**Doc No. FAD 24 (XXXX)WC**

**November 2024**

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| **BUREAU OF INDIAN STANDARDS** |
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| *भारतीय मानक मसौदा* |
| **बेकरी उत्पाद – विश्लेषण पद्धतियााँ** |
| (*आई एस 12711 का पहला पुनरीक्षण*) |
|  |
| *Draft Indian Standard* |
| **Bakery Products – Methods Of Analysis** |
| (*First Revision of IS 12711*) |
|  |
| ICS No. |
| Ready to Eat Foods and Specialized Products Sectional Committee, FAD 24  |  Last Date of Comments: |

**FOREWORD**

**1 SCOPE**

This standard prescribed the methods of analysis of bakery products.

**2 REFERENCES**

The standard given below contain provisions which, through reference in this text, constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of these standards.

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| --- | --- |
| *IS No.* | *Title* |
| IS 1070 : 2023 | Reagent grade water specification (*fourth* *revision*) |

**3 QUALITY OF REAGENTS**

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

NOTE – ‘Pure chemicals’ shall mean chemicals that do not contain impurities which affect test results.

**4 PREPARATION OF THE SAMPLE**

**4.1** In case of powdered or granular substances, mix the contents of a whole pack and, if necessary, further grind in a clean and dry mortar to convert it into homogenous powder. Store the ground sample in a clean and dry air-tight glass container.

**4.2** For low moisture crisp products, such as biscuits, cookies and rusks, etc. break the contents of the whole pack into small pieces and subsequently grind the pieces either in an electrically driven, clean, dry blender or in a clean and dry mortar to a near homogeneous powder. Store the powdered material in a dry air-tight glass container.

**4.3** For semi-moist products, such as, cakes, bread, buns, etc., cut the contents of pack into small piece with the help of clean dry scissors or a sharp-edged knife and further grind in an electrically driven dry blender taking care that the sample temperature does not rise above 45 °C in the entire operation. In the case of packs above 400 g, such as bread loaves, slice them uniformly into thin slices with the help of a sharp-edged knife and take two slices from the center and two from each end leaving the outermost end slices and proceed as described above.

**5 DETERMINATION OF MOISTURE CONTENT**

**5.1 Principle**

Air-ovens are electrically heated and the air within is at atmospheric pressure. Air circulation is by convection or forced draft. The loss of weight during drying is calculated as a percentage of the weight before drying and is taken as the percent moisture of the sample.

**5.2 Apparatus**

i) Moisture Dish *–* made of porcelain, silica, glass, aluminium or stainless steel;

ii) Oven *–* electric, maintained at 105 °C ± 2 °C; and

iii)Desiccator.

**5.3** **Procedure**

Weigh accurately about 5 g of the prepared sample in the moisture dish, previously dried in the oven at 105 °C and weighed. Place the dish in the oven maintained at 105 °C ± 2 °C for 4 hours. Cool in the desiccator and weigh. Repeat the process of drying, cooling and weighing at 30-minute intervals until the difference between two consecutive weighing is less than 1 mg. Record the lowest mass.

**5.3** **Calculation**

Moisture, percent by mass =

Where,

 *M1* = mass, in g, of the dish with the material before drying;

 *M*2 = mass, in g, of the dish with the material after drying to constant mass; and

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

**6 DETERMINATION OF TOTAL ASH**

**6.1 Principle**

The principle of muffle furnace is to burn off the organic matter and to determine the inorganic matter remaining. Heating is carried out in two stages - firstly to remove the water present and to char the sample thoroughly; and finally burning at 550 °C in a muffle furnace. This method is applicable to all food materials.

**6.2** **Apparatus**

i) Dish – Silica;

ii) Desiccator; and

iii) Muffle Furnace.

**6.3 Procedure**

Weigh accurately about 5 g of the prepared sample in a tared, clean and dry silica dish. Ignite the material in the dish with the flame of a suitable burner for about one hour. Complete the ignition by keeping in a muffle furnace at 550 ⁰C ± 10 ⁰C for 4 hours/ till insignificant difference between three subsequent readings. Until grey in colour. Cool in a desiccator and weigh. Repeat the process of igniting, cooling and weighing at one hour intervals until the difference between two successive weighing is less than 1 mg. Note the lowest mass. Preserve this ash for the determination of acid insoluble ash.

**6.4 Calculation**

Total ash (on dry basis), percent by mass =

Where,

 *M*2 = mass, in g, of the dish with the ash;

 *M* = mass, in g, of the empty dish;

 *M*1 = mass, in g, of the dish with the material taken for the test; and

 *W* = percent moisture in the sample.

**7 DETERMINATION OF ACID INSOLUBLE ASH**

**7.1 Principle**

Ignite in a muffle furnace at 550 °C ± 10 °C for one hour. Cool the dish in a desiccator and weigh. Heat the dish again at 550 °C ± 10 °C for 30 minutes, cool in a desiccator and weigh. Repeat this process of heating for 30 minutes, cooling and weighing until the difference between two successive weighing is less than 1 mg.

**7.2 Apparatus**

i) Dish – silica;

ii) Water – bath;

iii) Desiccator; and

iv) Muffle Furnace

**7.3 Reagents**

**7.3.1** *Dilute Hydrochloric Acid*

5N, prepared by diluting 1 volume of concentrated hydrochloric acid to 2.5 volumes with water.

**7.4 Procedure**

To the ash contained in the silica dish (*see* **6.2**), add 25 ml of dilute hydrochloric acid, cover with a watch-glass and heat on a water bath for 10 minutes. Allow to cool and filter the contents of the dish through Whatman filter paper No. 42 of its equivalent. Wash the filter paper with water until the washings are free from the acid. Keep it in an electric air-oven and heat it till it gets dried. Subsequently, ignite the contents of the dish over a burner till the contents get completely charred. Complete the ignition by transferring the dish to a muffle furnace 550 °C ± 10 °C until grey or white ash results. Cool the dish in a desiccator and weigh. Heat the dish again at 550 °C ± 10 °C for 30 minutes. Cool in a desiccator and weigh. Repeat the process of heating, cooling and weighing until the difference between two successive weighing is less than 1 mg. Record the lowest mass.

**7.4 Calculation**

Acid insoluble ash (on dry basis), percent by mass =

Where,

 *M*2 = mass, in g, of the dish with the acid insoluble ash;

 *M* = mass, in g, of the empty dish;

 *M*1 = mass, in g, of the dish with the material taken for the test; and

 *W* = percent of moisture content.

**8 DETERMINATION OF TOTAL SOLID CONTENT**

**8.1 Principle**

To obtain an accurate measurement of the moisture content or total solids of a food using evaporation methods it is necessary to remove all of the water molecules that were originally present in the food, without changing the mass of the food matrix.

 %Total solids = (100 – % Moisture).

This value shall be calculated from the moisture percent as 100 minus the moisture percent. The moisture content shall be determined as described under **5**.

**9 DETERMINATION OF VOLUME/MASS RATIO**

**9.1 Principle**

The volume of the loaf is measured by displacing a volume of rapeseeds equal to the volume of the loaf.

**9.2 Equipment**

**9.2.1** *Wooden Box*

Large enough to contain a loaf of bread in such a manner that the top surface of the loaf remains about 1.5 cm below the top level of the box when the load is placed over a thin layer of rape seeds or mustard seeds in the box.

i) Graduated Cylinder and Glass Beaker – 1 000 ml capacity;

ii) Rape Seeds (or Mustard Seeds);

iii) Weighing Scale – 5 kg capacity; and

iv) Glass Beaker –1 000 ml capacity.

**9.3 Procedure**

**9.3.1** *Determination of Density of Rape or Mustard Seeds*

Weigh the beaker on the weighing scale. Transfer 500 ml of rape seeds or mustard seeds from the measuring cylinder to the beaker and reweigh. Take the average of three readings and calculate the density of the seeds as follows:

Density of seeds (*D*), g/ml =

Where,

 *B* = average mass, in g, of the baker plus 500 ml of rape seeds or mustard seeds; and

 *A* = mass, in g, of the empty beaker.

**9.3.2** *Determination of Volume of Loaf*

Weigh the loaf after it is cooled to room temperature and record the mass. Fill the wooden box with rape or mustard seeds avoiding air packets and level the top surface of the seeds with a wooden plate. Weigh the box with the seeds. Take three such readings and record the average. Empty out the seeds leaving a thin layer at the bottom of the box. Place the loaf on this layer of seeds and fill the rest of the space in the box with rape or mustard seeds. Level off the surface of the seeds by a wooden plate. Weigh the box again. Take three such readings and record the average.

CAUTION **–** Do not press the loaf while keeping in the box. It should be placed on the layer of the seeds. The upper surface of the seeds should be levelled off with sides of the box.

NOTE – Testing shall be done without slicing the loaf.

 **9.4 Calculation**

Calculate the volume of the loaf in the following manner and determine the volume/mass ratio:

Volume, in ml, of the loaf =

Where,

 *C* = average mass, in g, of the box filled with seeds plus mass of the loaf;

 *E* = average mass, in g, of the box filled with loaf, with seeds in the residual space; and

*D* = density, in g/ml, of rape or mustard seeds (*see* **9.3.1**)

**10 DETERMINATION OF FAT**

**10.1 Principle**

The Soxhlet extraction uses the solvent reflux and siphon principle to continuously extract the solid matter by pure solvent, which saves the solvent extraction efficiency and high efficiency. The sold sample is placed on a thimble-shaped filter paper, positioned into Soxhlet extractor, and the device is assembled

**10.2 Soxhlet Extraction Method**

**10.2.1** *Soxhlet Extraction Apparatus*

**10.2.2** *Reagent*

**10.2.2.1** *Petroleum ether –* distilling between 40° C and 60° C

**10.2.3** *Procedure*

Weigh accurately about 5 g of the material in a suitable thimble and dry for 2 hours at 100 °C ± 2 °C. Place the thimble in the Soxhlet extraction apparatus and extract with the solvent for about 8 hours with speed of distillation not less than 5 drops per second.

**10.2.4** *Calculation*

Fat, percent by mass =

Where,

 *M*1 = mass, in g, of the Soxhlet flask with the extracted fat;

 *M2* = mass, in g, of the empty Soxhlet flask. clean and dry; and

**10.3 Acid Hydrolysis Method (AOAC 922.06)**

**10.3.1** *Principle*

Crude fat is determined by acid hydrolysis of the sample with HCl (25 + 11) followed by extraction of hydrolyzed lipid materials with mixed ethers. Ethers are evaporated, and lipid residue is heated to constant weight at 100 °C. Residue is expressed as % crude fat.

**10.3.2** *Apparatus*

i) 50 ml beaker;

ii) Mojonnier fat-extraction apparatus;

iii) Water bath;

iv) Oven;

v) Desiccator;

vi) Filter funnel; and

vii) Cotton pledget.

**10.2.2** *Procedure*

Weigh accurately 2 g of the prepared sample into a 50 ml beaker. Add 2 ml of alcohol and stir to moisten all particles. Add 10 ml of HCl (25+11) and mix well. Place the beaker in a water bath at 70 °C – 80 °C and stir frequently for 30-40 minutes. Add 10 ml of alcohol and cool. Transfer the mixture to a Mojonnier fat-extraction apparatus. Rinse the beaker with 25 ml of ether, added in three portions. Shake vigorously for 1 minute. Add 25 ml of redistilled petroleum ether (bp <60 °C) and shake vigorously again for 1 minute. Let stand until the upper liquid is clear, or centrifuge for 20 minutes at ca 600 rpm. Draw off the ether-fat solution through a filter into a weighed 125 ml beaker-flask. Re-extract the liquid remaining in the tube twice, each time with 15 ml of ether. Draw off the clear ether solutions into the same flask as before. Evaporate the ethers slowly in a steam bath. Dry the fat in an oven at 100 °C to constant weight (ca 90 minutes).

**10.2.3** *Calculation*

Fat percent by mass =

Where,

 *M2* = mass, in g, of the flask with the fat;

 *M* = mass, in g, of the empty flask;

 *M1* = mass, in g, of the sample taken for the test; and

 *M* = mass, in g, of material taken for the test.

**11 DETERMINATION OF ACIDITY OF EXTRACTED FAT (AS PERCENT OLEIC ACID)**

**11.1**

**Principle**

The acid value is determined by directly titrating the oil/fat in an alcoholic medium against standard potassium hydroxide/sodium hydroxide solution.

