LAB. MANUAL 4

MANUAL OF METHODS
OF
ANALYSIS OF FOODS

BEVERAGES (COFFEE, TEA, COCOA, CHICORY)
SUGAR AND SUGAR PRODUCTS &
CONFECTIONERY PRODUCTS

FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
MINISTRY OF HEALTH AND FAMILY WELFARE
GOVERNMENT OF INDIA
NEW DELHI
2012
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COFFEE - Definitions of different types of Coffee are given under section 2.10.2 of Food Safety and Standards (Food Product Standards and Food Additives) Regulations, 2011.

1.0 Roasted Coffee

1.1 Preparation of sample:

Grind the sample in a grinder to pass through No. 30 mesh sieve. Mix well to get a homogenous sample. Store sample in a tightly stoppered bottle. Withdraw portions for analytical determinations.

(Ref: A.O.A.C 17th edn, 2000 Official Method 920.91 Roasted Coffee Preparation of sample)

1.2 Determination of moisture: (Routine method)

Weigh accurately about 5 gms of sample in a tared aluminium dish. (about 7.5 cm in dia and 2.5 cm deep). Dry in an air oven at 100 ±2°C for 5 to 6 hours. Cool in a desiccator and weigh. Dry again for
30 minutes, cool in a dessicator and weigh. Repeat the process of heating and cooling in a dessicator until the difference in two successive weighings is less than 1 mg. Record the lowest weight. Carry out the determination in duplicate.

1.2.1 Calculation

\[
\text{Moisture (\%)} = \frac{W_1 - W_2}{W_1 - W} \times 100
\]

Where,

\(W\) = Weight in gms, of Aluminium dish.
\(W_1\) = Weight in gms, of Aluminium dish + sample before drying.
\(W_2\) = Weight in gms, of Aluminium dish + dried sample.

(Re :- I.S.1 Handbook of Food Analysis (Part IX) – 1984 page 51 / I.S 3077: 1972 Specification for roasted and ground Coffee)

1.2 A Determination of Moisture - Vaccum Oven method

(Reference method)

1.2A.1 Apparatus

1. Aluminium dish – 7 cm diameter and about 3 cm height with close fitting cover.
2. Dessicator
3. Vacum oven – connect with pump capable of maintaining partial vacum in oven A with pressure equivalent to 25 mm Hg and provided with thermometer passing into the oven in such a way that the bulb is near the test sample. Connect H2 SO4 gas drying bottle with oven to admit dry air when releasing vacum.

1.2A.2 Procedure

Accurately weigh about 5 gm of sample in a dish previously dried at 98 –100°C, cooled in dessicator and weighted with cover soon after attaning room temperature. Place in oven, lean cover against dish and heat to constant weight (about 5 1/2 hrs) at 98 – 100°C at pressure equal to 25 mm Hg. During heating admit slow current of air (about 2 bubbles / second) through H2SO4 into oven. Carefully admit dry air into oven to bring to atmospheric pressure. Cover dish, transfer to dessicator and weigh soon after room temperature is attained. Report percent loss in weight as moisture.

(Ref: - A.O.A.C 17th edn., 2000 Official Method 968.11 Moisture (Loss on Drying In Roasted Coffee, Vacuum Oven method 1)

1.3 Determination of total ash:

Weigh accurately about 5 gms of sample in a tared silica / platinum dish Char the material carefully on a burner and transfer the dish to a muffle furnace and ash at a temperature of 550 ±10 0 C until the ash is free of Carbon. Heat the dish again at 550 ± 10°C for 30
minutes Cool in a desiccator and weigh. Repeat this process of heating for 30 minutes, cooling in a dessicator and weighing until the difference between two successive weighing’s is less than 1 mg. Record the lowest weight.

\[
(W_2 - W) \times 100 \times 100
\]

Total ash (% on dry weight) = \( \frac{(W_1 - W) \times (100 - M)}{(W_1 - W) \times 100} \)

Where

\( W_1 = \) Weight in gms of Silica dish.+ sample
\( W_2 = \) Weight in gms of Silica dish + ash
\( W = \) Weight in gms of empty Silica dish.
\( M = \) Moisture % of the sample.

Note – Preserve the dish containing this ash for the determination of acid insoluble ash


1.4 Determination of water soluble ash:

Transfer the ash with the aid of about 25 ml distilled water into a beaker. Cover with a watch glass and boil for 5 minutes. Filter through an ashless filter paper (Whatman No. 42 or its equivalent). Collect the filterate in a 150 ml beaker, wash the filter paper 4 -5
times with hot water until the filtrate no longer turns red litmus blue and collect the washings in the same beaker. (Reserve the entire filtrate for the determination of alkalinity of soluble ash) Dry the ashless paper with residue in an oven and transfer to muffle furnace and ignite at 550°C for 2 hours. Cool in a desiccator and weigh (W3) Repeat the process till the difference in two consecutive weighings is less than 1 mg. Record the lowest weight.

\[
\text{Water in-soluble ash on dry wt basis(%) = } \frac{(W_2 - W) \times 100 \times 100}{(W_1 - W) \times (100 - M)}
\]

Where

\(W_2\) = Weight in gms of Silica dish + water insoluble ash.
\(W\) = weight in gm of empty dish
\(W_1\) = weight in gm of dish with material
\(M\) = Percentage of moisture

1.4.1 Calculation

Water soluble ash percent by wt = \(A - B\)

Where,

\(A\) = Total ash percent by wt
\(B\) = Water insoluble ash percent by wt
Water soluble ash of total ash = \frac{\text{Water soluble ash}}{\text{Total ash}} \times 100 \quad \text{(percent by wt)}


1.5 Determination of Ash insoluble in dilute HCl:

Boil total ash prepared as in sec. 1.3, with 25 ml of hydrochloric acid (1:2.5) for 5 minutes, covering the Silica dish with a watch glass to prevent spattering. Filter through ashless filter paper (Whatman No. 42 or equivalent). Wash the entire residue with hot water ( > 85°C) until the filtrate does not turn blue litmus paper to red. Dry the ashless paper with the residue and transfer to muffle furnace and ignite at 550°C for 2 hours. Repeat the process of igniting in the muffle furnace, cooling and weighing at ½ hr intervals until the difference in two successive weighings is less than 1 mg. Cool in a desiccator and weigh (W4).

1.5.1 Calculation

\[
\text{Ash insoluble in dilute HCl (\%)} = \frac{(W_2 - W) \times 100 \times 100}{(W_1 - W)x (100 - M)}
\]
Where,
\[ W_2 = \text{weight of dish} + \text{acid insoluble ash} \]
\[ W_1 = \text{weight of dish} + \text{sample} \]
\[ W = \text{weight of dish} \]
\[ M = \text{Percent moisture} \]


### 1.6 Determination of alkalinity of soluble ash:

To the filtrate reserved during the determination of water soluble ash (sec.1.4), add 3-4 drops of methyl orange indicator (0.1% in water) and titrate with 0.1N hydrochloric acid to an orange end point. Note down the titre value.

\[
\text{Alkalinity of soluble ash % per gm of sample (on dry wt.)} = \frac{\text{Titre value} \times \text{Normality of } HCl}{\text{Wt. of sample} \times (100 - M)}
\]

Where,
\[ W = \text{weight of empty dish} \]
\[ W_1 = \text{weight of dish} + \text{sample} \]
\[ M = \% \text{ Moisture of the sample} \]

1.7 Determination of aqueous extract:

Accurately weigh around 2 gms of sample and transfer to a 500 ml flask. Add 200 ml distilled water and connect the flask with a 50 cm long water jacketed condenser. Reflux for 1 hour over low flame with occasional mixing. Cool, and filter through Whatman No 1 filter paper or equivalent, wash 3 times with 10 – 15 ml water and finally make upto 250 ml in a volumetric flask. Shake well and pipette 50 ml of aliquot to a tared aluminium dish.

Evaporate on a steam bath and transfer to 100 ºC air oven and dry for 2 hours. Dry again for 30 minutes, cool in a desiccator and weigh. Repeat this process of heating for 30 minutes, cooling in dessicator and weighing until the loss in weight between two successive weighings is less than 1 mg.

Record the lowest weight

\[
\text{Aqueous extract (\%)} = \frac{(W_2 - W_1) \times 250 \times 100 \times 100}{W \times 50 \times (100 - M)}
\]

Where

\( W = \text{Weight of sample.} \)
\( W_1 = \text{Weight of empty aluminium dish.} \)
\( W_2 = \text{Weight of empty aluminium dish + dried extract.} \)
\( M = \text{Moisture \%.} \)
1.8 Determination of Caffeine Content (Bailey Andrew Method)

1.8.1 Procedure

Weigh accurately about 5 gm of sample, transfer to a 250 ml Erlenmeyer flask, add 3 gm of magnesium oxide and 100 ml of distilled water. Weigh the flask with contents and boil under a reflux condenser for 45 minutes, shaking occasionally. Cool and weigh the flask again and add water till the original weight is obtained. Mix well and filter through a dry filter paper directly into a 50 ml graduated flask until exactly 50 ml of the solution (equivalent to half the quantity of the sample taken for test) is obtained.

Transfer the solution to a 125 ml separator. Wash the graduated flask with 2 ml of water and add the washings to the separator. Add 4 ml of dilute Sulphuric acid (1:9). Extract with five 10 ml portions of chloroform shaking vigorously for 1 minute for each extraction. Let the emulsion break, then drain the chloroform into a 125 ml separator. Add 5 ml of Potassium hydroxide solution (1%). Shake vigorously for 1 minute, let the emulsion break and drain the chloroform through a cotton plug into a 100 ml Kjeldahl flask. Extract the Pot hydroxide solution with 5 ml of chloroform and add to the Kjeldahl flask. To the digestion flask add $1.3 \pm 0.5$ gm of potassium sulphate and $40 \pm 5$ mg
mercuric oxide. Rinse down the neck of the flask with 3 ml chloroform.

Place the flask on the digestion rack and concentrate chloroform to about 20 ml. Distill off chloroform. Add 2 ± 0.1 ml conc sulphuric acid of sp gr 1.84 digest for 1 hour after the acid begins to boil. Cool and add minimum quantity of water to dissolve the solids. Cool and place a thin film of vaselin at the rim of the flask. Transfer the digest with a few boiling chips to the distillation apparatus and rinse the flask 5 – 6 times with 1 – 2 ml water.

Place a 125 ml beaker containing a known quantity of standard sulphuric acid (0.05N). Add 6 ml of conc sodium hydroxide solution (1+ 2) carefully through the side of the still so that it does not mix, and assemble the distillation apparatus taking care that the dip tube extends well within the standard sulphuric acid solution contained in the beaker. Mix the contents of the distillation flask and distill until all ammonia has passed over into the standard sulphuric acid. Shut off the heater and immediately detach the flask from the condenser. Rinse the condenser thoroughly with water into the beaker. Wash the dip tube carefully so that all traces of the condensate are transferred to the beaker. When all the washings have drained into the beaker, add 2-3 drops of methyl red indicator and titrate with standard sodium hydroxide solution (0.1N).

Carry out a blank determination using reagents in the same proportion without the sample.
1.8.2 Calculation

\[
\text{Caffeine on dry basis} = \frac{486.96 \times (B - A) \times N}{W \times (100 - M)}
\]

Where,

- \(B\) = volume of standard sodium hydroxide used to neutralize acid in the blank determination
- \(A\) = volume of standard sodium hydroxide used to neutralize the excess acid in the test with the sample
- \(N\) = Normality of standard sodium hydroxide solution
- \(W\) = weight in gm of the sample in the aliquot
- \(M\) = Percentage of moisture in the sample

Note :- For soluble coffee (instant coffee) the quantity of sample taken for test should be 1 gm only


1.8A Alternate Chromatographic – Spectrophotometric Method:
Applicable to roasted, soluble (instant) and decaffeinated coffee

1.8A.1 Regents

a) Ammonium hydroxide solution – 1 : 2 (V/V)
b) Sulphuric acid – 4N

c) Diethyl ether – Water Saturated

d) Chloroform – Water Saturated

e) Celite 545

f) Caffeine standard solution – 10, 20, 30 µg /ml in Chloroform.

Accurately weigh 100 mg of caffeine (USP, anhydrous) into 100 ml volumetric flask, dissolve in CHCl₃ and make up to volume. Dilute 10 ml aliquot to 100 ml with chloroform. Further dilute 10, 20, and 15 ml aliquots to 100, 100 and 50 ml respectively with chloroform to obtain standard solutions of 10, 20, and 30 µg / ml

1.8A.2 Apparatus

a) Glass column – 25 x 250 mm size

b) Recording UV – VIS Spectrophotometer – To record 250 – 350 mm range with matched 1 cm cells.

