digest in the Kjeldahl flask and transfer the washings from Kjeldahl flask to 100 ml volumetric flask. Make the volume of the 100 ml volumetric flask to the mark using distilled water.

19.1.6.2. Distillation

A. Set up the distillation assembly and put on the burner to generate steam in the distillation assembly for cleaning the entire assembly.
B. Transfer 10 ml of digested solution from 100 ml volumetric flask to the distillation assembly using 10 ml measuring cylinder.
C. Rinse the cylinder with 5 ml distilled water and transfer the rinsings to distillation assembly.
D. Pipette 10 ml of 4% boric acid solution containing mixed indicator in a 150 ml conical flask and place the flask under the condenser of the distillation unit. The tip of the condenser should always be dipped in the boric acid solution so as to entrap the liberated ammonia.
E. Add 20 ml of 50% sodium hydroxide solution downside wall of distillation bulb and it should form a clear layer with diluted digest. Add 5-10 ml of water to the funnel to rinse the residual sodium hydroxide in the distillation assembly. Immediately pass the steam from the steam chamber in the distillation bulb to liberate ammonia. The purple colour of boric acid will change to green after absorbing liberated ammonia. Trap all the ammonia in boric acid using steam distillation and collect 60 - 70 ml distillate in 150 ml conical flask.
F. Remove the conical flask from the condenser, wash the condenser tip with 5 ml water (using wash bottle) and titrate the distillate using 0.02 N hydrochloric acid

solution. The change from green to purple colour will indicate the end point. Note the volume of standard hydrochloric acid used in the titration. Let it be V ml.

G. For blank, take 1 g of sucrose instead of milk and carry out the digestion and distillation (See Step 19.1.6.1 and 19.1.6.2) as done in case of the milk sample. Let the volume of standard hydrochloric acid used in case of blank is B ml.

19.1.6.3. Calculation

$$\text{Total nitrogen \% in milk} = \frac{14.007 \times (V-B) \times N \times 100}{1000 \times 10 \times W}$$

$$\text{Total Protein \% in milk} = \frac{14.007 \times (V-B) \times N \times 100 \times 100}{1000 \times 10 \times W} \times 6.38$$

Where

N = Normality of standard hydrochloric acid .

V = Volume in ml of standard hydrochloric used for milk sample.

B = Volume in ml of standard hydrochloric acid used for blank.

W = Weight in g, of the milk sample taken.

19.1.6.4. Nitrogen Recovery Test

Perform nitrogen recovery test as described in Section 19.1.2.3.4.

Derivation

1000 ml of 1 N HCl = 14.007 g of nitrogen

$\therefore$ 1 ml of 0.02 N HCl = 14.007/1000 x 50 g of nitrogen

Volume of 0.02 N HCl used for blank = B ml

Volume of 0.02 N HCl used for milk sample = V ml

$\therefore$ titre value of sample = (V-B) ml

Amount of sample taken for digestion = 5 g

Total volume made of the digested sample = 100 ml

Volume of the diluted digest taken for distillation = 10 ml

$$\text{Nitrogen present in 100 ml of digested material} = \frac{14.007 \times (V-B) \times 100}{1000 \times 50 \times 10}$$

Since, amount of sample taken is 5 g

$$\therefore \text{Nitrogen present in 5 g of sample} = \frac{14.007 \times (V-B) \times 100}{1000 \times 50 \times 10}$$

$$\therefore \text{Nitrogen present in 100 g of sample} = \frac{14.007 \times (V-B) \times 100}{1000 \times 50 \times 10} \times \frac{100}{5}$$

$$\text{Total Protein \% in milk} = \frac{14.007 \times (V-B) \times 100}{1000 \times 50 \times 10} \times \frac{100}{5} \times 6.38$$

19.2. Determination of Non-Protein Nitrogen (NPN) in Milk

In addition to caseins and whey proteins, milk also contains non-protein nitrogen (NPN) components, which are low molecular weight, and are not precipitated with the milk proteins by 12% trichloroacetic acid (TCA). NPN substances are metabolites of amino acids, and nucleic acid and their concentration in milk thus greatly depends on the protein content of the ration/feed given to the animal. The non-protein components are also known to be the end product of nitrogen metabolism in the animal body and are presumably introduced into milk directly from blood. The NPN content of milk is 250 – 350 mg/kg or about 6% of total nitrogen present in milk.