**11.1 Apparatus**

**11.1.1** *Soxhlet Apparatus –* with 250 ml flat-bottom flask.

**11.2 Reagents**

**11.2.1** *Petroleum Ether –* boiling point below 80° C.

**11.2.2** *Benzene*-*Alcohol-Phenolphthalein* Stock Solution – to 1 liter of distilled benzene, add 1 liter of alcohol or rectified spirit and 0.4 g of phenolphthalein. Mix the contents well.

**11.2.3** Standard Sodium Hydroxide Solution – 0.05 N.

**11.3 Procedure**

Weigh accurately about 5 g of prepared sample and transfer it to the thimble and plug it from the top with extracted cotton and filter paper. Dry the thimble with the contents for 15 to 30 minutes at 100 °C in an oven. Take the weight of empty dry Soxhlet flask. Extract the fat in the Soxhlet apparatus for 8 hours with speed of distillation not less than 5 drops per second and evaporate the solvent in the flask on a water bath. Remove the traces of the residual solvent by keeping the flask in the hot air oven at 100 °C for about half an hour and weigh.

Cool the flask and add 50 ml of mixed benzene-alcohol-phenolphthalein reagent and titrate the contents to a distinct pink color with sodium hydroxide solution taken in a 10 ml micro burette. If the contents of the flask become cloudy during titration, add another 50 ml of the reagent and continue titration. Make a blank titration of the 50 ml reagent. Subtract from the titre of the fat, the blank titre.

**11.4 Calculation**

Acidity of extracted fat (as oleic acid), percent by mass =

Where,

 *V* = volume of 0.05 N sodium hydroxide solution used in titration after subtracting the blank;

 *M*1 = mass, in g, of Soxhlet flask containing fat; and

 *M* = mass, in g, of empty Soxhlet flask.

**12 DETERMINATION OF *p*H OF THE AQUEOUS EXTRACT**

**12.1 Principle**

The principle of *p*H meters is wholly based on the ion exchange between the sample and the glass electrode's inner solution, which generates electrical voltage. The result of the principle of *p*H meters is based on the hydrogen ion concentration and the relation between electric voltage and the *p*H reading.

**12.1.1** *p*H of aqueous extract of the sample shall be determined either by the *p*H meter with glass electrode or by a suitable *p*H comparator provided with standard colour discs. In case of dispute, however, *p*H shall be determined by the *p*H meter.

**12.2 Apparatus**

**12.2.1** *p*H *Meter with Glass Electrode* or *p*H *Comparator* **–** The latter provided with standard *p*H color discs for the indicator solution given under **12.3.2**.

**12.3 Reagents**

**12.3.1** *Water*

Use distilled water of *p*H 6.2 to 7.0. Boil it for about 10 minutes and cool to room temperature immediately before use. Redistill the water in an all-glass apparatus if its *p*H not lie with in this range.

**12.3.2** *Indicator Solutions* – Universal indicator.

**12.4 Procedure**

**12.4.1** *Preparation of Aqueous Extract of the Material*

Grind to a fine paste about 10 g of the material in a glass mortar. Add 100 ml of water and mix thoroughly. Allow the mixture to stand for about 15 minutes. Filter the mixture and collect the filtrate in another beaker.

**12.4.2** *Determination of pH of Aqueous Extract*

Determine the *p*H of the solution by the *p*H meter, or by using the *p*H comparator as described in **12.4.2.1**.

**12.4.2.1** Clean the two glass tubes of the *p*H comparator with carbon tetrachloride to remove any oily or greasy film on them and allow the tubes to dry. Fill the two tubes with the aqueous extract to the 10 ml mark. Add 5 drops of universal indicator in the *p*H comparator. Place the two tubes in position in the comparator, the one without the indicator being placed behind the color discs and compare the color until the nearest color match is obtained. Record the approximate *p*H of the aqueous extract. Discard the contents of the glass tubes to which indicator had been added. Wash with water and dry. Clean the tube with carbon tetrachloride and dry it. Fill the tube with carbon tetrachloride and dry 10 ml mark, add 5 drops of the universal indicator solution and mix the contents well. Place this tube in position, replace the previous standard colour disc with the one provided for the indicator used and read the exact *p*H of the solution when the nearest colour match is obtained.

**13 DETERMINATION OF CRUDE FIBRE**

**13.1 Principle**

By treating a food material successively with petroleum ether, sulphuric acid and sodium hydroxide, all the lipids, carbohydrates etc. are removed/ hydrolysed leaving only the crude fibre along with some insoluble mineral matter. The insoluble residue is freed of the soluble materials by water washing and filtration, and ashed. The difference in weight of the alcohol washed residue (dried) and the ash give the weight of true crude fibre.

**13.1 Reagents**

i) Petroleum Ether;

ii) Dilute Sulphuric Acid – 1.25 percent (m/v), accurately prepared;

iii) Sodium Hydroxide Solution – 1.25 percent (m/v), accurately prepared; and

iv) Ethyl Alcohol – 95 percent by volume.

**13.2 Procedure**

Weigh accurately about 2.5 g of the moisture free sample after determining the total solid content and extract for about one hour with petroleum ether, using a Soxhlet apparatus. Transfer the fat-free material to a one litre flask. Take 200 ml of dilute sulphuric acid in a beaker and bring to boil. Transfer the whole of the boiling acid to the flask containing the fat-free material and immediately connect the flask with a water-cooled reflux condenser and heat so that the contents of the flask begin to boil within one minute.

Rotate the flask frequently, taking care not to allow the material to stick to the sides of the flask, and not to keep the material out of contact with the 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 acidic to litmus. Bring some quantity of sodium hydroxide solution to boiling under a reflux condenser. Wash the residue on the linen into the flask with 200 ml of 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 but compact layer of ignited asbestos. Wash the residue thoroughly first with hot water and then with about 15 ml of ethyl alcohol, 95 percent by volume. Dry the Gooch crucible and contents at 105 °C ± 2 °C in an air-oven to constant mass. Cool and weigh. Incinerate the contents of the Gooch crucible in an electric muffle furnace at 600 °C ± 20 °C until all the carbonaceous matter is burnt. Cool the Gooch crucible containing the ash in a desiccator and weigh.

**13.3 Calculation**

Crude fibre (on dry basis), percent by mass =

Where,

*M1*= mass, in g, of Gooch crucible and contents before ashing;

*M2* = mass, in g, of Gooch crucible containing asbestos and ash;

*M* = mass, in g, of the material taken for the test; and

*W* = percent of moisture content.

**14 DETERMINATION OF ALCOHOLIC ACIDITY**

**14.1 Principle**

Alcoholic acidity is defined as mg of H2SO4 required for 100 g of the sample to have the same alcohol soluble acids. Grains or their milled products on storage undergo physical, as well as, chemical changes.

**14.1 Reagents**

i) Neutral Ethyl Alcohol – 90 percent (v/v);

ii) Standard Sodium Hydroxide Solution – approximately 0.05 N; and

iii) Phenolphthalein-Indicator Solution – 60 mg of phenolphthalein dissolved in 100 ml rectified spirit.

**14.2 Procedure**

Weigh 5 g of sample into a stoppered conical flask and add 50 ml of neutral ethyl alcohol. Stopper, shake and allow to stand for 24 hours, with occasional shaking. Filter the alcoholic extract through a dry filter paper. Titrate the combined alcoholic extract against 0.05 N standard sodium hydroxide solution using phenolphthalein as indicator. calculate the percentage of alcoholic acidity as sulphuric acid.

**14.3 Calculation**

Alcoholic acidity (as H2SO4) in 90 percent alcohol, percent by mass =

Where,

*A* = volume, in ml, of standard sodium hydroxide solution used in titration;

*N* = normality of standard sodium hydroxide solution; and

*M* = mass, in g, for the material taken for the test.

**15 DETERMINATION OF FRUITS IN FRUIT BREAD/CAKE**

**15.1** The method determines both dry fruits (rasins, cashewnuts, almonds and walnuts) and preserved fruits (*PETHA* peel. *KARONDA*) and cherries) in fruit bread/cakes.

**15.2 Equipment**

i) Bread or Cake Knife;

ii) Brush; and

iii) Weighing Balance.

**15.3 Method**

Weigh accurately four cake/bread slices (approximately 100 g). Pick one by one each piece of preserved fruit and dry fruit and wipe them individually with a camel hair brush on a filter paper or clean cloth. Segregate preserved and dry fruits and weigh each type separately and calculate the percentage f fruits from the mass for fruits picked out separately for preserved fruits and dry fruits.

**16 DETERMINATION OF NON-FAT MILK SOLIDS IN MILK BREAD**

**16.1 Principle**

The method is a colorimetric oven for estimating non-fat milk solids in milk bread based on the orotic acid (2, 6-dihydroxypyrimidine-4-carboxylic aid) content. The mean orotic acid content of non-fat milk solids is 62.5 mg/100 g (range 48.0-74.5 mg/100 g).

**16.2 Apparatus**

i) Air-Drying Oven;

ii) Homogenizer;

iii) Pipettes – 5, 10 and 25/ml;

iv) Glass Stoppered Test-Tubes;

v) Volumetric Flask – 500, 100, 50 and 10 ml capacity;

vi) Water Bath; and

vii) Colorimeter.

**16.3 Reagents**

i) Zinc Sulphate – 23 percent (m/v) solution;

ii) Potassium Hexacyanoferrate – 15 percent (m/v) solution;

iii) p-Dimethylaminobenzaldehyde (DAB) – 3 percent (m/v) inpropanol;

iv) Standard Orotic Acid – Dissolve 50 mg orotic acid in a mixture of 1 ml of 0.88 ammonia and 10 ml water. Dilute to 500 ml with water. Take 10 ml aliquot and dilute to 100 ml with water. Further dilute 2.5, 5, 10 and 15 ml of this solution to 50 ml to produce solutions containing 2.5, 5, 10 and 15 µg orotic acid per 5 ml;

v) Saturated Bromine Water;

vi) Ascorbic Acid Solution – 10 percent;

vii) n-Butyl Acetate; and

viii) Anhydrous Sodium Sulphate.

**16.4 Procedure**

**16.4.1** Weigh the bread on receipt accurately, nearest to 0.1 g. Cut the bread into 2-3 mm slices, spread on paper and allow to dry in a warm room overnight so that the bread is crisp and brittle. The sample should be in equilibrium with the atmosphere so that the moisture content remains constant during grinding. Then return quantitatively the air-dried bread to the balance and re-weigh. Grind to pass through an 850 µm IS sieve mix and transfer to an air-tight container. Determine the total solids by drying 2 g at 130°C for 1 hour.

**16.4.2** Weigh 5 g of dried sample into the beaker of a homogenizer, add 100 ml water and mix at the maximum speed for 1 minute. Filter the supernatant liquor through a 15 cm Whatman No. 541 paper rejecting the first 10 ml. 5 ml is required for the determination.

**16.4.3** Into a series of glass-stoppered test tubes, add by pipette 5 ml of test solution (containing 2 µg -15 µg orotic acid) 5 ml of each of the standard orotic acid solutions and 5 ml of each of the standard orotic acid solutions and 5 ml of water to act as the blank. Add to each tube 1.5 ml of saturated bromine water and allow the mixture to stand at room temperature for not more than 5 minutes. As the addition for bromine water is made to the series for tubes, the times will vary slightly between each, the time of reaction is not critical provided it is between 1 and 5 minutes. Add 2 ml of 10 percent ascorbic acid solution to each tube and place the tubes in a water bath at 40° C for 5 minutes. Cool to room temperature, add to each tube 4.0 ml n-butyl acetate and shake vigorously for 15 seconds. Transfer the upper separated layers to dry test rubes containing 1 g anhydrous sodium sulphate. Mix gently. Add another gram of anhydrous sodium sulphate. Mix gently and allow to separate. Transfer the clear butyl acetate layer to a 1-cm cell and measure the optical density at 461 nm to 462 nm against the blank.

**16.5 Calculation**

Draw a calibration graph of the standard orotic acid solution plotting the optical density on the X-axis against the concentration of orotic acid on the Y-axis. Determine the orotic acid content in 5 ml of sample extract by interpolation of the colorimeter reading on the calibration graph, and hence the amount in the dry sample. For converting to milk, assume that skim milk powder contains 62.5 mg orotic acid per 100 g.

**17 CHARACTERIZATION AND ESTIMATION OF SYNTHETIC DYES**

**17.1 Principle**

Synthetic acidic colour(s) is dyed on to wool in acidic medium and extracted (stripped) from the wool into aqueous alkaline medium. The extracted colour(s) is developed (separated) by paper chromatography along with standard dyes using a suitable solvent system. Comparing their Rf values with that of standard colours identifies the sample colours. Quantification of the colours is done by spectrophotometry.