1.8A.3 Preparation of Sample

a) For roasted Coffee

Accurately weigh about 1 gm ground sample and transfer to 100 ml beaker. Add 5ml NH₄OH (1+2) and warm on boiling water-bath for 2 minutes. Cool, transfer to 100 ml volumetric flask and make up to volume with water. To 5 ml aliquot of the turbid solution add 6 g celite 545 and mix carefully.
b) For soluble Coffee

Proceed as in (a) taking 0.5 g sample and an aliquot of 3 ml

c) For decaffeinated roasted coffee

Accurately weigh 1 gm of ground sample. Transfer to 100 ml beaker, add 5 ml NH₄OH (1+2) and warm on boiling water bath for 2 minutes. Add 6 gm celite 545 and mix carefully.

d) For decaffeinated soluble coffee

Proceed as in c) taking 0.5 gm sample

1.8A.4 Preparation of Columns

a) Acid column:

Place fine glass wool and plug into the base of 25 x 250 mm column. Add 3 ml 4N H₂SO₄ to 3 g celite and mix well by kneading with spatula. Transfer into the tube and tamp using gentle pressure and place small glass wool above the surface.

b) Basic Column: Layer I:

Mix 3 g celite 545 and 2 ml 2N NaOH and place in 25 x 250 mm tube. Transfer over glass wool plug as in (a) into the column.
Layer II:

Transfer sample plus celite mixture in about 2 g portions to tube directly over layer I, tamping before adding mixture portion of sample until homogenous and compact layer is obtained. Dry wash beaker with about 1 g celite 545, transfer to tube and tamp to uniform mass. Dry wash beaker with wad of glass wool and transfer to top of basic column.

1.8A.5 Determination:

Mount basic column above acid column. Pass 150 ml water saturated ether sequentially through basic column to acid column and discard ether. Then pass 50 ml water saturated ether through acid column and discard ether. Place 50 ml volumetric flask under acid column. Pass 48 ml, water saturated CHCl₃ through acid column washing tip of basic column with first portions. Dilute contents of volumetric flask (100 ml) to volume with water saturated chloroform, mix, and read O.D. at 275 against water saturated chloroform CHCl₃ blank, by scanning from 350 to 250 nm. Determine O.D of standards and use this value to calculate the caffeine percentage.

(Ref:- A.O.A.C 17th edn, 2000 Official Method 979.11 Caffeine in Roasted Coffee, Chromatographic – Spectrophotometer method)
1.8 B Alternate HPLC method

1.8 B.1 Procedure

Dissolve 1.00 gm of sample in 100 ml hot water. Filter 20 ml through a Millipore filter (0.45µm) under vacuum and apply to a Bond Elut C 18 cartridge or equivalent under vacuum. Elute the caffeine with 5 ml of mobile phase (0.005 M Sodium acetate : tetrahydrofuran – 95 : 5 at pH 5). Collect in a 10 ml flask and make upto volume. Inject 20 ul into a Spherisorb ODS, C 18 , 5 um packed column 25 cm long x 4 mm internal dia. Elute with the mobile phase at 1 ml / min, observe absorbance at 280 nm, calibrate with standard Caffeine solution, 0 - 1 mg Caffeine in 10 ml mobile phase. For routine purposes the HPLC step can be eliminated and the absorbance of eluant from the cartridge measured at 280 nm in a spectrophotometer.

(Ref:- Pearson’s Composition and Analysis of Foods 9th edn,1991 , page373)

1.9 Microscopic Examination:

Boil about 1 gm of sample with 50 ml of 2% sodium hydroxide for about 2 - 3 minutes. Dilute and filter and wash the residue with water till the filtrate is free of alkali. Repeat till the residue gives no colour with water (treatment with calcium chloride solution and then washing with water may be done in case the decant still shows some
colouring matter. Place a drop of residue material in glycerine on a clear microscopic slide.

Place a cover slip on the drop of the suspension and see under microscope. Alternatively boil sample with water so that most of the colour is extracted. Drain and replace with chloral hydrate. Heat until sufficiently cleared. Wash out chloral hydrate and stain with phloroglucinol/hydrochloric acid. The microscopic structure as shown in the photomicrograph given below can be seen:
FIG. 5  Roasted Coffee Powder x 100. (Photomicrograph) Coffee Showing
Sclerenchymatous Fibres of Endocarp (Seed Coat)

FIG. 6  Roasted Coffee Powder x 100 (Photomicrograph) Coffee Showing
Masses of Sclereids
Note:-
Coffee is characterized by longitudinal and transverse schlerenchymatous fibres (from pericarp) Chicory has large vessels upto 115 microns across which have short pits. Roasted cereals such as barley, oats and wheat and soya may be mixed with coffee and coffee and chicory as coffee substitutes. Careful microscopic examination will reveal their presence.

1.9.1 Test for Chicory in Coffee:

Principle:

Chicory is a root and contains inulin, hydrolysis of which gives fructose. Coffee does not contain inulin. Therefore, this method can be used to test the presence of Chicory in coffee.

Reagents:

(a) Neutral lead acetate – Prepare 10% solution in water.
(b) Conc. HCl.
(c) Seliwanoff reagent – Dissolve 0.05 g of resorcinol in 100 ml of 1:2 HCl.

Procedure:

Clarify 25 ml of 2% aqueous extract of the sample with neutral lead acetate and filter. To 5 ml of filtrate add 5 ml of Seliwanoff reagent and 1 ml of conc HCl. Boil for 2 minutes. Appearance of distinct red colour on standing shows the presence of Chicory in coffee.

Note :-

In addition to microscopic examination and the positive reaction with Seliwanoffs reagent, the presence of chicory may be shown simply by sprinkling the powder onto water in a measuring cylinder. Coffee floats while chicory particles start sinking within a few seconds leaving behind a brown trail of caramel

(Ref :- F.A.O Manuals of Food Quality Control 14 / 8 pages317 and 318)
2.0 CHICORY AND COFFEE - CHICORY MIXTURE

2.1 Preparation of sample – Refer to clause 1.1

2.2 Determination of moisture – Refer to clause 1.2

2.3 Determination of total ash on dry basis – Refer to clause 1.3

2.4 Determination of ash insoluble in dil HCl – Refer to clause 1.5

2.5 Determination of aqueous extract – Refer to clause 1.7

2.6 Microscopic Examination – Refer to clause 1.9

2.7 Determination of Caffeine content – Refer to clause 1.8

3.0 SOLUBLE (INSTANT) COFFEE POWDER

3.1 Determination of Moisture – Refer to clause 1.2 A (vacuum oven method with the following changes)

(1) Keep temperature of oven at 70 ± 1 °C.
(2) Maintain vacuum at 37.5 mm Hg
(3) Dry for 16 hours
(4) Admit air at the rate of 1 bubble / second
(5) Calculate moisture (Loss on drying) as under

\[
\text{Moisture} \% = \frac{(M_1 - M_2)}{(M_1 - M_0)} \times 100
\]
Where

\[ M_0 = \text{Weight of empty dish} \]
\[ M_1 = \text{weight of dish + sample before drying} \]
\[ M_2 = \text{Weight of dish + sample after drying} \]


3.2 Determination of Total Ash = Refer to clause 1.3

3.3 Determination of Caffeine Content – Refer to clause 1.8.

3.4 Determination of Solubility in boiling water

3.4.1 Procedure

Weigh 2.5 gm of instant coffee powder / coffee-chicory powder in a 500 ml beaker. Then pour 150 ml of freshly boiled water, stir. The product should dissolve in 30 seconds.

(Ref :- I.S.I Hand book of Food Analysis (Part IX) – 1984 page 58)
4.0 COCOA POWDER

4.1 Preparation of Sample

Mix thoroughly and preserve in tightly stoppered bottle.

(Ref: A.O.A.C 17th edn, Official method 970.20 Cocoa products Preparation of Laboratory Sample)

4.2 Determination of moisture

Weigh accurately about 2 gm of sample in Platinum / stainless steel dish. Distribute the material as evenly as possible and place in an air oven maintained at 100°C. Dry to a constant weight. (aluminium dish may be used when ash is not determined on the same sample). Report loss in weight as moisture.


4.3 Determination of Cocoa Butter

4.3.1 Apparatus

(1) Soxhlet apparatus – with 250 ml flat bottomed flask
4.3.2 Reagents

(1) Petroleum ether – redistilled below 60 °C

4.3.3 Procedure

Weigh accurately 10 – 20 gm of the material and transfer it to the fat extraction thimble. Dry in an oven at 100 °C for 1 hour. Extract the fat with Petroleum ether. Continue extraction till 300 ml of petroleum ether have been circulated. Recover excess solvent and evaporate the last traces on a steam bath. Dry the fat in an air oven till the difference in two successive weighings is less than 1 mg.

4.3.4 Calculation

Cocoa butter (%) on moisture free basis = \( \frac{\text{Wt of extracted fat} \times 100 \times 100}{\text{Wt of sample} \times (100 - \text{Moisture})} \)

(Ref:- I.S.I Hand Book of Food Analysis (Part IX) – 1984 page 24)

(Also see A.O.A.C 17th edn, 2000 Official Method 963.15 Fat in Cocoa Products, Soxhlet Extraction method)

4.4 Determination of Total ash – Refer to clause 1.3

4.5 Determination of Ash insoluble in dil HCl – Refer to clause 1.5

4.6 Determination of Alkalinity of ash - Refer to clause 1.6
5.0 TEA

5.1 Preparation of sample: Ref. to clause 1.1.

5.2 Determination of moisture: Ref Clause 1.2

5.3 Determination of total ash : Ref Clause. 1.3


5.4 Determination of Water soluble ash:- Ref Clause. 1.4


5.5 Determination of Ash insoluble in dilute HCl: Ref Clause. 1.5


5.6 Determination Aqueous extract: Ref Clause. 1.7

5.7 Determination of alkalinity of soluble ash:

Determine as per the procedure given in clause 1.6 (under analysis of coffee).
Express the result as KOH (m/m) on dry basis:
(PFA standards for Tea – A.14, Notification No GSR 277 (E) dtd 9th May 2006)

\[
\text{Alkalinity of soluble ash} \% = \frac{0.0056 \times \text{titre value} \times \text{Normality HCl} \times 100 \times 100}{\text{Weight of sample} \times 0.1 \times (100 - \text{moisture} \%)}
\]


5.8 Determination of Crude Fibre:

Reagents:
1. 1.25 g Sulphuric acid/100 ml (1.25 percent w/v)
2. 1.25 g Caustic soda/100 ml free from sodium carbonate.(1.25 % w/v)

Apparatus:

(i) Condenser – Use condenser that will maintain constant volume of refluxing solutions.
(ii) Digestion flask – 700-750 ml Erlenmeyer flask is recommended.
(iii) Filtering cloth – Use filtering cloth of such character that no solid matter passes through when filtering is rapid. Fine linen or dress linen with about 18 threads/cm or 45
threads per inch (i.e. the aperture size 0.14 mm and thread thickness 0.42 mm) or its equivalent may be used (Whatman filter Paper No. 54 may also be used).

(iv) Muffle Furnace maintained at $525 \pm 20^\circ C$.

**Procedure:**

Weigh accurately about 2 gm of prepared sample and dry in an air oven maintained at $100 \pm 2^\circ C$ for 4 hours. Transfer to the digestion flask. Add 200 ml of boiling 1.25 percent sulphuric acid. Immediately connect to the condenser and heat (it is essential that the solution boils within one minute and boiling continues briskly for exactly 30 minutes). Rotate flask frequently until sample at sides is thoroughly wetted, taking care to keep material from remaining on the sides of the flask. Immediately filter through linen in fluted funnel, and wash with boiling water until washings are acid free.

Wash the residue back into the flask with 200 ml of boiling 1.25 percent sodium hydroxide solution using wash bottle marked to deliver 200 ml. Connect flask to reflux condenser and boil briskly exactly for 30 minutes. After 30 minutes remove flask immediately, filter through gooch prepared with asbestos mat and carefully transfer all the residue into the gooch with hot water. Wash the residue thoroughly with hot water until the filtrate is alkali free. Then, wash with about 10 ml alcohol. Dry the gooch crucible at 110$^\circ C$ to constant
weight. Cool and weigh \(W_1\). Transfer the gooch to a muffle furnace controlled at 525 - 550ºC and ash the material. Cool, weigh \(W_2\). Loss in weight represents crude fibre.

**Calculation:**

\[
\text{Crude fibre \%} = \frac{W_1 - W_2}{\text{Wt. of sample}} \times \frac{100 \times 100}{100 - \text{Moisture}}
\]

(Ref: I.S.I Hand book of Food Analysis (Part IX) – 1984 page 44)

5.9 Determination of Iron filings and size of the particles:

Spread the entire quantity of the sample in a thin and uniform layer on a polythene sheet. Run a powerful magnet over the sample repeatedly till no more iron filings cling to the magnet.

Collect the iron fillings in a clean dry and previously weighed petridish. Note down and express the weight of iron filings and calculate the content in ppm.

**Calculation:**

\[
\frac{\text{Weight of iron filings in gms \times 1000 \times 1000}}{\text{Weight of sample}} = \text{ppm}
\]
The method specified by Tea Board i.e. Total Iron And Non-Magnetic Iron By Colorimetry can be also be followed.

5.9.1 Method for the determination of the size of iron particles:

Calibrate an ocular scale against a known stage-micrometre scale. This is done by placing an ocular scale in the eye piece of a microscope. Focus the stage micrometre under a desired magnification. Count the number of ocular scales covering the number of stage micrometre scales and calculate the factor.

Example: If X number of ocular scales = Y No. of stage micrometer scale then 1 ocular scale (factor) = 1 Y/X mm.

Place iron particles under question on a slide and focus at same magnification. Bring individual particle under the ocular scale. Count the number of ocular scale covering the two farthest points of the particle. Multiply this number by the factor in order to get the size of iron particles.