The level of NPN in freshly drawn milk is fairly constant but does increases on ageing, especially if significant growth of psychrophilic bacteria occurs. Urea, the principal component of milk (20-50 mg/ 100 ml) is strongly correlated with the heat stability of milk; the urea content of milk from cows on pasture is twice as high as that from cows on dry feed and hence the heat stability of the former is considerably higher. The free amino acids may serve to support the growth of bacteria.

In this method, proteins are precipitated from milk by the addition of trichloroacetic acid solution. Final concentration of trichloroacetic acid in the mixture is about 12%. Precipitated milk proteins are removed by filtration. Filtrate contains NPN components of milk. Nitrogen content of filtrate is determined using Kjeldahl method.

19.2.1. Reagents

A. Trichloroacetic acid (TCA) solution (15%, w/v, aq): Dissolve 15.0 g of trichloroacetic acid (CCl_3COOH) in water and dilute to 100 ml in a volumetric flask.

Note: Do not use other concentrations of TCA and volumes of solutions than those specified in this procedure. Other concentrations or volumes of solutions will change the performance of the method with respect to its mean value and performance characteristics.

B. Standard hydrochloric acid solution: 0.01 ± 0.0001 N.

C. Other reagents as already described in Macro Kjeldahl method for the determination of total nitrogen content in milk (Method I).

19.2.2. Apparatus

Apart from the apparatus listed in Macro Kjeldahl method for the determination of nitrogen content in milk (Method I), the following apparatus required are as below.

A. Conical flask: 25 ml capacity.

B. Pipettes: 10, 20 ml capacities.

C. Filter funnel: 75 mm diameter.

D. Whatman filter paper: Grade 1 (nitrogen free).

E. Beaker: 50 ml capacity.

19.2.3. Procedure

19.2.3.1. Test portion Preparation

Pipette 10.0 ± 0.1 ml of the prepared test sample into a pre-weighed conical flask. Re-weigh the flask and its contents recording the weights to the nearest 0.1 mg.

19.2.3.2. Determination

19.2.3.2.1. Precipitation and Filtration

A. Add 40 ml of TCA solution to the conical flask. Weigh the flask and its contents again to the nearest 0.1 mg. Swirl to mix. Let the flask stand for approximately 5 min to allow the precipitate to settle.

B. Filter the contents of the flask through a Whatman filter paper Grade 1 and collect the entire filtrate in a clean, dry conical flask. The filtrate shall be clear and free of particulate matter. If it is not, repeat the process of precipitation of filtration with a new test portion. If duplicate tests are to be done, separate precipitations and filtrations should be carried out.

C. Swirl the filtrate to ensure the complete mixing. Pipette 20 ml of the filtrate into a 50 ml beaker and weigh. Pour the filtrate from the beaker into a Kjeldahl flask containing the appropriate amount of boiling aids, K_2SO_4, copper (II) sulfate solution and concentrated sulfuric acid as specified in the Macro Kjeldahl method for the determination of total nitrogen content in milk (Method I - Section 19.1.1.). Immediately re-weigh the empty beaker.

19.2.3.2.2. Digestion and Distillation

Continue with the digestion and distillation procedure as described in method for the determination of total nitrogen content in milk Macro Kjeldahl method (Method I - Section 19.1.1.).

19.2.3.2.3. Titration

Titrate the boric acid receiving solution with standard hydrochloric acid (0.01 N) to the first trace of pink colour. Take the burette reading to at least the nearest 0.05 ml. A lighted stir plate may aid visualization of the end point.

19.2.3.3. Blank Test

Simultaneously carry out a blank test. Digest, distil and titrate a blank comprising about 0.1 g of sucrose and 16 ± 0.5 ml of TCA solution according to the procedure as given above.