**17.2 Apparatus**

i) Glass Pestle and Mortar;

ii) Beakers *–* 250 and 100 ml capacity;

iii) Chromatographic Chamber *–* 30 cm x 20 cm x 10 cm;

iv) Test Tubes;

v) Spectrophotometer;

vi) Water Bath; and

vii) Porcelain Dish.

**17.3** **Reagents**

i) 2 Percent ammonia n 70 percent alcohol.

ii) White Knitting Wool – Boil pure white knitting wool in 1 percent sodium hydroxide solution and then in water to remove alkali. Wash repeatedly with distilled water and dry.

iii) Chromatographic Paper – Whatman No. 1.

iv) Hydrochloric Acid (0.1 N) – 8.5 ml of concentrated hydrochloric acid diluted to 1 litre with distilled water.

**17.4 Procedure**

Thoroughly grind 10 g of the powdered food material with 50 ml of 2 percent ammonia in 70 percent alcohol. Allow to stand for few hours and centrifuge. Pour the clear supernatant liquid in the disc and evaporate on the water bath. Dissolve the residue in 30 ml water acidified with acetic acid.

Add a 20 cm strip of pure white wool to the above solution and boil. When the wool takes up the colour fairly completely take out and wash it with tap water. Transfer the washed wool to a small beaker and boil gently with dilute ammonia (1:4) If the colour is stripped by the alcohol, the presence of an acid coal tar dye is indicated. Remove the wool. Make the liquid slightly acidic and boil. Add a fresh piece of wool and continue boiling until all colour is removed. Extract the dye from the wool again with a small volume of dilute ammonia, filter through small plug of cotton and evaporate to low bulk. (This double stripping technique usually gives a pure product but is not always necessary. Natural colours may also dye the wool during the first treatment but the colour is not removed by ammonia.) Transfer the solution to a volumetric flask and make the volume to 50 ml with water.

NOTE – Basic dyes can be separated by making the food alkaline with dilute ammonia, boiling with wool and then stripping with dilute acetic acid. At present, all the permitted water soluble colours are acidic in nature and hence an indication of the presence of a basic dye suggests the presence of a non-permitted colour.

**17.3.1** *Separation of Colours by Paper Chromatography*

Take Whatman No. 1 filter paper sheet (15 cm x 30 cm) and draw a line parallel to the bottom edge of the sheet and about 2 cm away from it. Pipette 0.5 ml of the extracted dye solution with the help of a graduated pipette and apply it on filter paper sheet in the form of a band on the line.

Prepare 0.1 percent solutions of the permitted water soluble dyes and with the help of capillary tubes, apply spots of all these dyes on the line leaving about 1.5 cm distance between two spots. Care should be taken to ensure that the solution does not spread n the filter paper. Allow the coloured spots to dry and subsequently suspend the paper sheet in the chromatography chamber such that the lower edge of the sheet remains dipped in the solvent placed in a solvent boat. Any of the following solvent systems may be used for the following solvent systems may be used for the separation of colours but solvent system at (5) has been found to give the best resolution:

i) 1 ml ammonia (sp. gr 0.85) + 99 ml water;

ii) 2.5 percent aqueous sodium chloride;

iii) 2 percent sodium chloride in 50 percent ethanol;

iv) Isobutanol: ethanol: water [1:2:1 (*v*/*v*)];

v) N-butanol: water: acetic acid (20:12:5); and

vi) Isobutanol : ethanol : acetic acid (3:12:5).

Close the chromatographic chamber tightly and allow the solvent to rise. When the solvent front has risen to about 20 cm from the base line, remove the filter paper sheet and allow it to dry at room temperature. Mark coloured bands and carefully cut coloured strips from the paper chromatogram. Cut coloured strips into small pieces and transfer them to a test tube and add about 1 ml 0.1 N-hydrochloric acid (HCI). Allow the colour to extract and decant the coloured extract in a volumetric flask. Repeat the process of extraction and decanting till all the colour is removed from the paper. Make up the volume Determine absorbance maximum and read the optical density at absorbances maximum and read the optical density at absorbance maximum against a blank prepared by cutting an equivalent strip of plain portion of the chromatogram and extracting it with 0.1 N HCI exactly as described in case of sample. From the absorbance values compute the concentration versus optical density (see 17.3.2)

**17.3.2** *Plotting of Standard Curve*

Prepare 0·1 percent solution of the dye in 0·1 N hydrochloric acid. Take 0.25, 0.50. 0·75, 1.0,

1·25 and 1·5 ml aliquots of this and dilute to 100 ml with 0·1 N hydrochloric acid. Read their

absorbance at respective absorbance maxima. Plot absorbance value against the concentration of the dye.

 **17.3.3** The *RI* values and absorbance maxima of the permitted water soluble dyes are given below which may be used as a guide in characterization of the dye and in determining their concentration. However chromatographic *Rf* values are known to vary slightly because of variation in temperature, solvent purity and solvent -saturation of the chromatography chamber, it is, therefore, essential that known dyes should be applied along with the sample for comparison of Rf values under actual conditions used in the test.

|  |  |  |
| --- | --- | --- |
| **Solvent System** | ***Rf Values*** | **Absorbance****Maximum 1 nm** |
| **(1)** | **(2)** | **(3)** | **(4)** | **(5)** | **(6)** | **(7)** | **(8)** |
| Ponceau 4 R | 0.95 | 0.36 | 0.42 | 0.29 | 0.33 | 0.29 | 505 |
| Carmoisine | 0.61 | 0.04 | 0.56 | 0.51 | 0.56 | 0.28 | 515 |
| Amaranth | 0.77 | 0.06 | 0.20 | 0.24 | 0.19 | 0.20 | 520 |
| Erythrosine | 0.23 | 0.00 | 0.70 | 1.00 | 1.00 | 0.38 | 525 |
| Fast red E | 0.45 | 0.00 | 0.60 | 0.45 | 0.54 | 0.60 | 505 |
| Sunset yellow FCF | 0.78 | 0.26 | 0.65 | 0.49 | 0.45 | 0.56 | 480 |
| Tartrazine | 1.00 | 0.26 | 0.30 | 0.26 | 0.18 | 0.22 | 430 |
| Inidigoi carmine | – | 0.07 | 0.30 | 0.28 | 0.21 | 0.27 | 615 |
| Brilliant blue FCF | – | – | – | – | – | – | 630 |
| Green S | 1.00 | 0.88 | 1.00 | 0.73 | 0.57 | 0.50 | 635 |
| Green FCF | – | – | – | – | – | – | 624 |

 **18 DETERMINATION OF SORBIC ACID**

Two methods, namely, colorimetric and spectrophotometric methods have been given. Any of the two can be used.

**18.1 Colorimetric Method**

**18.1.1** *Principle*

2,6-dichlorophenol indophenol dye, which is blue in alkaline solution and red in acid solution, is reduced by ascorbic acid to a colourlers form. The reaction is quantitative and can be performed by titration, this reaction is practically specific for ascorbic acid in fresh fruits and vegetables, Sulphur dioxide present in products like c~qua~hea cn deduce the dye and thus interferes in the estimation, condensing SO, with formaldehyde can eliminate this interference.

**18.1.2** *Apparatus*

i) Steam distillation apparatus;

ii) Volumetric flask – 50 ml and l litre capacity;

iii) Pipette – sand 20 ml capacity;

iv) Test tubes – 15 ml capacity;

v) Spectrophotometer – with 10 mm matched cells;

vi) Water bath; and

vii) Ice bath.

**18.1.3** *Reagents*

i) Sulphuric acid – 2 N and 0.3 N;

ii) Potassium dichromate solution – 147 mg potassium dichromate dissolved in distilled water and diluted to 100 ml;

iii) Thiobarbituric acid solution 0.5 percent – Dissolve 250 mg Thiobarbituric acid in 0.5 N sodium hydroxide solution in a SO ml volumetric flask by swirling in hot water. Add 20 ml distilled water, neutralize with 3 ml of I N hydrochloric acid and dilute to volume with distilled water. This solution should be prepared fresh before experimentation;

iv) Crystalline magnesium sulphate MgSO4.7H2O; and

v) Standard sorbic acid solution – Accurately weigh 134 mg potassium sorbate (equivalent to 100 mg sorbic acid) and dilute to 1 litre with distilled water. One ml of solution corresponds to 0.1 mg of sorbic acid. This solution is stable for several days when refrigerated.

**18.1.4** *Procedure*

**18.1.4.1** *Preparation of the sample*

a) *All types of bread and cakes not containing fruits*

Take one or a half loaf of bread or cake and cut it into slices of 2-3mmthickness. Spread the slices on the paper and let them dry in a warm place or at room temperature until sufficiently crisp and brittle to grind well. Grind entire sample to pass through 850micron sieve. Mix well and keep in an airtight container.

b) *Bread and cakes containing raisins and fruits*

Take one or a half loaf of bread or cake and cut it into slices of 2-3 mm thickness. Spread the slices on the paper and let them dry in a warm place or at room temperature until sufficiently crisp. Comminute by passing twice through a food chopper and dry the sample in an oven at 70° C under a pressure of less than 50 mm of mercury.

 **18.1.4.2** *Test portion*

Weigh 1.5 g to 2.0 g prepared sample into distillation tube containing silicon chips. Add 10 ml of 2 N sulphuric acid and 10 g magnesium sulphate. Steam distil the contents, maintaining 20 ml to 30 ml volume in distillation tube with small burner. Avoid charring. Collect 100 ml to 125 ml distillate in 250 ml volumetric flask within *45* minutes. Rinse the condenser with distilled water and dilute the distillate to volume and mix thoroughly.

**18.1.4.3** *Determination*

Pipette 2 ml of test portion and 2 ml of distilled water (for blank) into separate 15 m) test tubes. Add 1 ml of 0.3 N sulphuric acid and 1 ml of potassium dichromate solution and heat in boiling water bath exactly for five minutes. Immerse tubes in ice bath and add 2 ml thiobarbituric acid solution. Replace it in boiling water bath and boil it for 10 minutes. Cool and determine optical density of solution at 532 nm against blank using matched 1 cm cells.

**18.1.4.4** *Plotting of the calibration curve*

Pipette 5, 10, 15, 20 and 25 ml sorbic acid standard solutions into separate 500 ml volumetric flasks. Dilute each to volume and mix thoroughly and proceed as specified in **18.1.4.3.** Plot the optical density against µg sorbic acid/ml.

**18.1.5** *Calculation*

Calculate the sorbic acid content in the sample after reading the corresponding sorbic acid value of the optical density.

Percent sorbic acid in the sample =

Where,

 *A* = sorbic acid content obtained from calibration curve; and

*M* = mass of sample taken.

**18.2** **Spectrophotometric Method**

**18.2.1** *Principle*

In principle, preservatives from foods are extracted by steam distillation. The fraction is then purified by solvent extraction. Each acidified preservative from the extract solution is then measured at the specific absorption wavelength using UV spectrophotometer.

**18.2.2** *Reagents*

Metaphosphoric acid solution – 5g phosphoric acid dissolved in 250 ml distilled water and diluted to 1 litre with absolute alcohol;

ii) Mixed ethers – petroleum ether : anhydrous ether (1:1);

iii) Potassium permanganate solution – 15 g-potassium permanganate dissolved in distilled water, diluted to 100 ml and filtered through glass wool;

iv) Sorbic acid solution (stocks) – 200 mg of sorbic acid dissolved in 200 ml of mixed ethers;

v) Working solution – 10 ml stock solution diluted to 200 ml with mixed ether and this solution corresponds to 0·05 mg/ml; and

vi) Reference solution – Shake 100 ml mixed ethers with 10 ml metaphosphoric acid solution and dry supernatant ether fraction with 5 g anhydrous granular sodium sulphate.

**18.2.3** *Apparatus*

i) High speed blender;

ii) Separating funnels – 500 fill;

iii) Volumetric flasks;

iv) Graduated pipettes;

v) Ultraviolet spectrophotometer – Provided with a 0.5 mm monochromator with silica cells of 20 mm thickness fitted with ground lids.

 **18.2.4** *Procedure*

**18.2.4.1** *Preparation of the sample –* Proceed as in **18.1.4.1.**

**18.2.4.2** *Test portion*

Accurately weigh 10 g prepared sample in high speed blender cup. Add enough metaphosphoric acid solution to yield a total of 100 ml liquid in mixture. Blend for 1 minute and immediately filter through batman No. 3 paper. Transfer 10 ml filtrate to 250 ml separating funnel containing 100 ml mixed ethers and shake for 1 minute. Discard aqueous layer and dry the ether extract with 5 g anhydrous sodium sulphate.

**18.2.4.3** *Determination*

Place the ether solution in a silica cell with a ground lid of thickness 20 mm and measure the absorbance of this solution at 250 nm with respect to the reference solution in a similar silica cell.