5.9.2 Test for presence of added colouring matter:

Refer to manual on Food Additives

5.9.3 Microscopic Examination:

Follow the same procedure as in clause. 1.9 under analysis of Coffee.
SUGAR AND SUGAR PRODUCTS
(HONEY, SUGAR, BURA, JAGGERY, GLUCOSE SYRUP)

6.0 HONEY

6.1 Preparation of sample

If the sample is liquid or strained honey and is free from granulation, mix thoroughly by stirring or shaking. If granulated place the closed container in water bath without submerging and heat 30 minutes at 60 °C, then if necessary heat at 65 °C until liquefied. Occasional shaking is essential. Mix thoroughly and cool rapidly as soon as sample liquefies. Do not heat honey intended for hydroxyl methyl furfural or diastase determination.

If foreign matter such as wax, sticks, bees, particles of comb etc is present heat sample to 40°C in water bath and strain through cheese cloth in hot water funnel before sampling.

(Ref : A,O,A,C 17th edn ,2000 Official method 920.180 Honey (Liquid, strained or comb) Preparation of test sample )
6.2 Determination of Moisture:

6.2.1 Refractive Index Method:

Determine Abbe’s refractometer reading of the sample at a constant temperature near 20ºC and obtain corresponding moisture % from the following table. If the determination is made at a temperature other than 20ºC convert the reading to standard temperature of 20ºC according to the temperature corrections given below.

Relationship between refractive index and water contents of honey:

<table>
<thead>
<tr>
<th>Water Content %</th>
<th>Refractive Index at 20ºC</th>
<th>Water Content %</th>
<th>Refractive Index at 20ºC</th>
</tr>
</thead>
<tbody>
<tr>
<td>13.0</td>
<td>1.5044</td>
<td>19.0</td>
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<td>13.2</td>
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</tr>
<tr>
<td>14.2</td>
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<td>1.5007</td>
<td>20.4</td>
<td>1.4855</td>
</tr>
<tr>
<td>14.6</td>
<td>1.5002</td>
<td>20.6</td>
<td>1.4850</td>
</tr>
<tr>
<td>14.8</td>
<td>1.4997</td>
<td>20.8</td>
<td>1.4845</td>
</tr>
<tr>
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</tr>
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<tr>
<td>16.6</td>
<td>1.4951</td>
<td>22.6</td>
<td>4.4800</td>
</tr>
<tr>
<td>Temperature corrections to change refractive index</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Temperature above 20 °C - add 0.00023 per °C

Temperature below 20 °C - subtract 0.00023 per °C


6.2.2 Vacuum Oven Drying Method:

Place 20-25 gm pure quartz sand which passes through 500 micron I.S Sieve and is retained by I.S 180 micron I.S Sieve and a short glass rod in a 6 cm diameter aluminium flat dish. Dry thoroughly and cool in a desiccator and weigh. Accurately weigh about 5 gm of sample in a beaker and transfer completely to
aluminium flat dish by thorough washing with water. Mix well and heat on steam-bath for partial drying. Transfer the dish to vacuum oven and dry the sample at less than 70°C under 25 mm Hg pressure. After 2 hours remove the dish to a dessicator allow to cool and weigh. Replace the dish in the oven for a further period of 1 hour, cool and weigh again. Determine the difference in weight and determine the moisture %.

(Ref :- I.S.I Handbook of Food Analysis (Part II) – 1984 page 35)

6.3 Determination of specific gravity

6.3.1 Apparatus

Thermostatically controlled water bath maintained at 27 ± 1°C

Specific gravity bottle.

6.3.2 Procedure

Clean and thoroughly dry the sp. gravity bottle and weigh. Fill it up to the mark with freshly boiled and cooled distilled water maintained at 27 ± 1°C and weigh. Remove the water, dry the bottle again and fill it with honey sample maintained at the same temperature. Weigh the bottle again
6.3.3 Calculation

Specific gravity at 27 /27 °C = \frac{C-A}{B-A}

Where

C = wt of sp. Gravity bottle with honey sample

A = wt of empty sp. Gravity bottle

B = wt of sp. Gravity bottle with water

(Ref :- I.S.I Handbook of Food Analysis (Part II) page 35)

6.4 Determination of reducing sugars, total reducing sugars and sucrose in honey:

Principle:

Invert sugar reduces the copper in Fehling-A solution to a brick red insoluble cuprous oxide

Reagents:

Fehling A: Dissolve 69.28 g copper sulphate (CuSO4.5H2O) in distilled water. Dilute to 1000 ml. Filter and store in amber coloured bottle.

Fehling B: Dissolve 346 g Rochelle salt (potassium sodium tartrate)
(K Na C₄H₄O₆. 4H₂O) and 100 g NaOH in distilled water. Dilute to 1000 ml. Filter and store in amber coloured bottle.

**Neutral Lead Acetate:** Prepare 20% neutral lead acetate solution. (This reagent is used to clarify sugar solutions)

**Potassium Oxalate Solution:** Prepare 10% Potassium oxalate(K₂C₂O₄.H₂O) solution. This reagent is used to remove the excess lead used in clarification.

**Methylene Blue Indicator:** Prepare 1% of methylene blue solution in distilled water.

**6.4.1 Determination of Reducing Sugars:**

Weigh accurately 25 gms of sample and transfer to 250 ml volumetric flask. Add 10 ml of neutral lead acetate solution and dilute to volume with water and filter. Transfer an aliquot of 25 ml of the clarified filtrate to 500 ml volume flask containing about 100 ml water. Add potassium oxalate in small amounts until there is no further precipitation. Make up to volume. Mix the solution well and filter through Whatman No. 1 filter paper. Transfer the filtrate to a 50 ml burette.

**Preliminary Titration:** Pipet 5 ml each of Fehling A and B into 250 ml conical flask. Mix and add about 10 ml water and a few boiling chips or glass beads. Dispense solution. Heat the flask to boiling. Add
3 drops of methylene blue indicator. Continue the addition of solution dropwise until the blue colour disappears to a brick-red end point. (The concentration of the sample solution should be such that the titre value is between 15 and 50 ml). Note down the titre value.

**Final Titration:** Pipet 5 ml each of Fehling A and B. Add sample solution about 2 ml less than titre value of the preliminary titration. Heat the flask to boiling with in 3 minutes and complete the titration. Perform the titration duplicate and take the average. Calculate the reducing sugars % as shown below.

\[
\text{Reducing Sugars} \% = \frac{\text{Dilution} \times \text{Factor of Fehling (in gm)} \times 100}{\text{(as Invert Sugar)} \times \text{Weight of sample} \times \text{titre}}
\]

**6.4.2 Determination of Total Reducing Sugars:**

Pipette an aliquot of 50 ml of the clarified, de-leded filtrate to a 100 ml volumetric flask. Add 5 ml of conc. HCl and allow to stand at room temperature for 24 hours. Neutralise with conc. NaOH solution followed by 0.1N NaOH. Make up to volume and transfer to 50 ml burette having an offset tip and perform the titration on Fehlings solution similar to the procedure described in the determination of reducing sugars.
Total Reducing Sugars % = \frac{\text{Dilutions} \times \text{Fehling factor} \times 100}{\text{Weight of sample} \times \text{titre}}


6.4.3 Determination of Factor (for Invert Sugar) of Fehling Solution:

Accurately weigh around 4.75 gms of analar grade sucrose. Transfer to 500 ml volume flask with 50 ml distilled water. Add 5 ml conc. HCl and allow to stand for 24 hours

Neutralize with NaOH solution and make up to volume. Mix well and transfer 50 ml to a 100 ml volumetric flask and makeup to volume. Transfer to a burette having an offset tip.

Perform the titration of Fehling solution following the similar procedure as above:

\[
\text{Fehling Factor} \quad \text{(for Invert Sugar)} = \frac{\text{Titre} \times \text{Weight of sucrose in gm}}{500}
\]
6.4.4 Determination of Sucrose:

Calculation:

Sucrose %
= (Total reducing sugars / invert sugar % - reducing sugars %) x 0.95


6.5 Determination of Fructose: Glucose ratio

Principle:

Glucose % is determined iodimetrically in a weak alkaline medium and the value is subtracted from reducing sugars % to arrive at fructose % and fructose: glucose ratio.

Reagents:

1. 0.1N Iodine: Weigh 13 gm iodine and 20 gm potassium iodide together and dissolve in water and make up to 1 litre. Store in amber coloured bottle.
2. 0.2N Sodium bi-carbonate: Dissolve 3.5 gm sodium bicarbonate in 200 ml water.
3. 0.2N Sodium Carbonate: Dissolve 4.25 gm sodium carbonate in 200 ml water.
4. 25% H$_2$SO$_4$ (v/v).

5. 0.1N Sodium Thiosulphate: Dissolve 25 gm sodium thiosulphate in boiling distilled water. Cool make up to 1 litre. Filter and store in amber coloured bottle. Standardize against potassium dichromate.

**Procedure:**

Weigh 2 g of sample to 250 ml volumetric flask and make upto volume Mixwell and transfer an aliquot of 25 ml to a 250 ml of iodine flask. Pipet 50 ml of 0.1N Iodine and add 50 ml of 0.2N sodium carbonate and 50 ml of 0.2N sodium bicarbonate solution Allow to stand in dark for 2 hours Acidify with 12 ml of 25% H$_2$SO$_4$ and titrate with standard sodium thiosulphate using starch as indicator. Carry out blank simultaneously Subtract from titre value of blank the titre value of sample.

**Calculation:**

\[
\text{Glucose} \% = \frac{\text{Normality of thiosulphate } \times \text{ dilution } \times (B-S) \times 0.009005 \times 100}{0.1N \times \text{ weight of sample}}
\]

Fructose % = Reducing sugars % - glucose %

\[
\text{Fructose : Glucose ratio} = \frac{\text{Fructose} \%}{\text{Glucose} \%}
\]
6.6 Test for commercial Invert Sugar:

6.6.1 Fiehe’s Test:

Reagents:

1. 1% Resorcinol: Dissolve 1 gm Resorcinol in 100 ml conc. HCl.
2. Diethyl Ether, AR grade.

Procedure:

Dissolve around 2 gms of sample in 50 ml water and extract with diethyl ether in a separatory funnel. Collect the ether layer in a porcelain basin and evaporate the ether. Add 4-5 drops of freshly prepared resorcinol solution.

Appearance of cherry red colour indicates the presence of commercial invert sugar.

(Note: This test should not be taken as a conclusive test.)

(Ref: I.S.I. Handbook of Food Analysis (Part II) page 37)
6.6.2 Aniline Chloride Test:

Aniline Chloride Reagent: To 100 ml aniline, add 30 ml 25% HCl.

Procedure:

Introduce about 5 gm of sample into a porcelain basin and add while stirring 2.5 ml of freshly prepared aniline chloride reagent. In the presence of commercial invert sugar, the mixture assumes within 1 minute orange red colour turning dark-red. Yellow to salmon shades are to be disregarded.

(Ref :- I.S.I Handbook of Food Analysis (PartII) page 38)

6.7 Determination of Ash:

Weigh accurately 5 - 10 gm of sample in a previously dried and weighed silica dish. Char the sample on a burner and transfer the dish to muffle furnace maintained at 550 ºC. Cool in a desiccator and weigh. Incinerate to a constant weight and calculate the % ash.

(Ref :- I.S.I Handbook of Food Analysis (Part II) page 37)

6.8 Determination of Acidity

6.8.1 Reagents

(1) Standard Sodium Hydroxide solution – 0.05 N
(2) Phenolphthalein indicator – Dissolve 0.5 gm Phenolphthalein in 100 ml of 50% ethyl alcohol (v/v)

6.8.2 Procedure

Take 10 gm of the sample in a suitable titration flask and dissolve in 75 ml of carbon dioxide free water. Mix thoroughly. Titrate against standard sodium hydroxide solution using 4-6 drops of phenolphthalein indicator till pink colour persists for 10 seconds.

Determine blank on water and indicator and correct the volume of sodium hydroxide solution used.

6.8.3 Calculation

\[
\text{Acidity as formic acid (\%) by weight} = \frac{0.23 \times V}{M}
\]

Where,

\( V \) = corrected volume of 0.05 N sod. Hydroxide used

\( M \) = weight in gm of the sample taken for test

(Ref: I.S.I. Handbook of Food Analysis (Part II) 1984 page 37)
7.0 CANE SUGAR AND Refined SUGAR

7.1 Preparation of Sample

Grind if necessary and mix to a uniform mass. Thoroughly mix raw sugar (Gur, Jaggery) in minimum time. Break up any lumps on a glass plate or in a pestle and mortar. Transfer to a dry stoppered container

(Ref :- A.O.A.C 17th edn, 2000 Official method 920. 175 Preparation of Sample)

7.2 Determination of Moisture:

Transfer 5 g of the prepared sample in a previously dried, tared aluminium dish. Cover the dish with the lid and weigh accurately. Remove the lid and dry the sample at 105 °C ± 1 °C for 3 hours. Cool in a desiccator and weigh.

Redry 1 hour and repeat process until change in weight between two successive dryings is less than 2 mg. Report the loss in weight as moisture.

(Ref :- A.O.A.C 17th edn, 2000 Official method 925.45 (b) (except 105 °C temperature as per P.F.A) Moisture in Sugars)
Note :- The ICUMSA method (1974) requires a forced draft oven maintained at a temperature of 105 ± 1°C as measured 2.5 ± 0.5 cm above the dishes in the test. The oven is to be ventilated and the circulation fan fitted with an interlock switch which opens when the oven door is opened. The dishes with tight fitting lids should be 6-10 cm with a depth of 2-3 cm. Although the dish may be made of glass, platinum or Nickel, aluminium is recommended. The quantity of sample recommended is 20 – 30 gm, the depth of sample in the dish not to exceed 1 cm.