19.2.3.4. Calculation

Calculate the nitrogen content, expressed as a percentage by mass using the following formula

$$W_n = \frac{1.4007 \text{ x } (V_s - V_B) \text{ x } N}{W_f \text{ x } W_m/(W_t - 0.065\ W_m)}$$

Where,

W_n = nitrogen (NPN) content of sample, expressed as a percentage by mass;

V_S = volume in ml of the standard hydrochloric acid used for sample;

V_B = volume in ml of the standard hydrochloric acid used for blank test;

N = Normality of the standard hydrochloric acid expressed to four decimal places;

W_m = mass of test portion in g, expressed to nearest 0.1 mg.

W_f = mass of 20 ml filtrate in g, expressed to nearest 0.1 mg.

W_t = mass of the test portion plus 40 ml of TCA solution in g, expressed to nearest 0.1 mg.

Note: The factor 0.065 in the denominator assumes that milk contains about 3.5 % fat and 3.0% true protein (thus 0.035 + 0.030). The factor may need to be adjusted for other liquid dairy products e.g concentrated or fractionated skim or whole milk products.

Express the nitrogen content to four decimal places.

19.2.3.5. Calculation of Non-Protein Nitrogen Content

The non-protein nitrogen content, expressed as a percentage by mass, is obtained by multiplying the nitrogen content by 6.38. Express the result of non-protein nitrogen to three decimal places.

19.3. Determination of True Protein Nitrogen Content in Milk

In this method the protein in milk sample is precipitated by the addition of TCA such that the final concentration of TCA in the mixture is approximately 12%. The precipitated proteins are filtered and filtrate will contain the non-protein nitrogen contents. Then the nitrogen content of the precipitated proteins is determined by Macro Kjeldahl method which represents true protein nitrogen content of milk (Method I).

19.3.1. Reagents

A. Trichloroacetic acid (TCA) solution (15%, w/v, aq): Dissolve 15.0 g of trichloroacetic acid (CCl_3COOH) in water and dilute to 100 ml in a volumetric flask.

Note: Do not use other concentrations of TCA and volumes of solutions than those specified in this procedure. Other concentrations or volumes of solutions will change

the performance of the method with respect to its mean value and performance characteristics.

B. Other reagents as already described in Macro Kjeldahl method for the determination of total nitrogen content in milk (Method I - Section 19.1.1.).

19.3.2. Apparatus

Apart from the apparatus listed in Macro Kjeldahl method for the determination of nitrogen content in milk (Method I. Section -19.1.1.), the following apparatus required are as below.

A. Conical flask: 25 ml capacity.

B. Pipettes: 10, 20 ml capacities.

C. Filter funnel: 75 mm diameter.

D. Whatman filter paper: Grade 1 (nitrogen free).

E. Beaker: 50 ml capacity.

19.3.3. Procedure

19.3.3.1. Test Portion

Weigh approximately 5 ± 0.1 g, to the nearest 0.1 mg, of the prepared milk sample into a Kjeldahl flask. Immediately add 5.0 ±0.1 ml of water to the Kjeldahl flask, rinsing any milk on the neck of the flask to the bottom of it.

19.3.3.2. Determination

19.3.3.2.1. Precipitation and Filtration

A. Add 40 ± 0.5 ml of TCA solution to the flask and swirl to mix the contents. Let the flask stand for approximately 5 min to allow the precipitate to settle.

B. Pour the mixture from the Kjeldahl flask through a Whatman filter paper Grade 1 and collect the entire filtrate in a clean, dry conical flask. Some of the precipitate will remain in the Kjeldahl flask and some will be collected on the filter paper. It is not necessary to remove all of the precipitate from the flask. Immediately after pouring the mixture and so as not to allow any precipitate to dry on the neck of Kjeldahl flask, add by means of a pipette, 10 ml of TCA solution. Use the acid

solution also to rinse any precipitate from the neck of the flask down on to the bottom. Swirl to mix the contents. Pour the mixture from the flask through the same filter paper, adding the filtrate to that collected previously. Immediately rinse the neck of the flask with a further 10 ml of TCA solution again. Swirl to mix the contents and again pour the mixture from the flask through the same filter paper, adding the filtrate to that collected previously. This filtrate shall be clear and free of particulate matter.

Note: At this point, the filtrate is no longer needed and may be discarded in an appropriate manner.