**18.2.4.4** *Plotting of the calibration curve*

Into a series of four 100 ml volumetric flasks, add 1, 2, 4 and 6 ml of working standard sorbic acid solution and dilute to volume with mixed ethers. Determine absorbance of the solutions at 250 nm. Plot the absorbance against mg of sorbic acid/ml.

**18.2.5** *Calculation*

Calculate the sorbic acid content in the sample after reading the corresponding sorbic acid value from the calibration curve.

Percent sorbic acid in the sample = (mg sorbic acid/g sample) × (1/100 mg)×100 = mg sorbic acid/10.

Percent sodium sorbate = percent sorbic acid × 1.34.

**18.3 High Performance Liquid Chromatography Method (IS 17562 : 2021)**

**18.3.1** *Principle*

Extraction of benzoic acid and/or sorbic acid from a test portion using a mixture of ammonium acetate buffer solution and methanol, under acidic conditions. The concentration of benzoic and/ or sorbic acid is determined by means of high performance liquid chromatography (HPLC) using a reverse phase column and ultraviolet (UV) detector.

**18.3.2** *Reagents and materials*

Use only reagents of recognized analytical grade, unless otherwise specified, and water of HPLC grade.

i) Acetic acid (CH3COOH), glacial;

ii) Methanol (CH3OH), for HPLC;

iii) Ammonium acetate (CH3COONH4), 0,01 mol/l solution . Dissolve 0,771 g of ammonium acetate in 1 l of water;

iv) Ammonium acetate/acetic acid (CH3COONH4/CH3COOH), buffer solution. Mix 1 000 volume parts of ammonium acetate solution (3.3) with 1,2 volume parts of acetic acid (3.1);

v) Benzoic acid (C6H5COOH), stock solution. Dissolve 100 mg of benzoic acid in 40 ml of methanol (3.2) and make up to the mark with water in a 100 ml volumetric flask, to obtain the stock solution, *ρ*(C6H5COOH) = 1 g/l;

vi) Sorbic acid [CH3(CH:CH)2COOH], stock solution. Dissolve 100 mg of sorbic acid in 40 ml of methanol (3.2) and make up to the mark with water in a 100 ml volumetric flask, to obtain the stock solution, *ρ*[CH3(CH:CH)2COOH] = 1 g/l;

vii) Potassium hexacyanoferrate(II), trihydrate, K4[Fe(CN)6].3H2O. 2;

viii) Zinc sulfate, heptahydrate, (ZnSO4.7H2O), 300 g/l solution;

ix) Extraction solution Mix 60 volume parts of ammonium acetate/acetic acid buffer solution (3.4) with 40 volume parts of methanol;

x) Eluent for HPLC Mix 50 volume parts of ammonium acetate solution (3.4) with 40 volume parts of methanol for HPLC (3.2) and adjust to a pH of 4,5 to 4,6 with acetic acid (3.1). Filter the eluent over a membrane filter (4.2);

xi) Carrez solution I Dissolve 150 g of potassium hexacyanoferrate (II) (3.7) in water in a 1 000 ml volumetric flask. Dilute to the mark with water and mix the solution;

xii) Carrez solution II Dissolve 300 g of zinc sulfate (3.8) in water in a 100 ml volumetric flask. Dilute to the mark with water and mix the solution; and

xiii) Pleated filter paper, hard.

**18.3.3** *Apparatus*

Usual laboratory apparatus and, in particular, the following.

i) Ultrasonic bath;

ii) Membrane filters, of pore size 0,45 µm, for aqueous solutions (e.g. cellulose acetate); diameter dependent on the filter holder;

iii) Filter holder, for membrane filters with suitable aspirating and collection vessels;

iv) High performance liquid chromatograph, equipped with a UV-detector (variable wavelength) and recorder and/or integrator or computer with the appropriate integrating programme; and

v) Reverse phase separation column, e.g. reverse phase C8, 250 mm × 4,6 mm, particle size 5 µm.

**18.3.4** *Sample*

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

**18.3.5** *Procedure*

**18.3.5.1 Preparation of test solution**

Homogenize or mix the sample carefully. Concentrated juice should be diluted to single strength.

**18.3.5.2 Clear samples**

Dilute 500 ml to 10,00 ml (*V*1) of a sample in approximately 75 ml of extraction solution (3.9) in a 100 ml volumetric flask. Put the flask in the ultrasonic bath (4.1), mix the contents for at least 10 min and then dilute to the mark with extraction solution (3.9) at 20 °C. Filter the solution through a membrane filter (4.2).

**18.3.5.3 Cloudy samples**

Dilute 5,00 ml to 10,00 ml (*V*1) of a sample in approximately 75 ml of extraction solution (3.9) in a 100 ml volumetric flask. Put the flask in the ultrasonic bath (4.1) and mix the contents for at least 10 min. Then add 1,0 ml of Carrez solution I (3.11) and 1,0 ml of Carrez solution II (3.12) for clarification. Mix the solution carefully after each addition and dilute to the mark with the extraction solution (3.9) at 20 °C. Filter the solution over a paper filter (3.13); discard the first millilitres of filtrate. Filter the clear solution through a membrane filter.

**18.3.5.4 Preparation of the calibration curves**

Dilute benzoic acid stock solution (3.5) and/or sorbic acid stock solution (3.6) with extraction solution (3.9) at 20 °C to obtain standard solutions I, II and III with benzoic acid and/or sorbic acid concentrations of 10 mg/l, 25 mg/l and 50 mg/l, respectively. Inject 10 µl of each of the calibration solutions into the chromatograph (4.4), under the following conditions:

1. flow rate: approximately 1,2 ml/min;
2. wavelength for UV-detection: 235 nm (0,08 AUFS - absorbance unit full scale).
3. Prepare the calibration curves by plotting the peak areas against benzoic acid and/or sorbic acid concentration, in milligrams per litre.
	* + 1. **Determination**

Inject 10 µl of the test solution (6.1) into the chromatograph using the same conditions as for the preparation of the calibration graph. Identify the benzoic acid and/or sorbic acid peaks of the test solution by comparison with the peaks of the calibration solutions.

NOTE 1 For optimal separation of benzoic and/or sorbic acid, a slight change in the composition of the eluent may be necessary.

NOTE 2 Under the conditions described in this procedure, it is possible to determine the methyl, ethyl, and propyl esters of 4-hydroxybenzoic acid as well (see chromatogram in Annex A).

NOTE 3 Matrix peaks can cause interference with the analysis of benzoic acid in orange juice. In such a case, a suitable clean-up step is necessary.

NOTE 4 Identification of benzoic and sorbic acids in a sample is performed by comparing with the retention time of the standard solutions. It is possible to identify the analysed acids by using other methods of identification: spiking with single substances, viewing of absorption spectra at required wavelengths and measuring the absorption at different wavelengths.

NOTE 5 Quantification is carried by the external standard method with integration of peak area or measurement of peak heights. It is necessary to check linearity of the calibration function, e.g. with standard solutions I, II and III.

**18.3.6 Calculation**

Determine the concentration of benzoic and/or sorbic acid in the test solution directly from the calibration curve (6.2). Calculate the benzoic acid concentration of the sample, *ρ*A in milligrams per litre, using the following equation (external standard method).

Where,

*A*1 = the peak area or peak height of benzoic acid or sorbic acid in the test sample, expressed in area or length counts, respectively;

*A*2 = the peak area or peak height of benzoic acid or sorbic acid in the test standard solution, expressed in area or length counts, respectively;

*ρ*st = the concentration of the standard solution, in milligrams per litre; and

*V*1 = the volume of the test sample solution, in millilitres.

**19 ACETIC ACID, PROPIONIC ACID AND ITS SALTS**

This standard specifies two methods for the determination of acetic acid, propionic acid and its salts. namely, paper and column chromatographic methods. Paper chromatographic method shall be used for qualitative detection and column chromatographic method shall be used for quantitative estimation of acetic acid, propionic acid and its salts.

**19.1** **Paper Chromatographic Method**

**19.1.1** *Principle*

**19.1.1.1** Paper chromatography is a form of liquid chromatography where the basic principle involved can be either partition chromatography or adsorption chromatography.

**19.1.1.2** In paper chromatography separation of component is distributed between phases of liquid. Here, one phase of liquid is water that is held amidst the pores of filter paper and the other liquid is the mobile phase that travels along with the filter paper. Separation of the mixture is the result that is obtained from the differences in the affinities towards the water and mobile phase when travelling under capillary action between the pores of the filter paper.

**19.1.1.3** Though in a majority of paper chromatography applications, the principle is based on partition chromatography but sometimes, adsorption chromatography can take place where the stationary phase is the solid surface of the paper and the mobile phase is the liquid phase.

**19.1.2** *Apparatus*

i) Chromatographic tank;

ii) Pipettes – Graduated, 0·1 ml;

iii) Chromatographic paper – Whatman No.1, 20 cm x 20 cm sheets;

iv) Steam distillation apparatus; and

v) Beaker – 25 ml capacity.

**19.1.3** *Reagents*

i) Mobile solvent – Take two parts of acetone. One part of tertiary butyl alcohol, one part of n-butyl alcohol and one part of liquid ammonia and mix them. This solvent should always be prepared fresh;

ii) Chromogenic reagent – Add 200 mg each of methyl red and bromothymol blue to a mixture of 100 ml formalin and 400 ml absolute alcohol. Adjust to pH5.2 with 0.1 N sodium hydroxide;

iii) Sodium hydroxide – 0.1 N and 1 N;

iv) Phosphotungstic acid – 20 percent solution in distilled water;

v) Crystalline magnesium sulphate MgSO4.7H2O;

vi) Sulphuric acid;

vii) Acetic acids and propionic acid Standard solution – Pipette 1 ml of acetic acid/propionic acid into a 100 ml volumetric flask and dilute to volume with distilled water. Pipette 1 ml of this stock solution into 25-ml beaker and neutralize acid with 0.1 N sodium hydroxide using cresol red indicator avoiding excess alkali. Evaporate to 0.3 ml in a water bath; and

viii) Congo red indicator paper.

**19.1.4** *Procedure*

**19.1.4.1** *Sample preparation*

a) *All types of bread not containing fruits*

Take one or a half loaf of bread and cut it into slices of 2 mm to 3 mm thickness. Spread the slices on the paper and let them dry in warm room until sufficiently crisp and brittle to grind well. Grind entire sample to pass through 850 micron sieve; mix well and keep in air-tight container before proceeding for experimentation.

b) *Bread containing raisins and fruits*

Proceed as in 19.1.4.1 (a) except comminute by passing twice through food chopper instead of grinding and dry air-dried sample in an uncovered dish for 16 hours at 70°C under pressure of less than50mm of mercury.

**19.1.4.2** *Distillation*

Weigh accurately 10 8 of air-dried bread and transfer it to150ml distilling flask. Add 40 ml distillation water and 10 ml of 1 N sulphuric acid. Mix thoroughly and add 10 ml of 20 percent phosphotungstic acid solution. Mix the contents well and add 40 g magnesium sulphate. Swirl the contents well and make the solution acidic to congo red paper with 50 percent sulphuric acid. Connect the condenser and steam generator and distil 200 ml in 35 to 40 minutes. Immediately neutralize the distillate using cresol red and 0·1 N sodium hydroxide. Evaporate the solution to 0.5ml or evaporate just to dryness and then take up in 0.5ml distilled water.

**19.1.4.3** *Paper chromatography*

Take Whatman No. 1 *(see* iii) unwashed chromatographic paper and rule starting line 2.5 cm from bottom edge with hard pencil. Spot two 1 µl spots with 1 µl pipette on paper 2.5cm apart from each other, leaving at least 2.5 cm margin, the first spot being of acetic propionic acid standard solution and the second of unknown sample. Let the paper dry and clip it to a glass rod and suspend it in chromatographic tank with 50ml of mobile solvent in a trough. Do not saturate the tank with mobile solvent before inserting the paper. Seal the glass cover with cellophane or other suitable tape and let it develop until solvent reaches 2.5 cm from top of paper. Remove the paper from the tank and let it air dry.

**19.1.4.4** Spray chromogenic reagent on front side of the paper. Spraying should be uniform and rather heavy but not to the extent that chromogenic reagent runs or drips. Faint yellow spots indicate presence of propionic acid.

**19.1.4.5** To intensify acid spots, place paper in the atmosphere of ammonia fumes momentarily (by placing 50 ml ammonium hydroxide in a 2 litre beaker and exposing to fumes by placing each end in beaker momentarily), entire paper immediately turns green. Remove paper from ammonia fumes, acids gradually appear as red spots and presence of propionic/acetic acid in the sample may be determined by comparing their *Rf* values with that of the standard propionic/acetic acid. Since colours of the acids are not stable, mark the spot with the pencil as soon as they are completely developed.