7.3 Determination of Ash:

Follow the same procedure as given for honey under Sec. 6.0.

7.4 Determination of Sucrose:

Weigh exactly around 10 gm of prepared sample and make up to 250 ml volume. Determine the reducing sugars as described in the analysis of honey in Sec. 6.4.

Take an aliquot of 100 ml in a 500 ml volumetric flask and add 10 ml of HCl and let stand for 1½ days at 25°C and above. Dilute to 500 ml. Transfer an aliquot of 100 ml to a 250 ml volumetric flask, neutralize with NaOH and make up to volume and mix. Take this solution in a burette having an offset tip. Proceed with the titration against Fehling A and B similarly as described in the analysis of honey.
Calculation: See under honey analysis.

Sucrose %  
= \[\text{Reducing sugars \% after inversion} - \text{Reducing sugars \% before inversion}\] \times (0.95)

7.4.1 Alternate Method (Polarisation method)

7.4.1.1 Principle

An aqueous solution of the sugar sample (26 gm, i.e the normal weight of sucrose in 100 ml water.) is polarized by means of a saccharimeter which is calibrated to read 100° S on the ‘International Sugar Scale’ under specified conditions.

7.4.1.2 Apparatus

Saccharimeter, calibrated with quartz plates. Basis of calibration of 100° points on international sugar scale is polarization of normal solution of pure sucrose (26.000 gm / 100 ml) at 20° C in 200 mm tube using white light and dichromate filter defined by the commission. This solution polarized at 20° C must give saccharimeter reading of exactly 100° S. Temperature of sugar solution during polarization must be kept constant at 20° C.

Following rotations hold for normal quartz plate of international sugar scale:
NormalQuartz Plate =100° S = 40.690 ± 0.002 (\(\lambda = 546.1 \text{ nm}\)) at 20° C
In general make all polarizations at $20^\circ C$. For countries where mean temperature is above $20^\circ C$, Saccharimeters may be adjusted at $30^\circ C$ or any other suitable temperature, provided sugar solution is diluted to final volume and polarized at this temperature.

7.4.1.3 Procedure

In determining polarization use whole normal weight $(26\pm 0.002 \text{ gm})$ for 100 ml or multiple for any corresponding volume. Bring solution exactly to mark at proper temperature and after wiping out the neck of the flask with filter paper add minimum amount of dry basic lead acetate, shake to dissolve. Repeating addition till precipitation is complete Pour all clarified sugar solution on rapid air dry filter. Cover funnel at start of filtration. Reject first 25 ml filtrate and use remainder (must be perfectly clear) for polarization. In no case return whole solution or any part to filter. To remove excess lead used in clarification add anhydrous Pot or Sod Oxalate to clarified filtrate in small amounts until test for lead in filtrate is negative, then refilter Polarise in 200 mm tube. Other permissible clarifying and decolorizing agents are alumina cream or conc alum solution. Temperature correction for polarization of sugars. Polarisation when made at temperatures other than $20^\circ C$ may be calculated to polarization at $20^\circ C$ by the following formula:

$$P_{20} = P_t \left[ 1 + 0.0003 (t - 20) \right]$$

Where, $P_t =$ polarization at temperature read.
(Ref: A.O.A.C 17th edn, 2000 Official method 925.46 Sucrose in Sugars and syrups Polarimetric method)

7.4.1B Polarimetric method (Before and After inversion with HCl)

(a) Direct reading - Pipette 50 ml Pb free filterate into 100 ml volumetric flask, add 2.315 gm NaCl and 25 ml water. Dilute to volume with water at 20°C and polarize in 200 mm tube at 20°C. Multiply reading by 2 to obtain direct reading.

(b) Invert Reading - Pipette 50 ml aliquot Pb free filterate into 100 ml volumetric flask and add 20 ml water. Add little by little while rotating flask 10 ml HCl (sp. gr 1.109). Heat water bath and adjust heater to keep bath at 60°C. Place flask in water bath, agitate continuously for 3 minutes and leave flask in bath exactly 7 minutes longer. Place flask at once in water at 20°C.

When contents cool to 35°C dilute almost to mark,. Leave flask in bath at 20°C at least 30 minutes longer and finally dilute to mark.

Mix well and polarize in 200 mm tube provided with lateral branch and water jacket keeping temperature at 20°C. Multiply by 2 obtain invert reading.

Calculate Sugar % as follows

\[ \frac{100 (P - I)}{DRAFT} \]
S = \frac{132.66 - 0.0794(13-m) - 0.53(t - 20)}{1}

Where,

P = direct reading, normal solution
I = Invert reading, normal solution
t = Temperature at which readings are made
m = gm of total solids from original sample in 100 ml inverted solution

(Ref:- A.O.A.C 17th edn, 2000 Official Method 925.48 Sucrose in sugars and Syrups)

7.5 Determination of Sulphur Dioxide:

Sulphur dioxide is determined by the modified Monier-William’s Method

The apparatus as assembled is shown below:
7.5.1 Reagents

(a) Sodium Carbonate Solution - 10 percent (m/v). aqueous

(b) Bromophenol Blue Indicator Solution – Dissolve 0.1 g of bromophenol blue in 3.0 ml of 0.05 N sodium hydroxide solution and 5 ml of ethyl alcohol (90 percent by volume) by gently warming. Make up the volume of the solution with ethyl alcohol (20 percent v/v) to 250 ml in a volumetric flask.

(c) Hydrogen peroxide solution- Dilute a 30 percent (m/v) hydrogen peroxide solution with about twice its volume of water and neutralize the free sulphuric acid that may be present in the hydrogen peroxide solution with barium hydroxide solution, using bromophenol blue indicator solution. Allow the precipitate of barium sulphate to settle, and filter. Determine the concentration of hydrogen peroxide in the filtrate by titrating with standard potassium permanganate solution. Dilute the filtrate with cold water so as to obtain a 3 percent (m/v)
solution of hydrogen peroxide.

(d) Concentrated Hydrochloric acid- sp.gr. 1.16

(e) Carbon dioxide gas- from a cylinder.

(f) Standard sodium hydroxide solution- approximately 0.1 N, standardized at the time of the experiment using bromophenol blue indicator solution.

7.5.2 Procedure

Assemble the apparatus as shown above. Introduce into the flask C, 300 ml of water and 20 ml of concentrated hydrochloric acid through the dropping funnel E. Run a steady current of cold water through the condenser F. Boil the mixture contained in the flask G for a short time to expel the air from the system in current of carbon dioxide gas previously passed through the wash bottle A. Weigh accurately about 100 g of the material and mix with the minimum quantity of water so as to make the diluted material easily flow down to the dropping funnel. Introduce the diluted material into the flask C through the dropping funnel E. Wash the dropping funnel with a small quantity of water and run the washing into the flask C. Again boil the mixture contained in the flask C in a slow current of carbon dioxide gas (passed previously through the wash bottle A) for one hour. Just before the end of the distillation, stop the flow of water in the condenser. (This causes the condenser to become hot and drives over residual traces of sulphur dioxide retained in the condenser.) When
the delivery tube H, just above the Erlenmeyer flask j, becomes hot to touch, remove the stopper J immediately.

Wash the delivery tube H and the contents of the Peligot tube L with water into Erlenmeyer flask. Cool the contents of the Erlenmeyer flask to room temperature, add a few drops of bromophenol blue indicator and titrate with standard sodium hydroxide solution. (Bromophenol blue is unaffected by carbon dioxide and gives a distinct change of color in cold hydrogen peroxide solution). Carry out a blank determination using 20 ml of conc hydrochloric acid diluted with 300 ml of water.

7.5.3 Calculation

\[
\text{Sulphur dioxide, } \text{mg/kg} = \frac{0.032000 \times (V-v) \times 1000 \times 1000 \times N}{W}
\]

Where,

- \( V \) = volume in ml of standard sodium hydroxide solution required for the test with the material
- \( v \) = volume in ml of standard sodium hydroxide solution required for the blank determination;
- \( N \) = normality of standard sodium hydroxide solution; and
- \( W \) = weight in g of the material taken for the test

(Ref: - I.S.I Handbook of Food Analysis (Part II) -1984 page 8)
7.6 Determination of specific conductivity

Specific conductivity is determined by measuring the conductivity of a solution kept in a cell and multiplying it with the cell constant.

7.6.1 Apparatus
Conductivity bridge with magic eye indication for measuring the conductivity directly.

7.6.2 Reagents

(1) Chromic acid solution
(2) Conductivity water – of specific conductivity not more than $3.0 \times 10^6$
(3) Potassium Chloride solution – 0.02N, accurately prepared
(4) Chloroplatinic acid solution – Dissolve 3 gm of Chloroplatinic acid and 0.02 – 0.03 of lead acetate in 100 ml water.

7.6.3 Procedure

7.6.3.1 Platinizing the electrodes of the conductivity cell

Wash the electrodes of conductivity cell first with warm chromic acid solution and then several times with distilled water. Support the electrodes in an inclined position in the chloroplatinic acid solution and connect by way of a commutator to a 4 volt lead accumulator and rheostat. Adjust the current so that the evolution of
gas is slow. Reverse the current every 30 seconds. Thus continue to pass the current for 15 minutes. Disconnect the conductivity cell wash it with distilled water thoroughly and fill with dil solution of sulphuric acid. Electrolyze the solution of sulphuric acid for ½ hour to remove occluded gases, reversing the current every 30 seconds. Wash the cell wall with conductivity water.

Note: - The cleanliness of the cell is confirmed by determining the conductivity of the conductivity water, washing out the cell and making a second determination of the conductivity water. Two successive determinations shall give concordant measurement of the conductivity if the cell is clean.

7.6.3.2 Determination of the cell constant

Wash the conductivity cell with conductivity water. Then rinse with the standard Potassium chloride solution. Transfer sufficient quantity of Pot chloride solution so that the electrodes are well within the solution, taking care that no air bubbles are enclosed between the electrodes. Place the conductivity cell in a thermostat. Maintain the temperature of the thermostat at 35 ± 1°C. Ensure that all the connections made are with fairly thic copper wire and tight. When Pot chloride solution has attained the temperature of the bath, measure the observed conductivity of the solution. Report twice the measurement by replacing a fresh Pot Chloride solution.

Calculate the cell constant as follows
Where,

\[ K = \frac{C}{O_1} \]

\[ K = \text{cell constant} \]
\[ C = \text{specific conductivity of Pot chloride solution at } 35^0 \text{C, that is } 3.3.1 \times 10^3 \text{ Mhos/cm} \]
\[ O_1 = \text{Observed conductivity of Pot Chloride solution} \]

### 7.6.3.3 Determination of specific conductivity

Dissolve 10 gm of the material (accurately weighed) in 200 ml of conductivity water. Wash the conductivity cell thoroughly with distilled water and then with conductivity water and later rinse with test solution twice. Determine the observed conductivity at 35\(^0\) C. Repeat with a fresh sample of test solution and take the average value.

Determine the conductivity of conductivity water at 35 \(^0\)C in the same manner.

Calculate the specific conductivity \(x 10^6\) of 5 \(^0\text{w/ v}\) aqueous solution at 35 \(^0\text{C}\) as follows:

\[ S = [O_2 - (0.9 \times O_3)] \times K \times 10^6 \]

Where,
S = specific conductivity of test solution $\times 10^6$

$O_2$ = observed conductivity of test solution

$O_3$ = observed conductivity of conductivity water

$K$ = cell constant

(Ref: I.S.I. Handbook of food analysis (Part II) – 1984, page 7)
7.7 Determination of Calcium oxide

7.7.1 Apparatus

(1) Calibrated Brix spindle
(2) Brix Cylinder
(3) Conical flasks - 250 ml capacity
(4) Beakers – 100 and 200 ml capacity
(5) Funnels
(6) Pipettes- calibrated at 10 ml

7.7.2 Reagents

(1) EDTA solution – Weigh accurately 6.6473 gm EDTA into a beaker , dissolve in distilled water and make upto 1000 ml to obtain exactly M/56 solution
(2) Ammonia Liquor
(3) Lead Subacetate
(4) Potassium Ferrocyanide powder
(5) Potassium iodide
Eriochrome Black – T – weigh 0.1 eriochrome black T in a 100 ml volumetric flask and dissolve the same in rectified spirit or absolute alcohol. Make upto volume and use as indicator.

7.7.3 Procedure

Make a 15⁰ Brix solution of the sample. Transfer about 150 ml of the
solution to a conical flask. Clarify the solution with Lead subacetate. Transfer about 60 ml of the clarified solution to a dry conical flask or flask previously rinsed with the clarified solution. Add Potassium Ferrocyanide powder little by little till no further precipitate forms. Shake thoroughly and filter. Test the filterate with Pot. Iodide. Collect the lead free filterate in a conical flask Pipette out 10 ml of lead free filterate in a clean conical flask previously rinsed with distilled water and dried. Add 5 – 6 drops of liquor ammonia and 4-5 drops of indicator when a pink colour appears. Titrate against EDTA solution shaking the flask after each addition of EDTA solution. The end point is indicated by a sharp change of colour from red to blue. Note down the volume of the titrant.