C. Wearing gloves, carefully remove the filter paper from the filter funnel and fold the filter paper to enclose the precipitate. If any precipitate remains on either the inner or outer lip of the Kjeldahl flask, wipe with the folded filter paper so that any precipitate adheres to the paper and then drop the filter paper into the Kjeldahl flask.

19.3.3.2.2. Digestion and Distillation

Add the appropriate amount of boiling aids, K_2SO_4, copper (II) sulfate solution and concentrated sulfuric acid as specified in the Macro Kjeldahl method for the determination of total nitrogen content in milk (Method I - Section 19.1.1). Determine the nitrogen content of the precipitate and filter paper by the Macro Kjeldahl method (Method I Section 19.1.1.).

19.3.3.2.3. Titration

Titrate the boric acid receiving solution with standard hydrochloric acid solution (0.1 N) to the first trace of pink colour. Take the burette reading to at least the nearest 0.05 ml. A lighted stir plate may aid visualization of the end point.

19.3.3.3. Blank Test

Carry out a blank test as described above. Replace the test portion by a filter paper washed with TCA solution and record the blank value.

19.3.3.4. Calculation

The true protein nitrogen content, expressed as a percentage by mass is calculated by the following formula

$$W_{PN} = \frac{1.4007 \text{ x } (V_s - V_B) \text{x } N}{W_t}$$

Where,

W_{PN} = true protein nitrogen content of the sample, expressed as a percentage by mass;

V_S= volume in ml of the standard hydrochloric acid used for sample;

V_B= volume in ml of the standard hydrochloric acid used for blank test;

N = Normality of the standard hydrochloric acid expressed to four decimal places;

W_t= mass of the test portion in g, expressed to nearest 0.1 mg.

Express the results to four decimal places.

19.3.3.5. Calculation of True Protein Content in Milk Sample

The true protein content is obtained by multiplying the true protein nitrogen content by 6.38. Express the test result to three decimal places.

19.4. Determination of Protein content in milk and milk products by BIS method.

The steps recommended by Bureau of Indian Standards (BIS) in the Kjeldahl method for protein estimation (IS: 7219:1973) differs with other methods. In this method, the sample is oxidised in the presence of sulphuric acid and nitrogenous compounds are converted into ammonium sulphate. Mercury is added to the digestion mixture as a catalyst and alkali sulphate as a boiling-point elevator. Ammonia is liberated by adding and excess of alkali and is quantitatively distilled into a measured volume of standard hydrochloric or sulphuric acid. The acid not neutralized by ammonia is back-titrated with standards alkali. Please refer to procedure mentioned in IS: 7219-1973 for details.

References:

IS 7219-1973 (Reaffirmed 1995) Method for determination of protein in foods and feeds. Bureau of Indian Standards, New Delhi

ISO 8968-1/IDF 020-1:2001 - Milk - Determination of nitrogen content - Part 1: Kjeldahl method.

ISO 8968-2/IDF 020-2:2001 - Milk - Determination of nitrogen content - Part 2: Block-digestion method (Macro method).

ISO 8968-3/IDF 020-3:2004 - Milk - Determination of nitrogen content - Part 3: Block-digestion method (Semi-micro rapid routine method).

ISO 8968-4/IDF 020-4:2001 - Milk - Determination of nitrogen content - Part 4: Determination of non-protein-nitrogen content.

ISO 8968-5/IDF 020-5:2001 - Milk - Determination of nitrogen content - Part 5: Determination of protein-nitrogen content.