**19.2 Column Chromatographic Method**

**19.2.1** *Principle*

In column chromatography the stationary phase is packed into a glass or metal column. The mixture of analytes is then applied and the mobile phase, commonly referred to as the eluent, is passed through the column either by use of a pumping system or applied gas pressure. The stationary phase is either coated onto discrete small particles (the matrix) and packed into the column or applied as a thin film to the inside wall of the column.

As the eluent flows through the column the analytes separate on the basis of their distribution coefficients and emerge individually in the eluate as it leaves the column.

* + 1. *Apparatus*

i) Chromatographic tube – Approximately 15 mm x 250 mm constricted at lower end to 4 mm internal diameter;

ii) Steam distillation apparatus – All glass;

iii) Test tubes –16 mm x 150 mm;

iv) Pipettes;

v) Rubber bulb; and

vi) Micro funnel.

**19.2.3** *Reagents*

i) Sodium hydroxide – 0.1 N and 1 N;

ii) Phosphotungstic acid solution – 20 percent in distilled water;

iii) Crystalline magnesium sulphate – MgSO4. 7H2O;

iv) Sulphuric acid – 1 N;

v) Formic acid – 0.01 N;

vi) Alphamine red R indicator solution – 50 mg of monoammonium salt of 3-(4-anilino-1- naphthylazo)-2, 7-naphthalene disulphonic acid dissolved in 25 ml distilled water;

vii) Ammonium hydroxide – 1 N;

viii) Silicic acid – 100 mesh;

ix) Chloroform;

x) Butyl alcohol;

xi) Sulphuric acid – 50 percent;

xii) Absolute alcohol;

xiii) Butanol in chloroform 1 percent – Remove alcohol from chloroform by washing 3 times with water. Add 10 ml of n-butyl alcohol to 1 litre of washed chloroform in separating funnel, shake vigorously, add 25 ml of distilled water and shake again. Let it stand until the lower layer clears. Drain and discard the upper aqueous layer. Store it in contact with granular sodium sulphate;

xiv) Cresol red indicator – Dissolve 50 mg o-cresol-sulphonaphthalene in 20 ml of absolute alcohol, add 1·3 ml of 0.1 N sodium hydroxide and dilute to 50 ml with distilled water. Use 2 drops for each 25 ml of aqueous solution;

xv) Barium hydroxide standard solution – 0·01 N; and

xvi) Sodium acetate-sodium chloride solution – Dissolve 12 g of sodium chloride and 25 g of sodium acetate in distilled water and dilute to 500 ml.

**19.2.4** *Procedure*

**19.2.4.1** *Sample preparation -* As given in **19.1.4.1.**

**19.2.4.2** *Distillation*

Weigh accurately 10 g of air-dried sample and transfer it to 150 ml distilling flask. Add 40 ml distilled water and 10 ml of 1 N sulphuric acid. mix thoroughly and add 10 ml of 20 percent phosphotungstic acid solution. Mix the contents well and add 40 g magnesium sulphate. Swirl the contents well and make the solution acidic to congo red paper with *SO* percent sulphuric acid. Condenser and steam generator and distil 200 ml in 35-40 minutes. Transfer the distillate to 400-600 ml beaker. Add 10 ml 0.01 N formic acid, make alkaline to phenolphthalein with 1 N sodium hydroxide and evaporate to *5* ml. Transfer it into a 25 ml glass stoppered test tube rinsing the beaker with three portions of distilled water. If insoluble matter adheres to the beaker, rinse with t N sulphuric acid. Make this solution alkaline to phenolphthalein and evaporate just to dryness by inserting the test-tube in steam-bath.

**19.2.4.3** *Chromatographic separation*

a) *Preparation of partition column*

Take 5g silicic acid in glazed porcelain evaporating dish and add 1 ml of alphamine red R indicator solution and just enough 1 N ammonium hydroxide to give alkaline colour of the indicator (1 drop is enough). Add maximum amount of distilled water that the silicic acid will hold without becoming sticky or agglomerating in the butyl alcohol-chloroform solution (this amount shall be determined for each batch of silicic acid and usually varies from 50to 75percent of the weight of silicic acid). Homogenize the mixture thoroughly in a pestle. Add 25 ml of 1 percent butyl alcohol in chloroform and mix to form slurry that pours readily. Pour this slurry into a chromatographic tube containing small cotton plug in the neck of constricted end. To avoid air pockets, tilt the tube slightly while pouring. If air bubbles form while pouring. eliminate by stirring suspension in tube with long glass rod. Clamp the tube vertically in the ring stand. In the tube. Insert one-hole rubber stopper fitted with glass tube bent to 90*°* angle and held in place by a bunsen clamp against the pressure to be exerted. Connect bent glass tube to pressure. Adjust pressure to (0.35-0.70 kgf/cm2). 34.5 to 68.9 kPa so that excess solvent is forced through column dropwise. During removal of excess solvent, gel packs down. As column packs down. particles of gel adhere to the wall of the tube but eventually gel leaves wall of the tube relatively clean. This is the point of optimum density of column, and column is ready for use. Apply pressure until solvent reaches surface of the column. If solvent passes below surface causing drying or cracking of column or if air pockets are present, extrude packing from the tube, reslurry with the solvent and repack the column.

b) *Preparation of standard propionic/acetic acid solution*

Prepare stock solutions of propionic/acetic acid by diluting 5 ml of propionic/acetic acid to 250 ml with distilled water. Pipette 1 ml of stock solution into a 125 ml Erlenmeyer flask and titrate with 0·01 N sodium hydroxide using cresol red as indicator to pink colour persisting for 45 seconds.

Calculate concentration of the acids as follows:

mg acid/ml standard solution = ml 0.01 N sodium hydroxide × normality × *F*

Where,

 *F* = 7.41 for propionic acid and 6.01 for acetic acid .

e) *Preparation of known samples*

Pipette 50ml of standard solution into a 50 ml beaker and just neutralize with 0·01 N sodium hydroxide solution using phenolphthalein and add 10 drops in excess. Evaporate it to dryness on steam bath.

d) *Column separation*

To the dry residue, add 2 ml of I percent butyl alcohol in chloroform solution and while stirring with glass rod, add *SO* percent sulphuric acid dropwise until the sodium salts are converted to free acids (acid to congo red paper) and add 1g of anhydrous sodium sulphate. Place a 50 ml graduated cylinder under column as receiver. Decant the supernatant on to column, pouring it slowly down the side of the tube without disturbing level surface of column. Apply pressure until solvent reaches the surface of gel. Wash the beaker with 1ml solvent and pour it on to column. Apply pressure until solvent just disappears into sodium sulphate layer. Wash the beaker with another 1 ml of solvent. transfer to the column. wash inside of tube with 1 ml of solvent and apply pressure until solvent just disappears into sodium sulphate layer, Fill the tube with the solvent and apply pressure. Once the level reaches the point 2 to 5 mm above the narrowest portion of the construction of tube, record the volume and remove the receiver. Transfer the elute to a 125 ml Erlenmeyer flask. rinsing the cylinder with three 5 ml portion of distilled water. Add one drop of cresol red indicator and titrate with 0·1 N barium hydroxide solution. As its end point approaches, stopper flask and shake vigorously to completely extract acid from solvent phase. Correct titration for blank as follows. Collect 25 ml of butyl alcohol-chloroform mixture from column before the acid is transferred, add IS ml boiled and cooled distilled water and titrate as above with 0·01 N barium hydroxide solution.

**19.2.5** *Calculation*

Calculate the results for propionic acid and acetic acid to mg/100 g sample.

Propionic acid, mg/100 g = 7.40 × ml 0·01 N barium hydroxide solution

Calcium propionate = propionic acid x 1.256

acetic acid mg/100 g = 6·01 x ml 0·01 N barium hydroxide solution

**19.2.6** *Identification of Propionic/Acetic Acid*

Acid separated in butyl alcohol chloroform solution may be further identified by paper

chromatograph in **19.1.4.3.**

**20 ANTIOXIDANTS**

**20.1** **Propyl Gallate**

**20.1.1** *Reagents*

i) Petroleum ether reagent – mix 1 volume of 40° C to 60° C boiling-range petroleum ether with three volumes of 60° C to 100° C boiling range petroleum ether and shake the mixture for 5 minutes with one-tenth its volume of sulphuric acid. Discard acid layer. Wash several times with water, then once with 1 percent sodium hydroxide solution and followed again by water until washings are neutral. Discard washings and distil petroleum ether in an all-glass apparatus.

ii) Ammonium acetate solutions – 1.25, 1.67 and 10.0 percent aqueous solutions.

iii) Ferrous tartrate reagent – dissolve 0·10 g FeSO4.7H2O and 0.50 g Rochelle salt (sodium potassium tartrate) in water and dilute to 100 ml. The reagent should be used within 3 hours of preparation.

iv) Propylgallate standard solution – 50 mg of propylgallate dissolved in water and dilute to 1 litre with water.

**20.1.2** *Preparation of Standard Curve*

Place at least 7 aliquots of standard solution. covering range from 50 to 100 mg, in 50 ml conical flasks. Add 2.5 ml 10 percent ammonium acetate to each flask. Dilute to 24 ml with water and pipette 1 ml ferrous tartrate reagent into each flask. Let solutions stand for 3 minutes and measure absorbance at 540 nm against reagent blank prepared by taking 20 ml 1.25 percent ammonium acetate solution 4 ml water and 1 ml ferrous tartrate reagent. Plot propyl gallate against absorbance.

**20.1.3** *Procedure*

Accurately weigh 50 g powdered sample and shake with 50 ml petroleum ether reagent. Allow the petroleum ether layer to separate and carefully decant the extract in a 250 ml separating funnel. Add another 50 ml petroleum ether reagent to the residue and transfer the petroleum ether extract to a separating funnel. Repeat the process of extraction and decantation five times with 50ml portions of petroleum ether reagent. To the combined extract. add 20 ml of 1·67 percent ammonium acetate reagent and gently shake. Allow the two layers to separate and drain out aqueous layer into a 100 ml volumetric flask, being careful not to let any petroleum ether drop fall in the flask. Repeat the extraction twice with 20 ml portions of 1·67 percent ammonium acetate solution, combining aqueous layers in the volumetric flask. Finally, Extract with 15ml water. Allow the aqueous layer to separate and collect it along with ammonium acetate washings. Add 2.5ml of 10 percent ammonium acetate solution to the combined extract and dilute the volume to 100 ml with water. If the solution is turbid, filter through dry rapid filtering paper to remove turbidity. Pipette 20ml aliquots into 50ml glass test tubes. Add 4ml water and 1ml ferrous tartrate reagent and mix well. Measure absorbance at 540mm against reagent blank prepared by taking 20ml of 1.25 percent ammonium acetate, 4ml water and 1ml ferrous tartrate reagent. Calculate amount of propylgallate from the standard curve.

**20.2 Butylated Hydroxyanisole (BHA)**

BHA is extracted from bakery products with carbon disulphide (CS2) and determined by gas liquid chromatography (GLC) using flame ionization detection.

 **20.2.1** *Principle*

When the vapors of a sample mixture move between the stationary phase (liquid) and mobile phase (gas) the different components of a sample mixture will separate according to their partition coefficient between the gas and liquid stationary phase. Concentration of solute in liquid. It is general assumption that if partition coefficient is low the emergence of the component is fast and vice versa. The substances having low boiling point (B.P) i.e. more volatility and higher vapour pressure will have more concentration in the mobile phase and thus will elute or emerge first and so on.

**20.2.1** *Apparatus*

i) Gas chromatograph *–* A suitable equipment fitted with hydrogen flame ionization detection and integrator-cum-recorder. The suggestive operating conditions are given below, which may be changedaccording to the availability of instrument, provided standardization is done:

 a) Column: Glass, 122cm length and 4mm 1Dpacked with Apeizon L on Gas Chrom (80-100 Mesh); or Stainless steel, 183cm length and 4mm 1D packed with QF-1 silicon oil (fluoro-silicone, FS 1265) on Gas Chrom (80 -100 mesh).

b) Temperature:

ba) Column – 160 ℃;

bb) Injection –200 ℃; and

bc) Detector – 210 ℃.

 c) Gases:

ca) Carrier Gas – Nitrogen to elute BHA in 3-5 minutes;

cb) Fuel Gas and its flow rate – Hydrogen 40ml/min Apeizon column and 25ml/min for QF –1 column; and

cc) Air flow rate – 340ml/min.