7.7.4 Calculation

Calcium oxide mg / 100 gm = V X 100 mg per litre of diluted solution

(Ref :- I.S.I. Handbook of Food Analysis (Part II) – 1984 page 9)
8.0 BURA:

8.1 Determination of Ash insoluble in dilute HCl:

Weigh 5 gm of sample in a silica dish. Char the sample and ash in a furnace at 550°C. Add 25 ml of 1:2.5 Hydrochloric acid to the ash. Cover the dish with a watch glass and boil for 5 minutes. Cool, filter through an ashless filter paper (Whatman No. 42 or 41) and wash the residue well with hot water until acid-free. Return the filter paper to the silica dish and incinerate at 525°C. Cool, weigh and calculate as % acid insoluble ash.

(Ref :- I.S.I. Handbook of Food Analysis (Part IX) 1984) page 52)

8.2 Determination of total sugars expressed as sucrose:

Follow the procedure given under cane sugar for sucrose. Invert the solution with HCl. Conduct the titration and calculate as given under.

Total sugars % expressed as sucrose = Total reducing sugars % x 0.95
9.0 GUR OR JAGGERY:

9.1 Determination of Moisture:

Determine the moisture using a vacuum oven at 70°C.

9.2 Determination of extraneous matter insoluble in water:

Take 10 gm of sample, add 200 ml hot distilled H2O and bring to boiling. Allow to cool to room temperature. Filter through a tared gooch crucible having a bed of asbestos or sintered glass filter. Wash the residue with hot water till the filtrate is sugar-free (perform Molisch test). Dry the gooch crucible or sintered glass filter at 135 ± 2°C and weigh. Express as % insoluble matter.

(Ref:- I.S.I Handbook of Food Analysis (Part II) – 1984 page10)

9.3 Determination of total Ash:

Follow the procedure as given under analysis of Honey. (Sec. 3.5)

9.4 Determination of ash-insoluble in dilute HCl:

Follow the procedure as given under Analysis of Bura. (Sec 5.1).
10.0 Determination of Sulphated Ash in Dextrose:

Weigh accurately about 10 gm 5 g sample into a silica dish, add 0.5 ml of conc sulphuric acid or 5 ml of 10% (by weight) H\textsubscript{2}SO\textsubscript{4}. Heat on hot plate or burner to carbonize the sample (perform in a hood with exhaust facility). Then ash in the furnace at 550 °C. Cool, add again 2 ml of 10% H\textsubscript{2}SO\textsubscript{4}, evaporate on steam bath and dry on hot plate and again ash at 550 °C to constant weight. Express as % sulphated ash.

(Ref :- I.S.I. Handbook of Food Analysis (Part II) – 1984 page 18)

11.0 Determination of Starch in Icing Sugar:

If the iodine test for starch is positive, proceed as follows. Weigh suitable quantity of sample and dissolve with 100 ml of hot water. Cool, add equal volume of alcohol, stir and let stand for 2 hours. Filter the solution through Whatman No. 1 filter paper. Wash the precipitate with 50% alcohol until the washings does not answer the Molisch test for sugars.

Transfer the precipitate with 200 ml hot water into a flask. Add 20 ml HCl and connect the reflux condenser and heat in boiling water bath for 2.5 hours. Cool, neutralise with NaOH and dilute to 500 ml. Determine % of glucose by Lane Eynon’s method.

Calculation:

\[
\text{Starch} \% = \text{Glucose} \% \times 0.90
\]
12.0 Determination of Total solids in Liquid Glucose

12.1 Apparatus

(1) Moisture dish – approx 75 mm dia and 25 mm high
(2) Dessicator
(3) Vaccum oven
(4) Nickel scoop
(5) Glass stirring rod
(6) Diatomaceous earth – Neutral to litmus when moistened with water. If a commercial grade is used, wash it by percolation with water slightly acidulated with hydrochloric acid until the effluent is acidic to litmus. Then rewash with water (not acidulated) until the effluent is neutral to litmus. Finally dry in an oven at about 105°C,

12.2 Procedure

Place about 10 gm of diatomaceous earth and the glass rod in the weighing dish and dry them in an air oven at about 105°C. Cool in a dessicator and weigh. Repeat drying and cooling in dessicator and weighing till constant weight is achieved. Weigh accurately about 5 gm of the material in the nickel scoop, mix it with 5 ml of water and run on the diatomaceous earth using glass stirring rod. Wash the scoop...
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three times with 2 ml water and transfer the washings to diatomaceous earth working the contents of the dish into a thick paste with the stirring rod. Leave the stirring rod in the dish. Place the moisture dish in the vacuum oven and dry contents of dish at 100 °C. Cool the dish in a dessicator and weigh. Repeat drying in the vacuum oven, cooling and weighing till constant weight is obtained.

12.3 Calculation

Total solids % by wt = 100 - \[ \frac{100(M + A - B)}{M} \]

Where

\( M \) = weight in gm of the material taken for test

\( A \) = weight in gm of moisture dish with glass rod and diatomaceous earth.

\( B \) = weight in gm of moisture dish with glass rod, diatomaceous earth and dehydrated material.

PART B

CONFECTIONERY PRODUCTS

SUGAR BOILED CONFECTIONERY & LOZENGES

Sugar Boiled Confectionery - As per FSSA, Sugar boiled confectionery is a processed composite food article made from sugar with or without doctoring agents such as cream of tartar by process of boiling whether panned or not. It may be center filling or otherwise which may be in the form of liquid, semi-solid or solids with or without coating of sugar or chocolate or both.

Lozenges - As per FSSA, it is a confection made mainly out of pulverised sugar, or icing sugar with binding materials such as edible gums, edible gelatine, liquid glucose or dextrin and generally made from cold mixing which does not require primary boiling or cooking of the ingredients.

A.1 Preparation of sample

If composition of entire product is desired, grind and mix thoroughly. If product is composed of layers or of distinctly different portions and it is desired to examine these individually, separate with knife or other mechanical means as completely as possible, and grind and mix each test portion thoroughly.
A.2 Determination of Moisture - Vacuum Oven method

**Apparatus**

1. Aluminum dish – 75mm diameter and about 25mm height with close fitting cover
2. Dessicator
3. Vacuum oven

**Procedure**

1. Accurately weigh about 5 g of sample in a dish previously dried and weighed. Distribute the material as evenly as practicable over the bottom of the dish by gentle sidewise movements.
2. Place dish in vacuum oven, remove cover of dish and dry the material for two hours at 65 ± 1º C at a pressure not exceeding 50mm of Hg. During heating admit slow current of air into oven.
3. Cover dish, transfer to dessicator and weigh soon after room temperature is attained.
4. Redry for one hour and repeat the process till the difference between the two successive weighing is less than 2 mg. Report percent loss in weight as moisture %.
Calculations

Moisture content, % by mass  = \frac{(W3 - W2)}{W1} \times 100

Where,

W1 = Weight of prepared sample taken for test in g
W2 = Weight of empty moisture dish in g
W3 = Weight of (dish + dried sample) in g

[Ref: - IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]
A.3 Determination of Sulphated Ash

Reagents

(1) Sulphuric Acid - 10 percent (m/m)

Procedure

1. Accurately weigh about 5 g of the prepared sample into a 9-cm diameter platinum basin.
2. Add 5 ml of sulphuric acid to the material in the dish. Gently heat the dish on a hot plate until the material is well carbonized and then increase the heat until the evolution of sulphuric acid fumes ceases.
3. Ash the carbonized matter in a muffle furnace at 550 ± 25 °C.
4. Cool the ash and moisten it with 2-3 ml of sulphuric acid. Heat strongly on a hot plate until sulphuric acid fumes ceases to be evolved and finally ash in the muffle furnace at 550 ± 25 °C for two hours.
5. Cool in a desiccator and weigh.
6. Heat again in a muffle furnace for 30 minutes at 550 ± 25 °C. Cool in a desiccator and weigh.
7. Repeat the process of heating in the muffle furnace for 30 minutes, cooling and weighing till the difference between two successive weighing is less than 1 mg. Record the lowest mass.
Calculation

Sulphated ash, % by mass = \( \frac{M1 \times 100}{M2} \times 100 \)

Where,

M1 = mass in g of the sulphated ash

M2 = mass in g of the prepared sample taken for the test.

[Ref: - IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]

A.4 Determination of Sulphated ash on salt free basis

Procedure:

1. Determine Sodium Chloride content in the sample separately (as mentioned in A.4.1)
2. Convert the quantity of Sodium Chloride obtained to Sodium Sulphate \((2\text{NaCl} = \text{Na}_2\text{SO}_4)\) i.e. 117: 142
3. Ratio of \(\text{Na}_2\text{SO}_4 / \text{NaCl} = 142 / 117 = 1.21\)
4. Multiply the Sod. Chloride content obtained with 1.21.
5. Deduct the value thus obtained from the sulphated ash to obtain sulphated ash on salt free basis.
A.4.1 Titration method

**Apparatus**

1. Pipette — Graduated, 1 ml capacity
2. Conical flask — Glass-stoppered, 250 ml capacity

**Reagents**

1. Standard AgNO$_3$ Solution — 0.1 N
2. Potassium chromate indicator solution — 5% solution in water

**Procedure**
1. Weigh appropriate sample quantity in a conical flask.
2. Add 100 ml of boiling water.
3. Let it shake for 30 minutes to 1 hour, ensuring complete dissolution of salt present in sample.
4. Filter the contents through a filter paper.
5. Titrate the filtrate thus obtained against standard 0.1 N AgNO$_3$ using Potassium chromate as indicator, till brown colored end point persists for 30 seconds.
6. If the colour of the sample solution interferes in titration, an alternative potentiometric method can be used.
Calculation

\[ 54.5 \times N \times (A-B) \]

Sodium chloride, % by mass \[=\] \[\frac{\text{---}}{\text{---}}\]

Where,

N = normality of AgNO\(_3\) solution,

A = volume, in ml, of AgNO\(_3\) solution in the blank titration

B = volume, in ml, of AgNO\(_3\) solution in the sample titration, and

W = weight, in g, of the sample.

[Ref: - A.O.A.C 18th edn, 2005 Official Method 960.29 Salt in Butter
& A.O.A.C 18th edn, 2005 Official Method 971.27 Sodium chloride in canned vegetables]

A.5 Determination of Ash Insoluble in dilute HCl

Reagents

(1) Dilute hydrochloric acid - Approx 5 N (445ml in 1litre)
Procedure

1. Weigh accurately about 5 g of the prepared sample in a tared, clean and dry platinum basin of 100ml capacity.
2. Carbonize the material in the dish with the flame of a burner.
3. Complete the ignition by keeping in a muffle furnace at 550 ± 25ºC until gray ash results.
4. Cool in a desiccator.
5. To the ash, add 25ml of the dilute hydrochloric acid, cover with a watch glass and heat on a small flame of a burner to near boiling.
6. Allow it to cool and filter the contents of dish through Whatman filter paper No. 42 or its equivalent. Wash the filter paper with hot water until the washings are free from chlorides (To check this, add few drops of 2M Nitric acid and 0.1 M Silver nitrate solution to the filtrate obtained. No precipitate or milky turbidity should occur in the solution, if it is chloride-free.)
7. Return the filter paper and the residue to the dish. Keep it in an air oven maintained at 105 ±2ºC for about three hours. Ignite in the muffle furnace at 550 ±25ºC for one hour.
8. Cool the dish in a desiccator and weigh.
9. Heat again for 30 minutes in the muffle furnace, cool and weigh.
10. Repeat this process of heating for 30 minutes, cooling and weighing till the difference between two successive weighing is less than one milligram. Note the lowest mass.
**Calculation**

Acid insoluble ash, percent by mass = \( \frac{100 \times M_1}{M_2} \)

Where,

- \( M_1 \) = mass in g of the acid insoluble ash
- \( M_2 \) = mass in g of the prepared sample taken for the test

[Ref: - IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]

**A.6 Test for the presence of added synthetic colour**

**Paper Chromatographic Separation of Synthetic Food Colours**

The general scheme for identifying synthetic food colours present in foods normally involve preliminary treatment of the food, extraction of the colour from the prepared solution of the food, separation of colours in case of mixtures and identification of the separated colours.

**Apparatus**

1. Pipette
2. Beaker
3. Flask
**Reagents**

(1) White knitting wool: Extract pure white wool in a soxhlet extractor with petroleum ether for 2-3 hrs to remove fat. Boil in very dilute solution of sodium hydroxide and then in water to free it from alkali.

(2) Paper: Whatman No. 1 chromatographic paper.

(3) Solvents

- 1 ml (0.88 sp. gr) ammonia + 99 ml water
- 2.5% aqueous sodium chloride
- 2% sodium chloride in 50% ethanol
- Acetic acid solution in water (1:3)
- Iso-butanol-ethanol-water (1: 2 : 1, v/v)
- n-butanol-water-glacial acetic acid (20 : 12 : 5, v/v)
- Iso-butanol-ethanol-water (3 : 2 : 2, v/v). to 99 ml of this add 1ml of
  - (0.88 sp gr.) ammonia
  - 80 gm phenol in 20 gm water.

**Procedure**

1. *Preliminary treatment of food*: Assuming that an acidic colour is present, the preliminary treatment involves removing interfering substances and obtaining the dye in acid solution prior to boiling with wool.
Non-alcoholic beverages e.g. soft drinks: As most foods in this group are acidic they can be usually treated directly with wool, otherwise, slightly acidify the food with acetic acid.

Alcoholic liquids (e.g. Wine): Boil to remove alcohol and acidify if necessary as in (a).