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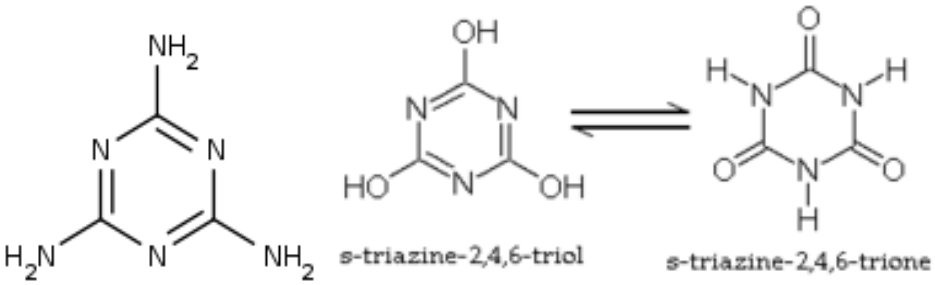
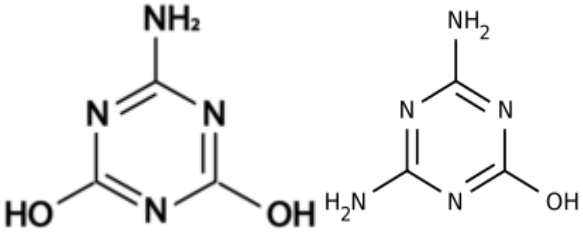
20. DETERMINATION OF MELAMINE AND CYANURIC ACID IN MILK, MILK PRODUCT AND INFANT FORMULAE - GUIDELINES

Melamine (2,4,6- triamino-1,3,5 triazine) is not a natural constituent of milk and thus its presence in milk products indicates either adulteration or contamination. It is widely believed that the melamine adulteration in milk and milk products by unscrupulous traders and producers is done to mislead the nitrogen-based protein estimation standard tests, such as the Kjeldahl and Duma tests. As in these methods of protein estimation, nitrogen is essentially estimated; the presence of melamine increases the estimated nitrogen and thus calculated protein content fraudulently. Melamine contains 66% nitrogen by mass and thus its addition to milk products can falsely elevate the crude protein content estimated by Kjeldahl method. Commercially produced melamine may contain structural analogues (Fig 1) such as cyanuric acid (1,3,5-triazinane-2,4,6-trione), ammelide (6-amino-2,4-dihydroxy-1,3,5-triazine) and ammeline (4,6-diamino-2-hydroxy-1,3,5 triazine). Many countries have now specified the limit for the melamine in milk and milk products. In 2008, FSSAI has also issued advisory to the state health authorities on monitoring melamine contamination of foods (Advisory No.: 2/FSSAI/2008).



Melamine Cyanuric acid



Ammelide Ammeline

Fig 1. Melamine and its structural analogues

20.1 Method 1. Detection of Non-Protein Nitrogen (NPN) Containing Substances in Milk

The Annexure 1 of the FSSAI Advisory has also stated a method for Detection of Non-Protein Nitrogen (NPN) Containing Substances in Milk. The method can be applied to suspected milk samples. The method is based on the concept that NPN content in milk is fairly constant and any abrupt increase in NPN content indicates abnormality. A step-wise procedure for detection of non-protein nitrogen containing substances in milk is given below. This procedure will provide approximate amount of non-protein nitrogen rich substances which have been mixed with milk as adulterant. The procedure is based on the determination of total protein in milk directly in Milkoscan (Milkoscan gives the values of total protein in milk directly), and estimation of total nitrogen in the milk sample (which may include nitrogen of protein and non-protein substances present in milk), which is then converted into the equivalent amount of protein by using the accepted conversion factor, and then comparing these two values of protein so obtained.

The procedure is as follows:

A. Take a sample of milk (reconstituted milk in case of milk powder) and divide it into two parts.

B. Analyse the Part 1 of the sample in Milkoscan for total protein content. The result would give the total protein (milk proteins as well as non-milk proteins, if mixed with

milk). The test method is provided in Annex 1.

C. Analyse Part 2 of the sample through *Kjeldahl* method for total nitrogen content. The nitrogen content so obtained would include nitrogen from all the sources – protein and non-protein. Convert the nitrogen content into protein content by using the approved conversion factor of 6.38. The standard ISO method (ISO/TS 15495:2010) involving LC-MS/MS, which can be used for quantitative estimation of melamine and cyanuric acid is given below (Method 3).

D. Compare the protein content obtained directly in Step B with the protein content obtained in Step C. If the protein content obtained through Step C is appreciably higher than that analyzed in Step B then it is an indication that the milk has been adulterated with non-protein nitrogen rich substance, which could be urea, melamine or other sources. If the difference in the values of proteins obtained in Step B and Step C is not substantial then there is no indication of adulteration of milk with nonprotein nitrogen substances. Small differences could be due to acceptable variations inherent in the analysis by these methods.