Prepare column and column material by washing the inside of column tubes and glass wool with methyl alcohol and drying. Slowly sprinkle about 50 g chrome into 800 ml beaker filled with carbon tetrachloride. Remove fine particles that remain on surface with vacuum line and trap. Decant carbon tetrachloride and dry at 150 ℃. Transfer 20g dried gas chrome in 500 ml round-bottom flask. Add 100 ml chloroform or methylene dichloride and mix gently. Dissolve 1g Apeizon L or 2 g QF-1 in 50 ml chloroform or methylene dichloride transfer to the flask, and mix gently. Evaporate to dryness using rotary vacuum evaporator. Carefully plug exit of column with small plug of fibre glass wool and through-hole septum. Apply vacuum to the exit port and slowly add coated support through the other end, tapping very gently to aid compaction. Pack to within 1cm of area heated by injection port. Plug with find glass wool and condition for about 3 days at 200 ℃ with slow stream of nitrogen or until steady baseline is obtained.

ii) Chromatographic tube *–* 25 mm γ 200 mm glass tube with small drip tip (4 mm internal diameter, 6 mm outer diameter and 50 mm long) with or without medium porosity fitted disc, with close fitting tamping rod.

iii) Fine glass wool *–* Wash with carbon disulphide and dry.

**20.2.2** *Reagents*

i) Carbon disulphide – Reagent grade, nearly, colourless. If it is distinctly yellow distil before use.

ii) BHA and di-BHA standard mixture – 0.02 µg/ml each of BIIA di-BHA in carbon disulphide. Dissolve 1 mg of BRA in a small amount of carbon disulphide, add 10 ml internal standard solution and dilute to 50 ml with carbon disulphide. Prepare fresh and store in low actinic glassware.

iii) Internal Standard – 0.1 µg di-BHA/µl carbon disulphide (CS2). Prepare fresh and store in low-actinic glassware.

iv) Dichlorodimethylsilane solution – 5 ml diluted to 100 ml with toluene.

**20.2.3** *Method*

Grind sample to pass through 850 micron IS Sieve and mix well. Place small plug of glass wool at bottom of chromatographic tube and add 20 g sample to column using tamping rod to pack it firmly without solvent. Put another plug of glass wool at the top. Add three 5 ml portions of carbon disulphide to column letting each portion sink into the column before adding next. Elute carbon disulphide at a rate of 5 ml/minute and collect in a 50 ml graduated cylinder. Continue eluting by adding 10 ml portions of carbon disulphide at a time till 50 ml elute is collected. Rinse tip of the column with small amount of carbon disulphide. Accurately add di-BHA to elute to obtain concentration after evaporation of 0.02 µg di-BHA/µl, final solution. Evaporate elute under gentle stream of nitrogen in a hood at room temperature to small volume (5.0 ml). Accurately dilute evaporated sample to appropriate volume (5 ml), Inject 3 to 9 µl sample, using 10 µl syringe into gas chromatograph. Before and after each series of sample chromatographs, inject 3.0 to 9.0 µl standard mixture, and average standard values for calculations. Measure each pack height in mm.

**20.2.4** *Calculation*

Calculate mg/kg antioxidants present, correcting for internal standards as follows :

BHA = (HX /HS) (CS /CX) (HS1 /HX1) (CX1 /CS1)

Where,

*HX* and *H*S = height in mm of sample and standard peaks, respectively;

*HX1* and *H*S1 = heights in mm of internal standard peaks in sample and standard,

respectively;

*CX* and *C*S = concentrations of samples (µg/µl) and standard µg/µl, respectively; and

*CX1* and *C*S1 = concentrations (µg/µl) of internal standard in sample and standard solution, respectively.

**21 MEASUREMENT OF PEROXIDATION IN STORED BAKERY PRODUTS**

**21.1** During storage, fat or shortening used in bakery projects, such as biscuits, cookies cakes,etc, undergo auto-oxidation which is often followed by stored odours. Though there is nofoolproof method to determine the exact level of antioxidant, peroxide value and TBA value are widely used for estimating peroxidation and rancidity in foods.

**21.2 Peroxide Value**

**21.2.1** *Reagents*

i) Sodium thiosulphate stock solution 0.1 N – dissolve 24.88 g in 1 litre distilled water. Determine its exact normality by titrating against standard potassium dichromate solution.

ii) Sodium thiosulphate 0.01 N – pipette 25 ml of stock solution in 250 ml volumetric flask and dilute to volume.

iii) Starch solution (1 percent) – take 1 g soluble potato starch and add about 5 ml water in 250 ml beaker and mix to form a paste. Add 100 ml distilled water and boil for 5 minutes.

iv) *Saturated potassium iodide solution –* take about 25 ml distilled water in 50 ml conical flask and add about 5 g potassium iodide and shake. If it completely dissolves, add more potassium iodide until undissolved crystals remain at the bottom. Solution should be prepared just before use.

 **21.2.2** *Procedure*

Take 50 to 100 g powdered sample in a stoppered flask, add 100 ml chloroform and shake for about half an hour. Filter through a dry filter paper and transfer 20 ml aliquots to 250 ml iodine flask. Add 30 ml glacial acetic acid and 1 ml saturated potassium iodide solution. Stopper the flask and keep aside for 5 minutes. Run a blank by taking 20 ml chloroform instead of food extract. After S minutes add 50 ml water to each flask and mix the contents well. Add 1 ml starch solution (1 percent) as indicator and immediately titrate against 0.01 N sodium thiosulphate solutions. For determining the fat concentration in the extract, evaporate 10 ml aliquots in tared aluminium dishes in an air oven at 80˚C to constant mass.

**21.2.3** *Calculation*

Peroxide value (milliequivalent of oxygen per kg fat) =

Where,

*V1* = volume of thiosulphate solution consumed for sample;

*V2* = volume of thiosulphate solution required for blank titration;

*N* = normality of sodium thiosulphate solution; and

*W* = weight of rat content in the 20 ml chloroform extract of the sample.

**21.3 TBA Value**

**21.3.1** *Reagents*

i) Hydrochloric acid (3 N) – Take 250 ml concentrated hydrochloric acid in 1 litre measuring flask and dilute to volume.

ii) Thiobarbituric *acid solution* (0.67 percent) – Dissolve 0.67g thiobarbituric acid in 100ml glacial acetic acid by slightly warning, if necessary. Prepare fresh solution daily.

**21.3.2** *Procedure*

Take 10g powdered sample in a 250ml round-bottomed flask, add 7.5ml and 3N hydrochloric acid and 75ml distilled water. Steam distill the contents, collecting 50ml of the distillate in 10 minutes. Accurately, pipette 20ml aliquots in 50-ml boiling tubes, add 2ml of 0.67 percent thiobarbituric acid in glacial acetic acid and heat the tubes in a boiling water bath for 30 minutes. Run a blank experiment by taking 20ml distilled water in place of sample distillate and treating exactly as in the experiment. Measure the colour intensity at 532mm against the blank. Calculate the concentration of malonaldehyde from the standard curve.

**21.3.2.1** *Standard curve*

Weigh accurately about 250 mg of tetramethoxy propane in a glass curette and transfer the curette to a glass stoppered flask and heat at 50 ℃ for 3 hours. Transfer the contents quantitatively to 1litre volumetric flask and make up the volute to 1 litre. Pipette 1 ml to 5 ml aliquots of the solution in 50ml boiling tubes and make up the volume to 20 ml with water. Add 2 ml of 0.67 percent thiobarbituric acid in glacial acetic acid and heat the tubes in boiling water for 30 minutes. Measure the absorbance at 532 mm against reagent blank prepared by taking 20 ml distilled water in place of malonaldehyde (164 tetramethoxy propane = 72 g malonaldehyde).

**22 DETERMINATION OF CHOLESTEROL(AOAC 954.03 )**

**22.1** *Principle*

The digitonin method for fat determination is based on the principle of selective precipitation of cholesterol and other sterols from a solution containing fat. Digitonin, a saponin, forms complexes with sterols in the presence of alcohol, facilitating their precipitation. After precipitation, the sterol complex is isolated, dried, and weighed to determine the fat content in the original sample. This method leverages the solubility properties of digitonin and the specific interaction with sterols to quantify fat content accurately.

**22.2** *Apparatus*

i) 300 ml Erlenmeyer flask 22.1.2 Steam bath;

ii) Bell jar;

iii) Knorr-type (Allihn) extraction tube;

iv) Fritted glass disk;

v) Porcelain chip;

vi) Gooch crucible (10 ml capacity); and

vii) Glass fiber filter.

**22.3** *Procedure*

Weigh 5 g of the ground sample into a 300 ml Erlenmeyer flask. Add 15 ml of HCl (1 + 1) and heat on a steam bath for 30 minutes, shaking frequently. Cool and add 15 g of KOH pellets, swirling to ensure complete hydrolysis. Add 20 ml of alcohol and heat on a steam bath for 45 minutes with an air condenser, shaking frequently. Add 25 ml of water and 50 ml of ether, and swirl vigorously for 1 minute. Transfer the mixture to a 500 ml separator and wash with 25 and 10 ml portions of ether and 50 ml of 1% KOH solution. Drain the soap solution into a 250 ml separator and rinse with 5 ml of 1% KOH solution. Add 25 ml of ether to the smaller separator and shake vigorously for 1 minute. Drain the aqueous layer and add the ether solution to the larger separator. Wash the ether solution with three additional 50 ml portions of 1% KOH solution and twice with 50 ml of water. Drain the aqueous layer and add porcelain chip to the flask. Transfer the ether solution to the flask and rinse the separator with three 5 ml portions of ether. Add the rinsings to the flask and evaporate the ether on a steam bath. Dissolve the residue in 5 ml of acetone and filter through a Knorr-type extraction tube into a 100 ml centrifuge tube or test tube. Wash the flask and tube with three 4 ml portions of acetone and rinse with a few ml of acetone. Add 5 ml of freshly prepared digitonin solution androtate to mix. Place the tube in a steam bath and evaporate nearly to dryness. Add 50 ml of hot water and stir well to disperse the precipitate. Place the tube in a boiling water bath and hold for several minutes with frequent stirring. Cool to ca 60 °C, add 25 ml of acetone, and mix well. Cool to room temperature and decant into a previously dried and weighed Gooch crucible containing a glass fiber filter covered with ca 1 g of washed and ignited sand. Finally rinse crucible with acetone to dissolve any fat-like material, rinse with 5 ml. ether, dry 30 min at 100 °C, and weigh

**22.4** *Calculation*

Cholesterol content, percent by mass = (Weight of residue x 0.243)

**23 DETERMINATION OF DIETARY FIBER (AOAC 985.29)**

**23.1 Principle**

Duplicate test portions of dried foods, fat-extracted if containing >10% fat, are gelatinized with Termamyl (heat-stable a-amylase), and then enzymatically digested with protease and amyloglucosidase to remove protein and starch. (When analyzing mixed diets, always extract fat prior to determining total dietary fiber.) Four volumes of ethyl alcohol are added to precipitate soluble dietary fiber. Total residue is filtered, washed with 78% ethyl alcohol, 95% ethyl alcohol, and acetone. After drying, residue is weighed. One duplicate is analyzed for protein, and other is incinerated at 525°C and ash is determined. Total dietary fiber = weight residue - weight (protein + ash).

**23.2 Apparatus**

i) Fritted crucible – Porosity No. 2 (Pyrex No. 32940, coarse, ASTM 40-60 ms of Corning No. 36060 Büchner, fritted disc, Pyrex, 60 ml, ASTM 40-60 µm). Clean thoroughly, heat disk, 25°C, and soak and then rinse in H₂O. Add ca 0.5 g Celite to air- dried crucibles and dry at 130°C to constant weight (21 h). Cool and store in desiccator until used.

ii) Vacuum source – Vacuum pump or aspirator equipped with in-line double vacuum flask to prevent contamination in case of H.O backup.

iii) Vacuum oven 70°C – Alternatively, 105°C air oven can be used.

iv) Desiccator.

v) Muffle furnace.

vi) Water baths – (1) Boiling (2) Constant temperature – Adjustable to 60 °C, with either multistation shaker or multistation magnetic stirrer to provide constant agitation of digestion flasks during enzymatic hydrolysis.

vii) Beakers – Tall-form, 400 or 600 ml.

viii) Balance – Analytical, readability to 0.1 mg.

ix) *p*H meter – Standardized with *p*H 7 and *p*H 4 buffers.