Starch based foods (e.g. cakes, custard powder etc): Grind 10 g of sample thoroughly with 50 ml of 2% ammonia in 70% alcohol, and allow it to stand for an hour and centrifuge. Pour the separated liquid into a dish and evaporate on water bath. Take up the residue in 30 ml dilute acetic acid.

Candied fruits: Treat as in (c).

Products with high fat content (e.g. Sausages, meat, fish paste): De-fat the sample with light petroleum and extract the colour with hot water (acidify etc. as usual). Note that oil soluble colours tend to give coloured solutions in organic solvents.

If the extraction is difficult treat with warm 50-90% acetone or alcohol (which precipitates starch) containing 2% ammonia. The organic solvent should be removed before acidifying as in (c).

2. Extraction of the colour from the food: Introduce about 20 cm length of woollen thread into a beaker containing about 35 ml of the prepared acidified solution of the sample and boil for a few minutes till the woollen thread is dyed. Take out the woollen thread and wash it with tap water. Transfer the washed woolen thread to a small beaker containing dilute ammonia and heat again. If the colour is stripped by the alkali, the presence of an acid coal-
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Remove the woollen thread. Make the liquid slightly acidic and boil with a fresh piece of woollen thread. Continue boiling until the colour is taken by the woollen thread. Extract the dye from the woollen thread again with a small volume of dilute ammonia, filter through a small plug of cotton and concentrate the filtrate over a hot water bath. This double stripping technique usually gives a pure colour extract. Natural colours may also dye the wool during the first treatment, but the colour is not usually removed by ammonia. Basic dyes can be extracted by making the food alkaline with ammonia, boiling with wool and then stripping with dilute acetic-acid. At present, all the permitted water soluble coal-tar dyes are acidic, hence an indication of the presence of a basic dye suggests that an unpermitted colour is present.

3. Identification of the separated food colours by paper chromatography:

Draw a pencil-line parallel to the bottom edge of the paper (Whatman No.1) at about 2 cm distance. Spot the concentrated solution of the unknown dye on the line together with a series of spots (about 2 cm apart) of aqueous solutions of standard permitted dyes of similar colour and dry. Run the chromatogram, by ascending technique, using a selected solvent. Solvent No.5 is often helpful for general purposes. Identify the colour in the sample by matching its spot with the spot of the standard colour and confirm by co-spotting.
4. **Determination of Synthetic food colours in food products**:

(1) For samples containing single colour

(a) Preparation of standard curve: Stock solution: Weigh 0.1 g m of each reference colour and dissolve in 0.1N HCl in separate 100 ml volumetric flasks and make up the volume with 0.1N HCl in each case.

Working standard: Pipette 0.25, 0.5, 0.75, 1.0, 1.25 and 1.5 ml of stock solution of each of the reference colours into series of clean and dry 100 ml volumetric flasks and dilute to volume with 0.1N HCl. Determine the optical densities of each of the reference colours at the respective wave length of maximum absorption (refer table) Obtain the standard curve for each colour by plotting optical density against concentration.

(b) Determination in sample by column chromatography: Transfer a known weight of the sample (approximately 5 - 10 gm) into a glass stoppered separatory funnel. Extract the colour with 70% acetone. Shake acetone extract with petroleum ether (40-60ºC) in order to remove carotenoids and other natural pigments, if any. Continue extraction with petroleum ether until petroleum ether extract is colourless. Pass the acetone extract containing only coal-tar food colours through a column (2.1 · 45 cm) containing aluminium oxide acidified with 1% HCl. Elute the adsorbed colour with 1% ammonia. Evaporate the eluate to dryness on a hot waterbath, dissolve the residue with 0.1 N
HCl, transfer quantitatively to a 100 ml volumetric flask and make up the volume with 0/1N HCl. Determine the optical density of the dye solution at the wavelength of maximum absorption. Calculate the dye concentration from the standard curve.

**TABLE SHOWING ABSORPTION MAXIMA OF PERMITTED FOOD COLOURS**

<table>
<thead>
<tr>
<th>Name of the Colour</th>
<th>Absorption maxima (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carmosine</td>
<td>516</td>
</tr>
<tr>
<td>Ponceau 4 R</td>
<td>507</td>
</tr>
<tr>
<td>Erythrosine</td>
<td>527</td>
</tr>
<tr>
<td>Green FCF</td>
<td>624</td>
</tr>
<tr>
<td>Indigo Carmine</td>
<td>609</td>
</tr>
<tr>
<td>Brilliant Blue FCF</td>
<td>630</td>
</tr>
<tr>
<td>Tartrazine</td>
<td>427</td>
</tr>
<tr>
<td>Sunset yellow FCF</td>
<td>482</td>
</tr>
</tbody>
</table>
(2) For samples containing mixture of colours

(a) Paper Chromatography:

Extract the colours present in the samples and isolate as described under column chromatography. Make up the purified dye solution to a known volume with water (5 ml).

Spot an aliquot (approximately 0.5 to 1.0 ml) of the purified dye on Whatman No.1 filter paper as a band and develop the chromatogram using butanol : acetic acid : water (20:5:12) solvent system. After drying, cut out the coloured spots on the chromatogram and elute with 0.1 N HCl. Prepare a blank by cutting an equivalent strip from plain portions of the chromatogram and elute with 0.1 N HCl. Make up the eluate to a known volume (100 ml) with 0.1 N HCl and determine the dye content as described under column chromatography.

(b) Thin Layer Chromatography:

Extract the colours present in the sample as described under column chromatography. Concentrate the eluate and make up to known volume with water (5 ml).

Preparation of TLC Plate:

To 50 g of silica gel without binder add 50 ml of starch solution (0.6 gm of soluble starch dispersed in 100 ml or glass distilled water heated to boiling to gelatinise starch) and 50 ml of 1.25% solution of
disodium salt of ethylene diamine tetra acetic acid, Mix the slurry well. Spread slurry using applicator on glass plates (20 · 20 cm) to thickness of 0.5 mm. Allow the plate to air dry and then dry at 120°C for two hours. Spot an aliquot of the purified dye on TLC plate and develop the chromatogram using isoamyl : glacial acetic acid : water (40 : 20 : 20) solvent system. Remove the plate and dry. Scrape out the colour spots on the plate and transfer to test tubes. Elute the colour using 0.1N HCl. Prepare a blank by scraping from the plain portions of the plate. Make up the eluates to a known volume (100 ml) with 0.1N HCl. Determine the dye content as described under column chromatography.

(Ref:- Manual Methods of Analysis for Adulterants and Contaminants in Food, I.C.M.R 1990 Page 56 )

A.7 Determination of Total Protein  (in Milk Toffee)

Apparatus

(1) A recommended distillation assembly is shown below - The assembly consists of a round bottom flask A of 1000 ml capacity fitted with a rubber stopper through which passes one end of the connecting bulb tube B. the other end of the bulb B is connected to the condenser C which is attached, by means of a rubber tube, to a dip tube D which dips into a known quantity of standard sulphuric acid contained in a beaker E of 250 ml capacity.

(2) Kjeldahl flask – 500 ml capacity
Reagents

(1) Anhydrous Sodium sulphate

(2) Copper Sulphate

(3) Concentrated Sulphuric Acid- sp gr 1.84

(4) Sodium Hydroxide Solution- Dissolve about 225 g of sodium hydroxide in 500 ml of water

(5) Standard Sulphuric Acid - 0.1 N

(6) Methyl red indicator solution- Dissolve one g of methyl red in 200 ml of Rectified spirit (95 percent v/v)

(7) Standard sodium hydroxide solution -0.1N
**Procedure**

1. Transfer carefully about one to two grams of the sample accurately weighed, to the Kjeldhal flask, taking precaution to see that particles of the material do not stick to the neck of the flask.

2. Add about 10 g of anhydrous sodium sulphate, 0.2 to 0.3 g of copper sulphate and 20 ml of concentrated sulphuric acid.

3. Place the flask in an inclined position. Heat below the boiling point of the acid until frothing ceases. Increase heat until the acid boils vigorously and digests for 30 minutes after the mixture becomes clear and pale green in colour. Cool the flask.

4. Transfer quantitatively to the round-bottomed flask with water the total quantity of water used being about 200 ml. Add a few pieces of pumice stones to avoid bumping. Add about 50 ml of Sodium hydroxide solution (which is sufficient to make the solution alkaline) carefully through the side of the flask so that it does not mix with the acid solution but forms a separate layer below the acid layer.

5. Assemble the apparatus as shown above taking care that the dip tube extends below the surface of the standard sulphuric acid solution contained in the beaker.

6. Mix the contents of the flask by shaking and distill until all the ammonia has passed over into the standard sulphuric acid.
7. Shut off the burner and immediately detach the flask from the condenser. Rinse the condenser thoroughly with water into the beaker. Wash the dip tube carefully so that all traces of the condensate are transferred to the beaker.

8. When all the washings have been drained into the beaker, add two or three drops of methyl red indicator solution and titrate with the standard sodium hydroxide solution. Carry out a blank determination using all reagents in the same quantities but without the sample to be tested.

**Calculation**

\[
\text{Total Protein (N x 6.25), \% by mass} = \frac{8.75 \times (B-A) \times N}{M}
\]

Where

- \( B \) = volume in ml of the standard sodium hydroxide solution used to neutralize the acid in the blank determination
- \( A \) = volume in ml of the standard sodium hydroxide solution used to neutralize the excess of the acid in the test with the material
- \( N \) = Normality of the standard sodium hydroxide solution
- \( M \) = mass in g of the material taken for the test

[Ref: - IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]
A.8 Determination of Fat

(in Milk Toffee and Butter Toffee)

A.8.1 Simple Extraction (in Butter Toffee)

**Apparatus**

(1) Mojonnier fat extraction tube or any other similar apparatus
(2) Flasks

**Reagents**

(1) Diethyl ether
(2) Petroleum ether

**Procedure**

1. Dissolve 10 gm sample in 10 ml warm water, and introduce into Mojonnier fat extraction tube or similar apparatus.

2. Add 25 ml peroxide free ethyl ether.

3. Cork the tube and shake vigorously for 1 minute.

4. Add 25 ml of Petroleum ether and shake again for 30 seconds.

5. Let stand for 30 minutes or until separation is complete.
6. Draw off the ether layer containing fat in a previously dried and weighed flask.

7. Repeat the extraction twice.

8. Pool the ether extract, recover excess solvent and dry the fat for 1 hour at 100°C. Cool and weigh.

9. Fat must be dried by keeping the flasks for 30 minutes and weighed, till constant mass is achieved.

**Calculation**

\[
\text{Fat, \% on dry basis} = \frac{\text{M1} \times 100 \times 100}{\text{M2} \times (100 - \text{M})}
\]

Where,

- \(\text{M1}\) = Weight in g of the fat
- \(\text{M2}\) = Weight in g of sample taken
- \(\text{M}\) = Moisture \% in the sample

[Ref: IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]
A.8.2 Extraction of Fat by Rose Gottleib Method (in Milk Toffee)

**Apparatus**

(1) Mojonnier fat extraction tube or similar apparatus.

**Reagents**

(1) Concentrated Ammonia - sp.gr. 0.88  
(2) Ethyl Alcohol 95 to 96 percent (v/v)  
(3) Diethyl ether - sp.gr. 0.720 (peroxide free)  
(4) Petroleum ether – boiling range 40 to 60 C, recently distilled

**Procedure**

1. Introduce 4 g sample into a Mojonnier extraction tube or similar apparatus. Dilute to 10 ml with water.  
2. Add 1.2 ml Ammonia solution and mix thoroughly. Add 10 ml alcohol and mix.  
3. Then add 25 ml ether and shake vigorously for about 30 seconds and finally add 25 ml petroleum ether and shake again for about 30 seconds.  
4. Let stand for 20 minutes or until separation of liquids is complete.  
5. Draw off as much as possible of ether fat solution (usually 0.5 to 0.8 ml is left) into a weighed flask through a small rapid filter.  
6. Again extract liquid remaining in tube, this time with 15 ml each of ether and petroleum ether; shake vigorously for about 30 seconds with each solvent and let settle. Proceed as above, washing
mouth of tube and filter with a few milliliters of mixture of equal parts of two solvents.

7. For accuracy, repeat extraction. If previously solvent-fat solution has been drawn off closely, third extraction usually yields approximately up to 1 mg fat or about 0.02 percent with 4 gm sample.

8. Slowly evaporate solvent on steam bath and then dry fat in an oven maintained at 100°C to constant mass.

9. Test purity of fat by dissolving in a little petroleum ether. If residue remains, wash out fat completely with petroleum ether, dry the residue, weigh and calculate the mass of the fat.

**Calculation**

$$\text{Fat, \% by mass} = \frac{M_1}{M_2} \times 100$$

Where

- $M_1$ = Weight in g of the fat
- $M_2$ = Weight in g of sample taken

[Ref: IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]
A.9 Determination of Reducing Sugars:

Reagents:

(1) Fehling A: Dissolve 69.28-g copper sulphate (CuSO\textsubscript{4}·5H\textsubscript{2}O) in distilled water. Dilute to 1000 ml. Filter and store in amber coloured bottle.

(2) Fehling B: Dissolve 346 g Rochelle salt (potassium sodium tartrate) (K Na C\textsubscript{4}H\textsubscript{4}O\textsubscript{6}· 4H\textsubscript{2}O) and 100 g NaOH in distilled water. Dilute to 1000 ml. Filter and store in amber coloured bottle.