Notes:

1. It is important that the Milkoscan has been calibrated with pure, unadulterated milk using *Kjeldahl* method.
2. Theoretically, the above suggested procedure will be able to detect non-protein nitrogen from melamine content above 0.1% in milk. (0.1% of melamine in milk will translate into a difference of 0.4% protein as determined by *Kjeldahl* method and using Milkoscan.)
3. The test method using Milkoscan provided in Annex 1 is for guidance only. The exact procedure for using particular model of Milkoscan provided in the manufacturer supplied operation manual should be used.

20.2 Method 2. ELISA based Methods

One of the approaches in the detection of melamine involves the use of commercial enzyme linked immunosorbent assay (ELISA) technology. Garber (2008)

evaluated three commercially available ELISA kits. These include Melamine Plate kit (Abraxis, Warminster, Pa, USA.), an atrazine ELISA test kit (Abraxis, Warminster, Pa, USA.) and the EnviroGard Triazine Plate kit (Strategic Diagnostics, Inc., Newark, Del, USA). The atrazine and triazine kits cross-react with melamine. All these ELISA kits involved use of a competitive ELISA design in which the target analyte (i.e. melamine) in the test sample competes with a labeled antigen, supplied with the test kit, to bind with the capture antibody, as a result of which there is a decrease in response when antigen is present in the sample. All three ELISA kits can detect melamine in food samples, however, only the Melamine Plate Kit manufactured by Abraxis could detect melamine at concentrations sufficiently low enough to surpass other validated methods and LOD was 0.009 ppb and 1 ppb in PBS buffer and pet foods respectively. The recovery of melamine spiked in pet food was around 74%. Garber (2008) has concluded that Abraxis ELISA for melamine provides a rapid, reliable, sensitive and high throughput method for detection of melamine in food matrix. The price of the kit is around $400.00 plus shipping (approximately $160.00) and one kit contains 96 wells.

20.3 Method 3. ISO Method

A method described by ISO (ISO/TS 15495:2010) which gives guidance for the quantitative determination of melamine and cyanuric acid content in milk, powdered milk products, and infant formulae by electrospray ionization liquid chromatography tandem mass spectrometry (LC-MS/MS) can be followed.

In this method, the sample is made homogenous reconstituted (optional) in the case of powdered samples. A suitable solvent is added to the test sample to precipitate proteins from the matrix and to extract melamine and cyanuric acid. After centrifugation, an aliquot of the supernatant is analysed by LC-MS/MS. For details, refer to ISO document (ISO/TS 15495:2010).

Limits of detection (LOD) of ISO method

The LODs (signal-to-noise ratio equals 3) for melamine content are 0.08 mg/kg in milk-based infant formula and 0.07 mg/kg in cow milk, respectively.

The LODs for cyanuric acid content are 0.10 mg/kg in milk-based infant formula and 0.07 mg/kg in cow milk, respectively.

Limits of quantitation (LOQ) of ISO method

The LOQs (lowest validated levels) for melamine content are 0.11 mg/kg in milk-based infant formula and 0.10 mg/kg in cow milk, respectively.

The LOQs for cyanuric acid content are 0.13 mg/kg in milk-based infant formula and 0.10 mg/kg in cow milk, respectively.

References

1. *Advisory no.: 2/FSSAI/2008. Advisory to the state health authorities on monitoring melamine contamination of foods. FSSAI, India (http://www.fssai.gov.in/Portals/0/Pdf/AdvisoryMelamineinFoods[1].pdf).*
2. *Garber E A E (2008) Detection of melamine using commercial enzyme-linked immunosorbent assay technology. J Food Protection **71**(3): 590-94.*
3. *ISO/TS 15495/IDF/RM 230 (2010) Milk, milk products and infant formulae — Guidelines for the quantitative determination of melamine and cyanuric acid by LC-MS/MS. International Organization for Standardization (ISO), Geneva.*

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*The methods mentioned in the manual needs to be verified/ validated before they are put in use by the laboratory.

Food Safety and Standards Authority of India
(Ministry of Health and Family Welfare)
FDA Bhawan, Kotla Road,
New Delhi-110002
www.fssai.gov.in