**23.3** **Reagents**

**i)**  95% Ethanol.-v/v. Technical grade;

**ii)** 78% Ethanol. Place 207 ml H₂O into 1 L volumetric flask. Dilute to volume with 95% ethyl alcohol. Mix and dilute to volume again with 95% ethyl alcohol if necessary. Mix. One volume H₂O mixed with four volumes 95% ethyl alcohol will also give 78% ethyl alcohol final concentration;

**iii)** Acetone;

**iv)** Phosphate buffer – 0.08 M, pH 6.0. Dissolve 1.400 g sodium phosphate dibasic, anhydrous (Na, HPO) (or 1.753 g dihydrate) and 9.68 g sodium phosphate monobasic monohydrate (NaH, PO, HO (or 10.94 g dihydrate) in ca 700 ml H,O. Dilute to 1 L with H.O. Check ph with the ph meter;

**v)** a-amylase (heat stable) – Termamyl (1) Store in refrigerator.Based on Nelson/Somogyi reducing sugar with soluble starch as substrate.-10000+1000 units/ml (I unit is defined as the amount of enzyme required to release 1 µmole reducing sugar equivalent/min at *p*H 6.5 at 40 °C). (2) Based on Ceralpha method lens p-nitrophenyl-monosaccharide as substrate in the presence of a thermostable a-glucosidase.-3000 + 300 Ceralpha units/ml of (1 unit of enzyme is required to release 1 µmole p-nitrophenyl/m *p*H 6.5 and 40°C);

**vi)** Protease – Keep refrigerated.

a) Casein assay.- 300-400 Units/ml. [1 protease unit is defined as the amount of enzyme required to hydrolyze (and solubilize in TCA) 1 µmole tyrosine equivalents/min from soluble casein at *p*H 8.0 and 40°C]; 7-15 units/mg (1 unit will hydrolyze casein to produce color equivalent to 1.0 µmole tyrosine/min at *p*H 7.5 and 37°C). Color by Folin-Ciocalteu Reagent.

b) Azo-casein assay-300-400 Units/ml [1 unit endo-peptidase activity is defined as the amount of enzyme required to hydrolyze (and solubilize in TCA) 1 µmole tyrosine equivalents/min from soluble casein at *p*H 8.0 and 40°C].

**vii)** Amyloglucosidase. Keep refrigerated.

a) Starch/glucose oxidase-peroxidase method. 2000-3300 Units/ml (1 unit enzyme activity is defined as the amount of enzyme required to release 1 µmole glucose/min at pH 4.5 and 40°C).

b) PNPBM p-nitrophenyl B-maltosidase) method, -130-200 Units/ml. [1 unit enzyme activity (PNP unit) is the amount of enzyme, which in the presence of excess levels of B-glucosidase, will release 1 µmole p-nitrophenyl from p-nitrophenyl B-maltosidase/min at 40 °C). The only enzyme which has been found to be significantly contaminated with interfering activities is amyloglucosidase. Thermostable a-amylase and protease from commercial sources have been found to be generally free of interfering enzymes. Low levels of B-glucanase have been detected in protease preparations, but at levels well below that which would interfere with total dietary fiber analysis. The major contaminant in amyloglucosidase preparation was shown to be an endo-cellulase and resulted in endo-depolymerization of mixed-linkage B-glucan from barley and oats, with resultant underestimation of this dietary fiber component. The contamination of amylogucosidase with endo- cellulase (B-glucanase) can be easily detected. Alternatively, there are kits containing all three enzymes (pretested) available from a number of companies.

**viii)** Sodium hydroxide solution – 0.275 M. Dissolve 11.00 g NaOH ACS in ca 700 ml H₂O in 1 L volumetric flask. Dilute to volume with H.O.

**ix)**  Hydrochloric acid solution – 0.325 M. Dilute stock solution of known titer, e.g., 325 ml 1 M HCl, to 1 L with H₂O.

**x)**  Celite – Acid-washed.

**23.3 Enzyme Purity**

To ensure absence of undesirable enzymatic activity in enzymes used in this procedure, run materials listed in Table 985.29 through entire procedure each time lot of enzymes is changed, or at maximum interval of 6 months to ensure that enzymes have not degraded.

**23.4 Test Portion Preparation**

Determine total dietary fiber on dried test sample. Homogenize test sample and dry overnight in 70 °C vacuum oven, cool in desiccator, and dry-mill test sample to 0.3 mm to 0.5 mm mesh. If test sample cannot be heated, freeze-dry before milling. If high fat content (>10%) prevents proper milling, defat with petroleum ether (3 times with 25 ml. portions/g test sample) before milling. Record loss of weight due to fat removal and make appropriate correction to final % dietary fiber found in determination. Store dry-milled test sample in capped jar in desiccator until analysis is carried out.

**23.5** **Procedure**

Run blank through entire procedure along with test portions to measure any contribution from reagents to residue Weigh duplicate 1 g test portions, accurate to 0.1 mg. into 400 ml tall-form beakers. Test portion weights should not differ >20 mg. Add 50 ml *p*H 6.0 phosphate buffer to each beaker. Check *p*H and adjust to *p*H 6.0 ± 0.2 if necessary. Add 0.1 ml. Termamyl solution. Cover beaker with Al foil and place in boiling water bath 15 min. Shake gently at 5 min intervals. Increase incubation time when number of beakers in boiling water bath makes it difficult for beaker contents to reach internal temperature of 95 °C to100 °C. Use thermometer to indicate that 15 min at 95 °C to100 °C is attained. Total of 30 min in water bath should be sufficient. Cool solutions to room temperature. Adjust to *p*H 7.5 ± 0.2 by adding 10 ml 0.275 M NaOH solution. Add 5 mg protease. [Protease sticks to spatula, so it may be preferable to prepare enzyme solution (50 mg in 1 ml phosphate buffer) and pipet 0.1 ml to each sample just before use). Cover beaker with Al foil. Incubate 30 min at 60 °C with continuous agitation. Cool. Add 10 ml 0.325 M HCI solution. Measure *p*H and dropwise add acid if necessary. Final *p*H should be 4.0 to 4.6. Add 0.3 ml amyloglucosidase, cover with Al foil, and incubate 30 min at 60 °C with continuous agitation. Add 280 ml 95% ethyl alcohol preheated to 60 °C (measure volume before heating). Let precipitate form at room temperature for 60 min. Weigh crucible containing Celite to nearest 0.1 mg, then wet and redistribute bed of Celite in crucible by using stream of 78% ethyl alcohol from wash bottle. Apply suction to draw Celite onto fritted glass as even mat. Maintain suction and quantitatively transfer precipitate from enzyme digest to crucible, wash residue successively with three 20 ml. portions of 78% ethyl alcohol, two 10 ml portions of 95% ethyl alcohol, and two 10 ml portions of acetone. Gum may form with some products, trapping liquid. If so, break surface film with spatula to improve filtration. Time for filtration and washing will vary from 0.1 to 6 h, averaging 0.5 h per sample. Long filtration times can be avoided by careful intermittent suction throughout filtration. Dry crucible containing residue overnight in 70 °C vacuum oven or 105 °C air oven. Cool in a desiccator and weigh to the nearest 0.1 mg. Subtract crucible and Celite weight to determine weight of residue. Analyze residue from 1 test portion of set of duplicates for protein by 960.52 (*see* 12.1.07), using N 6.25 as conversion factor, except in cases where N content in protein is known. Incinerate second test portion of duplicate 5 h at 525 °C. Cool in desiccator and weigh to nearest 0.1 mg. Subtract crucible and Celite weight to determine ash.

**23.6** **Calculations**

Determination of blank:

B = blank, mg = weight residue – PB - AB

Calculate TDF as follows:

TDF, % = [(weight residue - P-A - B)/weight test portion] × 100

where weight residue = average of weights (mg) for duplicate blank determinations; and P and A = weights (mg) of protein and ash, respectively, in first and second test portion residues; and weight test portion = average of two test portion weights (mg) taken.

**24 Determination of Protein Content (AOAC 920.87)**

**24.1 Principle**

The Kjeldahl method determines nitrogen content by digesting the sample with sulfuric acid, potassium sulfate or sodium sulfate, and a mercury catalyst to convert organic nitrogen into ammonium sulfate. The digested mixture is then diluted, cooled, and neutralized with sodium hydroxide, and mercury is precipitated using sulfide or thiosulfate. Ammonia is distilled from the alkaline mixture into a standard acid solution. The amount of ammonia is determined by titration with sodium hydroxide, and the nitrogen content is calculated to determine the protein content of the sample.

**24.2 Apparatus**

i) Digestion flask;

ii) Condenser;

iii) Distilling bulb;

iv) Receiver;

v) Burette; and

vi) Volumetric pipettes.

**24.3 Reagents**

i) Mercury(II) oxide (HgO);

ii) Metallic mercury (Hg);

iii) Potassium sulfate (K₂SO₄);

iv) Sodium sulfate (Na₂SO₄);

v) Sulfuric acid (H₂SO₄);

vi) Sodium hydroxide (NaOH);

 vii) Sulfide or thiosulfate solution;

 viii) Standard acid (e.g. hydrochloric acid, HCl);

 ix) Standard sodium hydroxide solution (NaOH); and

 x) Indicator solution (e.g. methyl red or bromothymol blue).

**24.4 Procedure**

Place a weighed test portion (0.7 g to 2.2 g) in a digestion flask. Add 0.7 g HgO or 0.65 g metallic Hg, 15 g powdered K₂SO₄ or anhydrous Na₂SO₄, and 25 ml H₂SO₄. If the test portion is greater than 2.2 g, increase the H₂SO₄ by 10 ml for each gram of test portion. Place the flask in an inclined position and heat gently until frothing ceases; then boil briskly until the solution clears and for at least 30 minutes longer (2 hours for test portions containing organic material). Cool, add approximately 200 ml H₂O, cool to less than 25°C, and add 25 ml of sulfide or thiosulfate solution to precipitate Hg. Add a few Zn granules to prevent bumping, tilt the flask, and add a layer of NaOH without agitation. Immediately connect the flask to the distilling bulb on the condenser and, with the tip of the condenser immersed in standard acid and 5 to7 drops of indicator in the receiver, rotate the flask to mix the contents thoroughly. Heat until all NH₃ has distilled (at least 150 ml distillate). Remove the receiver, wash the tip of the condenser, and titrate the excess standard acid in the distillate with standard NaOH solution. Correct for blank determination on reagents.

**24.5 Calculation**

N % = [(ml standard acid x molarity acid) - (ml standard NaOH x molarity NaOH)] × 1.4007/g test portion

Multiply percent N by **5.7** to obtain percent protein.

**25 Determination of Iron Content (944.02)**

**25.1 Principle**

The methods described aim to determine iron content in flour through distinct approaches: dry ashing and wet digestion.

**25.1.2** *Dry Ashing Method* – This involves ashing a sample in a dish until carbon-free, followed by dissolution in hydrochloric acid (HCl) and subsequent complexation with hydroxylamine hydrochloride (HNOH-HCl), buffer solution, and a complexing agent like o-phenanthroline or dipyridyl. Absorbance is measured and iron content is quantified using a standard curve or equation.

**25.1.3** Wet Digestion Method – Here, the sample is digested in a Kjeldahl flask with sulfuric acid (H₂SO₄) and nitric acid (HNO₃), followed by dilution and filtration. An aliquot is then complexed with HNOH-HCl, sodium acetate buffer, and o-phenanthroline. Absorbance is measured after pH adjustment, and iron concentration is determined based on absorbance readings and corrections for blanks and dilutions.

**25.2 Reagents**

Note – Rinse all flasks, beakers, funnels, etc., with H₂O before use and filter all reagents to remove suspended matter.

i) o-Phenanthroline solution – Dissolve 0.1 go-phenanthroline in ca 80 ml H2O at 80°C, cool, and dilute to 100 ml;

ii) a,a-Dipyridyl solution – Dissolve 0.1 ga,a-dipyridyl in H and dilute to 100 ml. Reagents (a) and (b) kept in cool, dark place will remain stable several weeks;

iii) Iron standard solution – 0.01 mg Fe/ml

a) Dissolve 0.1g analytical grade Fe wire in 20 ml HCl and 50 ml H₂O, and dilute to 1 L. Dilute 100 ml of this solution to 1 L or

b) Dissolve 3.512 g Fe(NH) (SO₂), 6H₂O in H₂O, add two drops HCl, and dilute to 500 ml. Dilute 10 ml of this solution to 1 L;

iv) Hydroxylamine hydrochloride solution – Dissolve 10 g HNOH-HCI in H₂O and dilute to 100 ml;

v) Magnesium nitrate solution – Dissolve 50 g Mg(NO₂), 6H,0 in H₂O and dilute to 100 ml;

vi) Acetate buffer solution – Dissolve 8.3 g anhydrous NaC,H,O, (previously dried at 100°C) in H₂O, add 12 ml acetic acid, and dilute to 100 ml. (It may necessary to redistill acetic acid and recrystallize NaC2​H4​ depending on amount Fe present);

vii) Sodium acetate solution – 2 M. Dissolve 272 g NaC2​O4.3H₂O in H₂O and dilute to 1 L; and

viii) Buffer solution, *p*H 3.5 – Dilute 6.4 ml 2 M NaC2​O4​.H₂O solution, (g), and 93.6 ml 2 M HC,H,O, (120g / L) to 1 L with H₂O.