(3) Carrez 1 – Add 21.9 g Zinc acetate and 3 ml acetic acid in a 100 ml volumetric flask. Make up the volume with water.

(4) Carrez 2 – 10.6 % aqueous solution of Potassium ferrocyanide.

(5) Methylene Blue Indicator: Prepare 1% of methylene blue solution in distilled water.

Procedure

1. Weigh accurately about 5 g sample, transfer to a 200 ml volumetric flask dissolve in warm water, dilute to about 150 ml.

2. In case solution is not clear, add 5 ml of Carrez 1 solution followed by 5 ml of Carrez 2 solution.
3. Make up to 200 ml. Filter through a dry filter paper.

4. Titrate the solution obtained as such to determine % Reducing sugars.

**Preliminary Titration:**

Pipet 5 ml each of Fehling A and B into 250 ml conical flask. Mix and add about 10 ml water and a few boiling chips or glass beads. Dispense solution. Heat the flask to boiling. Add 3 drops of methylene blue indicator. Continue the addition of solution dropwise until the blue colour disappears to a brick-red end point. (The concentration of the sample solution should be such that the titre value is between 15 and 50 ml). Note down the titre value.

**Final Titration:**

Pipet 5 ml each of Fehling A and B. Add sample solution about 2 ml less than titre value of the preliminary titration. Heat the flask to boiling with in 3 minutes and complete the titration. Perform the titration duplicate and take the average. Calculate the reducing sugar % as shown below.

\[
\text{Reducing Sugars} \% = \frac{\text{Dilution} \times \text{Factor of Fehling (in gm)}}{\text{Weight of sample} \times \text{Titre value}} \times 100
\]
A.10 Determination of Sucrose

Take an aliquot of the filtrate obtained in Reducing sugar method and invert it with Hydrochloric acid in a water bath at 60º C by keeping for 10 minutes. Cool immediately and neutralize with sodium hydroxide and finally with sodium carbonate. Make up to volume and determine reducing sugar by Lane and Eynon method.

\[
\text{Reducing Sugars \%} = \frac{\text{Dilution x Factor of Fehling (in gm)}}{\text{Weight of sample x Titre value}} \times 100
\]

**Determination of Factor (for Invert Sugar) of Fehling Solution:**

Accurately weigh around 4.75 g of analar grade sucrose. Transfer to 500 ml volume flask with 50 ml distilled water. Add 5 ml conc. HCl and allow to stand for 24 hours. Neutralize with NaOH solution and make up to volume. Mix well and transfer 50 ml to a 100 ml volumetric flask and makeup to volume. Transfer to a burette having an offset tip.

Perform the titration of Fehling solution following the similar procedure as above:

\[
\text{Fehling Factor (as Invert Sugar)} = \frac{\text{Titre x Weight of sucrose in gm}}{500}
\]
**Calculation:**

Sucrose %

\[ \text{Sucrose }\% = \left( \frac{\text{Total reducing sugars}}{\text{invert sugar }\% - \text{reducing sugars }\%} \right) \times 0.95 \]

[Ref: - IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]

**A.11 Determination of Sulphur Dioxide**

Sulphur dioxide is determined by the modified Monier-William’s Method —The apparatus as assembled is shown below:

![Assembly for determination of Sulphur dioxide](attachment:image.png)
Reagents

(1) Sodium Carbonate Solution - 10 percent (m/v) aqueous

(2) Bromophenol Blue Indicator Solution – Dissolve 0.1 g of bromophenol blue in 3.0 ml of 0.05 N sodium hydroxide solution and 5 ml of ethyl alcohol (90 percent by volume) by gently warming. Make up the volume of the solution with ethyl alcohol (20 percent v/v) to 250 ml in a volumetric flask.

(3) Hydrogen peroxide solution - Dilute a 30 percent (m/v) hydrogen peroxide solution with about twice its volume of water and neutralize the free sulphuric acid that may be present in the hydrogen peroxide solution with barium hydroxide solution, using bromophenol blue indicator solution. Allow the precipitate of barium sulphate to settle, and filter. Determine the concentration of hydrogen peroxide in the filtrate by titrating with standard potassium permanganate solution. Dilute the filtrate with cold water so as to obtain a 3 percent (m/v) solution of hydrogen peroxide.

(4) Concentrated Hydrochloric acid - sp.gr. 1.16

(5) Carbon dioxide gas - from a cylinder

(6) Standard sodium hydroxide solution - approximately 0.1 N, standardized at the time of the experiment using bromophenol blue indicator solution.
Procedure

1. Assemble the apparatus as shown above.
2. Introduce into the flask C, 300 ml of water and 20 ml of concentrated hydrochloric acid through the dropping funnel E.
3. Run a steady current of cold water through the condenser F.
4. Boil the mixture contained in the flask G for a short time to expel the air from the system in current of carbon dioxide gas previously passed through the wash bottle A. Check the pressure of carrier gas entering the receiving flask by monitoring the gas bubbling rate.
5. Weigh accurately about 100 g of the material and mix with the minimum quantity of water so as to make the diluted material easily flow down to the dropping funnel. Introduce the diluted material into the flask C through the dropping funnel E. Wash the dropping funnel with a small quantity of water and run the washing into the flask C.
6. Again boil the mixture contained in the flask C in a slow current of carbon dioxide gas (passed previously through the wash bottle A) for one hour. Just before the end of the distillation, stop the flow of water in the condenser. (This causes the condenser to become hot and drives over residual traces of sulphur dioxide retained in the condenser.)
7. When the delivery tube H, just above the Erlenmeyer flask J, becomes hot to touch, remove the stopper J immediately. Wash the delivery tube H and the contents of the Peligot tube L with water into Erlenmeyer flask.
8. Cool the contents of the Erlenmeyer flask to room temperature, add a few drops of bromophenol blue indicator and titrate with standard sodium hydroxide solution. Bromophenol blue is unaffected by carbon dioxide and gives a distinct change of color in cold hydrogen peroxide solution). Carry out a blank determination using 20 ml of conc. hydrochloric acid diluted with 300 ml of water. Blank must be carried out, treating the same way as sample, with same boiling time and gas bubbling rate.

**Calculation**

\[
\text{Sulphur dioxide, mg/kg} = \frac{0.032000 \times (V - v) \times 1000 \times 1000 \times N}{W}
\]

Where

- \(V\) = volume in ml of standard sodium hydroxide solution required for the test with the material
- \(v\) = volume in ml of standard sodium hydroxide solution required for the blank determination;
- \(N\) = normality of standard sodium hydroxide solution; and
- \(W\) = weight in g of the material taken for the test

[Ref :- I.S.I Handbook of Food Analysis (Part II) -1984 page 8]
A.12 Lead, Copper and Zinc

**Equipment:**

1. Atomic absorption spectrophotometer (Flame)
2. Crucibles
3. Hot plate
4. Calibrated weighing balance
5. Muffle Furnace

**Reagents:**

1. Nitric acid
2. Water
3. Hydrochloric acid — (1:1)
4. Standard stock solution (Certified Reference Materials) – 1000 mg/L of Pb, Cu & Zn (traceable to NIST)

**Procedure:**

1. Sample preparation: Homogenize product, if necessary using non-contaminating equipment.
2. Weigh about 10-20 g of the sample to nearest 0.01g in a crucible, and dry on a hot plate at 100°C.
3. Place the dish in muffle furnace at initial temperature not higher than 100°C. Increase temperature at a maximum rate of 50°C per hour to 450°C. Let dish stand for at least 8 hours or overnight.
4. Take crucible out of furnace and let cool.
5. Wet ash with 1-3 ml of water and evaporate on hot plate.
6. Put crucible back in furnace at no more than 200˚C and raise temperature (50-100˚C per hour) to 450˚C. Proceed for 1-2h or longer.
7. Repeat procedure until product is completely ash i.e. ash should be white/ grey or slightly covered.
8. Add 5 ml (1:1) HCl to crucible ensuring that all ash comes into contact with acid. Evaporate acid on hot plate.
9. Dissolve residue in 10-30 ml 0.1M HNO₃. Cover with watch glass and let stand for 1-2 hours. Make up to a particular volume.
10. Treat blank in the same way as prepared sample.
11. Standard preparation: Add 10 ml of standard stock solution into 100 ml volumetric flask and make up the volume using deionized water. (Pb/Cu/Zn 100 mg/L solution)
12. Preparation of standard working solution: To a series of 100 ml one-mark volumetric flasks, pipette 0.2, 0.5, 1.0, 2.0 and 5.0 ml of the standard working solution and 1ml of concentrated nitric acid and then dilute to mark with deionized water. These solutions then contain lead concentration 0.2, 0.5, 1.0, 2.0 and 5.0 µg of Pb/Cu/Zn per ml of the solution.
13. Aspirate both standard & sample solutions into the AAS.
**Calculation:**

Determine the Pb/Cu/Zn concentration in the sample solution by extrapolating the value from the standard curve plotted using conc. of different standard solution vs. absorbance reading.

\[
\text{Pb / Cu / Zn, mg/kg} = \frac{(S - B) \times \text{Dilution}}{\text{Weight of sample}}
\]

Where,

- \( S \) = Concentration of Pb/Cu/Zn in sample
- \( B \) = Concentration of Pb/Cu/Zn in blank

[Ref:- A.O.A.C 18th edn, 2005 Official Method 999.11]

**A.13 Filth in candy**

**Apparatus**

1. Microscope
2. Magnetic stirrer – hot plate
3. Sieve
4. Wire basket – 8 cm diameter and 3 cm height, made from No. 8 screen and with wire handles.
**Reagents**

(1) HCl (1+70)
(2) Turgitol anionic 7 – sodium heptadecyl sulphate (SIGMA chemical co.)
(3) Mineral oil – Paraffin oil, white, light, 125/135 Saybolt universal viscosity (38), sp. gravity 0.840-0.860 (24)
(4) Isopropanol
(5) Chloroform
(6) Floatation liquid – mineral oil and heptane (85+15)
(7) Dichloromethane

**Procedure**

(1) *In hard candy, gum drops, gum, starch or pectin based candies*

1. Dissolve in boiling HCl.
2. Filter through rapid paper on hirsch funnel.
3. And examine microscopically.

(2) *In hard boiled candy difficult to filter and in all water insoluble candy except those containing confectioners corn flakes, wheat bran, or other cereal fillers, and those whose major constituents, excluding chocolate coating, consists primarily of ground nutmeats (eg. Peanut butter, almond paste etc.)*
1. Weigh 225g test portion into 1.5 to 2 litre beaker.
2. Add 1 litre 5% solution of turgitol and heat in steam bath for 10 minutes. Stir 5 to 10 minutes on magnetic stirrer – hot plate.
3. Sieve portion wise on number 230 sieve. If residue on sieve is small, transfer directly to ruled filter paper; otherwise, transfer quantitatively to 2 litre trap flask, using 40% isopropanol.
4. Bring volume to 1 litre with 40% isopropanol and add 50 ml HCl.
5. Gently stir on magnetic stirrer – hot plate, while heating to full boil.
6. Immediately transfer flask to cool stirring unit and add 40 ml light mineral oil.
7. Stir magnetically for 2 minutes, let it stand for 1 minute; then slowly fill flask with 40% isopropanol by running liquid down stoppered rod while top of stopper is maintained just above liquid. After filling flask, gently stir settled plant material for 5 to 10 seconds with stoppered rod.
8. Let stand undisturbed for 2 minutes and immediately trap off.
9. Add 25 ml light mineral oil, stir by hand gently for 30 seconds, and let stand for 10 minutes. Repeat trapping. Wash flask neck thoroughly with isopropanol and transfer washings to beaker containing trappings.
10. Filter onto ruled paper and examine microscopically.

(3) In water insoluble candies containing confectioners corn flakes, wheat bran, or other cereal fillers, and those whose major
constituents, excluding the chocolate coating, consists primarily of finely ground nutmeats (eg. Peanut butter, almond paste etc.)

1. Proceed as mentioned in (2); through sieving on number 230 sieve.
2. Wash residue on sieve with isopropanol.
3. Form filter paper around 600 ml beaker, moistening with water to make paper pliable. Insert paper into 91 mm buchner, wash with isopropanol, and aspirate to near dryness.
4. Quantitatively transfer residue on sieve to filter paper cup with isopropanol and add enough isopropanol to cover residue.
5. After 1 minute apply vacuum until dripping ceases.
6. Place paper cup containing sieved residue in 1 litre beaker, add 200-ml chloroform, and boil 5 minutes on steam bath.
7. After few minutes of cooling, lift paper, drain, and transfer to 200ml fresh chloroform. Repeat for 5 minutes, boil and drain.
8. Return paper cup to buchner and apply vacuum until dripping ceases. Cover residue with isopropanol for 5 minutes, reapply vacuum, and continue to aspirate for 5 minutes after visible dripping ceases.
9. Transfer quantitatively to 2 litre trap flask, using 40% isopropanol. Bring volume to 1 litre with 40% isopropanol and add 50 ml HCl.
10. Gently stir on magnetic stirrer – hot plate, while heating to full boil.

11. Immediately transfer flask to cool stirring unit and add 40 ml floatation liquid.

12. Stir magnetically for 2 minutes, let it stand for 1 minute; then slowly fill flask with 40% isopropanol by running liquid down stoppered rod while top of stopper is maintained just above liquid. After filling flask, gently stir settled plant material for 5 to 10 seconds with stoppered rod.