**25.3 Preparation of Standard Curve**

Construct 10-point standard curve, plus zero, preparing solutions containing 0.0 (zero), 2.0, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 40.0, and 45.0 ml, respectively, of final diluted Fe standard solution, A(c), plus 2.0 ml HCl, in 100 ml H₂O. Alternatively, construct a 5-point curve (5.0, 15.0, 25.0, 35.0, and 45.0 ml), plus zero, after correction for reagent blank. Using 10 ml of each of these solutions, proceed as in C, beginning "... add 1 ml H,NOH-HCl...". Plot concentration against scale reading.

**25.4 Procedure**

**25.4.1** *By dry ashing* – Ash 5.00 g test portion in Pt, SiO₂, or porcelain dish (ca 60 mm diameter, 35 ml capacity) as in 923.03 (see 32.1.05). (Porcelain evaporating dishes of ca 25 ml capacity are satisfactory. Do not use flat-bottom dishes of diameter >60 mm.) Cool and weigh if percent ash is desired. Continue ashing until practically C-free. To diminish ashing time, or for products that do not burn practically C-free, use one of following ash aids: Moisten ash with 0.5 ml to 1.0 ml Mg (NO3)2 solution or with redistilled HNO, Dry and carefully ignite avoiding spattering. (White ash with no C results in most cases.) Do not add these ash aids to self- rising flour (products containing NaCl) in Pt dish because of vigorous action on dish. Cool, add 5 ml HCl, letting acid rinse upper portion of dish, and evaporate to dryness on steam bath. Dissolve residue by adding 2.0 ml HCl, accurately measured, and heat 5 min on steam bath with watch glass on dish. Rinse watch glass and dilute residue solution to 100 ml with H₂O, if necessary (undissolved particles visible in residue solution), filter diluted residue solution through ashless paper and discard first 15 ml to 20 ml filtrate.

**25.4.1.1** Pipet 10 ml aliquot into 25 ml volumetric flask and add 1 ml. H,NOH-HCI solution; let stand 5 min and then add 5 ml buffer solution, A(f), and 1 ml o-phenanthroline, A(a), or 2 ml dipyridyl solution, A(b), and dilute to volume. Determine absorbance, A, in spectrophotometer or photometer at ca 510 nm. From reading. determine Fe concentration from equation of line representing standard points or by reference to standard curve for known Fe concentration. If further dilution is required to maintain test solution absorbance reading below highest standard point on curve, pipet smaller aliquot into 25.0 ml flask, dilute to 10.0 ml with 2% HCl solution (v/v) and continue as described in C, paragraph 3. Determine blank on reagents and make correction. Calculate Fe in flour as mg/lb.

**25.4.2** *By wet digestion* – Transfer 10.00 g test portion to 800 ml. Kjeldahl flask, previously rinsed with dilute acid, then with H₂O, add 20 ml H₂O and mix, pipet 5 ml H,SO, into flask and mix, add 25 ml HNO, and mix well. After 2-3 min, heat flask very gently at brief intervals (to avoid foaming out of flask) until heavy evolution of NO, fumes ceases. Continue to heat gently until material begins to char; then cautiously add 2-3 ml HNO, at intervals until SO, fumes evolve and colorless or very pale yellow liquid is obtained (60 ml to 65 ml HNO, total in ca 2 h). Cool, add 50 ml H₂O and one glass bead, and heat to SO, fumes; cool, add 25 ml H₂O, and filter quantitatively through 11 cm paper into 100 ml volumetric flask; cool, and dilute to volume.

**25.4.2.1** Pipet 10 ml into 25 ml volumetric flask, add 1 ml H,NOH-HCI solution, rotate flask, and let stand 2-3 min. Add 9.5 ml 2 M NaC,H,O, solution, A(g), and 1 ml o-phenanthroline solution, dilute to volume, and mix. Let stand 25 min and determine A in spectrophotometer or photometer at ca 510 nm. With self-rising flour, the 9.5 ml 2 M NaC,H,O, solution, A(g), may be reduced to 8.0 ml. To determine exact amount of buffer solution, A(h), needed to adjust each digest to most desirable *p*H range, mix 10 ml aliquot of test solution with measured amount of buffer solution, dilute with H₂O to 25 ml, and determine *p*H either potentiometrically or colorimetrically.

**25.4.2.2** For colorimetric determination, add five drops bromophenol blue indicator (grind 0.1 g powder with 1.5 ml 0.1 M NaOH and dilute to 25 ml with H₂O) to solution and compare color with that of equal volume of *p*H 3.5 buffer solution, A(h), also treated with five drops indicator. Although color develops in range *p*H 2-9, avoid *p*H <3.0 and preferably work at *p*H 3.5 to 4.5. With cereal products, 9.5 ml buffer solution, A(h), is satisfactory. With test portions high in Fe, aliquot of 5 ml instead of 10 ml may be used with 4.8 ml. buffer solution, A(h).

**25.4.2.3** Conduct digestion so as to avoid contamination with Fe and determine blank. After correction for blank, calculate as mg Fe/lb (453.6 g).

**26 DETERMINATION OF SUGARS**

**26.1 Reducing Sugars (AOAC 906.3)**

**26.1.1** *Principle*

**26.1.1.1** The Munson-Walker method is used to determine the concentration of reducing sugars in a sample. The principle of the method involves the reduction of copper (II) ions (Cu²⁺) to copper (I) oxide (Cu₂O) in an alkaline medium by the reducing sugars present in the sample. The reducing sugars react with an alkaline copper reagent, which contains copper sulfate, in the presence of heat. The copper(II) ions are reduced to copper(I) oxide, which forms a precipitate. The amount of precipitated copper(I) oxide is then quantitatively measured to determine the concentration of reducing sugars.

**26.1.1.2** In essence, the reducing sugars in the sample reduce the blue copper (II) ions to a red or orange precipitate of copper (I) oxide. The reduction reaction is directly proportional to the amount of reducing sugars present. This method provides a reliable way to quantify the amount of reducing sugars, such as glucose, in various food products, including bakery items.

**26.1.2** *Reagents*

**26.1.2.1** Asbestos Digest asbestos, amphibole variety with HCI (13) 2-3 days. Wash acid-free, digest for a similar period with 10% NaOH solution, and then treat for a few hours with hot alkaline tartrate solution, (b)(2), (alkaline tartrate solution that may have stood for some time may be used for this purpose.) Wash alkali-free, digest for several hours with HNO, (13), wash acid-free, and shake with water to a fine pulp (because of the unavailability of asbestos, use a fine porosity fritted glass crucible).

**26.1.2.1.1** To prepare the gooch crucible, make a film of asbestos ca 6 mm thick and wash thoroughly with water to remove the fine particles. If precipitated Cu, O is to be weighed as such, wash crucible with 10 ml. alcohol and then with 10 ml. ether, dry 30 min at 100 °C, cool in desiccator, and weigh.

**26.1.2.2** Soxhlet modification of Fehling solution

i) Copper sulfate solution – Dissolve 34.639 g CuSO4 SH2O2 dilute to 500 ml, and filter through glass wool or paper. Determine copper content of solution (preferably by electrolysis) (*see* **31.044**, 14th Ed.) and so adjust the solution that it contains 440.9 mg copper/25 ml.

ii) Alkaline tartrate solution – Dissolve 173 g potassium sodium tartrate 4H2O (Rochelle salt) and 50 g NaOH in water, dilute to 500 ml, let stand 2 days, and filter through prepared asbestos, (i).

**26.1.2.3** Invert sugar standard solution 1% to a solution of 9.5 g pure sucrose, add 5 ml. hydrochloric acid and dilute with water to 100 ml. Store several days at room temperature (about 7 days at 12 °C to 15 °C or 3 days at 20 °C to 25 °C); then dilute to 1 L. Acidified 1% invert sugar solution is stable for several months. Neutralize aliquot with approximately 1 M sodium hydroxide solution and dilute to desired concentration immediately before use.

**26.1.3** *Precipitation of Cuprous Oxide*

Clarify solutions with neutral lead acetate solution, 925.46 B(d) (*see* **44.1.07**) (never with basic lead acetate solution). Remove excess Pb with dry sodium oxalate.

Transfer 25 ml each of CuSO4 and alkaline tartrate solutions to 400 ml. beaker of alkaline-resistant glass, and add 50 ml reducing sugar solution; or if smaller volume of reducing sugar solution is used, add water to make final volume of 100 ml. Heat beaker, covered with a watch glass for entire period, at such a rate that boiling begins at 4 min and continue boiling for exactly 2 min. (It is important that these directions be exactly observed. To regulate heater, make preliminary tests with 50 ml reagents and 50 ml. water before proceeding with actual analyses).

Filter hot solution at once through the asbestos mat in the porcelain gooch, using suction. Wash the precipitate of cuprous oxide thoroughly with water at about 60 °C and either weigh directly as Cu,O, 929.09 (*see* **44.1.17**), or determine the amount of reduced copper by one of the methods described in 31.040-31.044 (14th Ed.). Conduct a blank determination, using 50 ml reagent and 50 ml. water, and if weight Cu2O obtained is > 0.5 mg, correct results of reducing sugar determination accordingly. The alkaline tartrate solution deteriorates on standing ad Cu2O in the blank increases.

**26.1 Determination of Sucrose (AOAC 925.05)**

**26.1.1** *Apparatus*

i) 250 ml volumetric flask;

ii) Steam bath or water bath;

iii) Funnel;

iv) 100 ml volumetric flask;

v) Beaker;

vi) Centrifuge (optional);

vii) Filter paper;

viii) Burette; and

ix) Litmus paper

**25.2 Reagents**

i) Calcium carbonate (CaCO₃);

ii) 50% alcohol by volume;

iii) Neutral 95% alcohol;

iv) Saturated neutral lead(II) acetate solution Pb(CH₃COO)₂;

v) Anhydrous sodium carbonate (Na₂CO₃) or potassium oxalate;

vi) Hydrochloric acid (HCl); and

vii) Sodium carbonate (Na₂CO₃).

**25.3** **Procedure**

Place 10 g test portion in a 250 ml volumetric flask. If the material is acid, neutralize by adding 1g to 3 g CaCO₃. Add 125 ml 50% alcohol by volume, mix thoroughly, and boil on a steam bath or by partially immersing the flask in a water bath for 1 hour at 83 °C to 87 °C, using a small funnel in the neck of the flask to condense vapor. Cool and let the mixture stand for several hours, preferably overnight. Dilute to volume with neutral 95% alcohol, mix thoroughly, let settle or centrifuge for 15 minutes at 1500 rpm, and decant closely. Pipet 200 ml of the supernate into a beaker and evaporate on a steam bath to 20-30 ml. Do not evaporate to dryness. Transfer to a 100 ml volumetric flask and rinse the beaker thoroughly with H₂O, adding the rinsings to the flask. Add enough saturated neutral Pb(CH₃COO)₂ solution (ca 2 ml) to produce a flocculent precipitate, shake thoroughly, and let stand for 15 minutes. Dilute to volume with H₂O, mix thoroughly, and filter through dry paper. Add enough anhydrous Na₂CO₃ or potassium oxalate to the filtrate to precipitate all Pb, again filter through dry paper, and test the filtrate with a little anhydrous Na₂CO₃ or potassium oxalate to make sure that all Pb has been removed. Place 50 ml of the prepared test solution in a 100 ml volumetric flask, add a piece of litmus paper, neutralize with HCl, add 5 ml HCl, and let inversion proceed at room temperature as in 925.48(c) (*see* **44.1.09**). When inversion is complete, transfer the solution to a beaker, neutralize with Na₂CO₃, return the solution to the 100 ml flask, dilute to volume with H₂O, filter if necessary, and determine reducing sugars in 50 ml solution (representing 2 g test portion) as in 906.03B (*see* **44.1.16**). Calculate results as invert sugar.

**25.4 Calculation**

Sucrose, % = [percent total sugar after inversion - percent reducing sugars before inversion (both calculated as invert sugar)] × 0.95

Correct for the volume of insoluble material by multiplying all results by 0.97.