13. Let stand undisturbed for 2 minutes and immediately trap off.

14. Add 25 ml floatation liquid, stir by hand gently for 30 seconds, and let stand for 10 minutes. Repeat trapping. Wash flask neck thoroughly with isopropanol and transfer washings to beaker containing trappings.

15. Filter onto ruled paper and examine microscopically.

(5) In chocolate candy coating

1. Heat 400ml dichloromethane in 800ml beaker to 30-35 °C and keep at this temperature.
2. Place test portion of candy in wire basket.
3. Move basket up and down through dichloromethane until chocolate coating dissolves. Rinse each candy center with fine stream of dichloromethane from wash bottle and save center. Repeat with balance of test sample.
4. Stir dichloromethane-chocolate suspension and pour through No. 140 sieve.

5. Transfer residue from sieve to filter paper and examine microscpically. Examine candy centers by appropriate method as in (1), (2) and (3).

[Ref:- A.O.A.C 18th edn, 2005 Official Method 971.34 Filth in candy (Floatation method)]

A.15 Starch in Confectionery

Reagents

(1) Malt extract – Prepare infusion of freshly ground malt just before use. For every 80 ml malt extract required digest 5 g ground malt with 100 ml water at room temperature 2 hours or 20 minutes if mixture can be stirred by electric mixer. Filter to obtain clear extract, refiltering first portion of filterate if necessary. Mix infusion well.

Procedure

1. Measure 25 ml of solution of uniform mixture (representing 5 g test portion) into 30 ml beaker, or add to beaker 5 g finely ground test portion (previously extracted with ether, if test sample contains much fat); add enough water to make 100 ml; heat to 60°C
(avoiding, if possible gelatinizing starch); and let stand for 1 hour, stirring frequently to secure complete solution of sugar.

2. Transfer to wide-mouth bottle, rinse beaker with little warm water and cool.

3. Add equal volume alcohol and mix and let stand for less than 1 hour.

4. Centrifuge until precipitate is closely packed on bottom of bottle and decant supernate through hardened filter.

5. Wash precipitate with successive 50 ml portions of alcohol, 50% by volume, by centrifuging and decanting through filter until washings are sugar-free by following test:

Add to test-tube few drops of washing, 3-4 drops 20% alcoholic α-naphthol solution, and 2 ml water. Shake well, tip tube, let 2 to 5 ml H₂SO₄ flow down sides of tube, and then hold tube upright. If sugar is present, interphase of two liquids is coloured faint to deep violet; on shaking, whole solution becomes blue-violet.

6. Transfer residue from bottle and hardened filter to beaker with 50 ml water.

7. Immerse beaker in boiling water, and stir constantly 15 minutes or until, all starch is gelatinized.

8. Cool to 55°C, add 20 ml malt extract, and hold at this temperature for 1 hour, or until residue treated with I₂ solution shows no blue tinge upon microscopic examination. Cool, dilute to 250 ml and filter.
9. Place 200 ml of filterate in flask, add 20 ml HCl (sp. Gr. 1.125), connect with reflux condenser, and heat in boiling water-bath 2.5 hours.

10. Cool, nearly neutralize with 10% NaOH solution, finish neutralization with Na$_2$CO$_3$ solution, and dilute to 500 ml.

11. Mix solution thoroughly, pour through dry filter, and determine glucose in aliquots by Munson – Walker method. Conduct Blank determination on same volume of malt extract as used with test portion and correct weighed glucose accordingly.

\[
\text{Weighed glucose obtained} \times 0.925 = \text{Weighed Starch}
\]

A.16 Paraffin in Confectionery

**Procedure**

1. To solvent extract in flask (as obtained in the above method for Ether Extract), add 10 ml alcohol and 2 ml NaOH solution (1+1); connect flask with reflux condenser and heat 1 hour on water-bath or until saponification is complete.

2. Remove condenser and keep flask on bath until alcohol evaporates and residue is dry.

3. Dissolve residue as completely as possible in approx. 40 ml water and heat on bath, shaking frequently.

4. Wash into separator, cool, and extract with 4 successive portions of petroleum ether, collecting extracts in weighed flask or capsule.
5. Evaporate petroleum ether and dry to constant weight at 100°C. Any phytosterol or cholesterol present in fat could be extracted with the paraffin, but the amount is so insignificant that it may generally be disregarded.

A.17 Shellac in Confectionery

Procedure
1. Place 50 g test portion in 400 ml beaker.
2. Add 50 ml mixture of Benzene and absolute alcohol (1+1), and cover with watch-glass. Heat to boiling point on steam bath, and simmer few minutes, stirring occasionally.
3. Decant liquid into tared, round 100 ml glass dish with flat bottom approx. 7 cm diameter.
4. Extract once more with Benzene-alcohol mixture, and finally rinse with two 25 ml portions of absolute alcohol, simmering and stirring each time. With moist sugar candy, avoid over heating to prevent pieces from sticking together. Add each extract to glass dish previously placed on steam bath.
5. Evaporate until alcohol is just removed, rotating dish as it goes to dryness in order to spread extract uniformly on the bottom surface. Avoid baking shellac on dish. If fat appears to be present, wash with three 15 ml portion of petroleum ether, stirring and warming. Decant through rapid filter.
6. Add mixture of 25 ml iso-amyl alcohol (B.P. – 129-132°C) and 25 ml Benzene to filter, and filter back to dish.
7. Heat on steam bath with stirring, cool somewhat, and transfer solution with suspended matter to 125 ml separator. Rinse dish with 25 ml hot (approx. 60°C) water, and add to separator; shake well and filter and wash water if necessary.

8. Repeat washings with water twice (or until washings are colourless), rinsing dish well around sides with first portion of liquid.

9. Finally, filter solution of shellac into tared dish, rinsing separator and filters with little absolute alcohol.

10. Evaporate to dryness on steam bath, rotating dish to give uniform film.

11. If much fat was extracted in original benzene extraction, wash final shellac residue with 25 ml petroleum ether, warming and stirring. Decant, dry on steam bath and in 100°C oven and weigh.

12. After weighing, check for complete removal of sugars by thoroughly rinsing dish and surface of shellac with hot water, warming on steam bath, decanting, rinsing down with alcohol and evaporating with care to give uniform film on dish.

13. Dry and reweigh.

A.18 Alcohol in syrups

Procedure

1. Collect n beaker syrup from enough pieces of confectionery to yield 30 – 50 g, strain into weighed beaker and weigh.
2. Place syrup in 250-300 ml distillation flask, dilute with half its volume of water, attach flask to vertical condenser and distill almost 50 ml, or as much of liquid as possible without causing charring. Foaming may be prevented by adding little tannin or piece of paraffin approx. size of pea.

3. Cool distillate, dilute to volume with water, and mix well.

4. Determine specific gravity of distillate.

Calculate % alcohol by weight or volume in candy filling by using Table - % by volume at 15.56°C of ethyl alcohol corresponding to apparent specific gravity at various temperatures & % by weight corresponding to various percentages by volume at 15.56°C in mixtures of ethyl alcohol and water.

A.19 Determination of Total Plate Count (CFU/gm)

Materials and equipment

The following media and reagents (1-4) are commercially available and are to be prepared and sterilized according to the manufacturer's instructions.

(1) Plate count agar (PC)/Nutrient Agar (NA)
(2) Peptone water diluent (0.1%)(PW)
(3) Sodium 2, 3, 5 triphenyltetrazolium chloride, TTC (0.1%)
    (optional)
(4) 1N HCl and 1N NaOH
(5) pH meter or paper capable of distinguishing to 0.3 to 0.5 pH units within a range of 5.0 to 8.0

(6) Stomacher, blender or equivalent for sample preparation/homogenization.

(7) Incubator capable of maintaining the growth temperature required for the specific type of aerobic bacteria being enumerated (i.e. for psychrophilic bacteria: 15 – 20°C, for mesophilic bacteria: 30 – 35°C, and for thermophilic bacteria: 55°C) and 45°C waterbath

(8) Colony counting device (optional)

**Procedure**

The test shall be carried out in accordance with the following instructions:

**1) Handling of Sample Units**

1. During storage and transport, the following shall apply: with the exception of shelf stable products, keep the sample units refrigerated (0-5°C). Sample units of frozen products shall be kept frozen.

2. Thaw frozen samples in a refrigerator or under time and temperature conditions which prevent microbial growth or death.

3. Analyze sample units as soon as possible after receipt in the laboratory.
(2) **Preparation of Media**

1. Prepare plate count/Nutrient agar and dispense in appropriate quantities. Sterilize.
2. Clean surface of working area with a suitable disinfectant.
3. Clearly mark the duplicate Petri plates.

(3) **Preparation of Dilutions**

1. Prepare sterile 0.1% peptone water diluent.
2. To ensure a truly representative analytical unit, agitate liquid or free flowing materials until the contents are homogeneous. If the sample unit is a solid, obtain the analytical unit by taking a portion from several locations within the sample unit.
3. Prepare a 1:10 dilution of the food by aseptically homogenizing 25 g or mL (the analytical unit) into 225 mL of the required diluent. If a sample size other than 25 g or mL is used, maintain the 1:10 sample to dilution ratio, such as 11 g or mL into 99 mL.

**NOTE:**

1. Check the pH of the food suspension. If the pH is outside the range of 5.5-7.6, adjust the pH to 7.0 with sterile NaOH or HCl.
2. Prepare succeeding decimal dilutions as required, using a separate sterile pipette for making each transfer.
3. Shake all dilutions immediately prior to making transfers to ensure uniform distribution of the microorganisms present.
(4) Plating

1. Agitate each dilution tube to re-suspend material that may have settled out during preparation.
2. Pipette 1 mL or 0.1 mL of the required dilutions to appropriately marked duplicate Petri plates.
3. In the case of products that tend to adhere to the bottom of the plates, add the inoculum to 1.0 mL of sterile diluent previously placed in the Petri plate.
4. Pour 12-15 mL of tempered agar into each plate, and mix by rotating and tilting.
5. Allow to solidify. Plates should be poured not more than 15 min after preparation of dilutions.

(5) Incubation

Incubate plates in the inverted position for 48 h ± 4 h. Incubation temperature is dependent on the growth temperature requirements of the target organisms (for psychrophilic bacteria: 15 – 20°C, for mesophilic bacteria: 30 – 35°C, and for thermophilic bacteria: 55°C). The plates used to enumerate psychrophilic and thermophilic bacteria may be incubated up to 5 days.

(6) Counting Colonies

1. Count colonies promptly after the incubation period.
2. If possible, select plates with 15-300 colonies (including pinpoint colonies). If counts do not fall within this range select plates that fall nearest to the 15-300 range.
For counting of colonies in cfu/gm or cfu/ml the formula is as follows:

\[ N = \frac{\sum c}{(n1+0.1n2)d} \]

Where,
- \( n \) = the number of counts
- \( n1 \) = the number of plates counted in the first dilution
- \( n2 \) = the number of plates counted in the second dilution
- \( d \) = the dilution from which the first counts were obtained (for example, 10-1)

Round the results obtained with above formula to two significant figures.

(7) Differentiation of Colonies from Interfering Particles

Food particles such as meat, milk powder, etc., often interfere with the enumeration of the plates. This can be eliminated by making one extra plate of each dilution containing interfering particles and holding it under refrigeration as a control for comparison during counting.

Alternatively, after incubation flood plates with 2 mL of 0.1% 2,3,5, triphenyltetrazolium chloride. Gently rock plates from side to side to cover the entire area with solution. Pour off excessive solution and allow the plates to remain at room temperature for 3
hrs. in an inverted position. The bacteria reduce the indicator to a formazan which colours the colonies red and aids in distinguishing the food particles. Colonies cannot be picked for isolation after this method has been used.

(8) Reporting:
Total Plate Count: cfu/g

A.20 Determination of Coliform Count (CFU/gm)

Materials and equipment
The following media and reagents are commercially available and are to be prepared and sterilized according to the manufacturer's instructions.

(1) Violet Red Bile Agar
(2) Peptone water diluent (0.1%)(PW)/N-Saline
(3) pH meter or paper capable of distinguishing to 0.3 to 0.5 pH units within a range of 5.0 to 8.0
(4) Stomacher, blender or equivalent for sample preparation/homogenization.
(5) Incubator capable of maintaining the growth temperature required for the specific type of aerobic bacteria being enumerated i.e. at 35°C.
(6) Colony counting device (optional)
**Procedure**

The test shall be carried out in accordance with the following instructions:

1. **Handling of Sample Units**
   1. During storage and transport, the following shall apply: with the exception of shelf-stable products, keep the sample units refrigerated (0-5°C). Sample units of frozen products shall be kept frozen.
   2. Thaw frozen samples in a refrigerator or under time and temperature conditions which prevent microbial growth or death.
   3. Analyze sample units as soon as possible after receipt in the laboratory.

2. **Preparation of Media**
   1. Prepare VRB agar and dispense in appropriate quantities.
      Sterilize.
   2. Clean surface of working area with a suitable disinfectant.
   3. Clearly mark the duplicate Petri plates.

3. **Preparation of Dilutions**
   1. Prepare sterile 0.1% peptone water diluent.
   2. To ensure a truly representative analytical unit, agitate liquid or free flowing materials until the contents are homogeneous. If
the sample unit is a solid, obtain the analytical unit by taking a portion from several locations within the sample unit.

3. Prepare a 1:10 dilution of the food by aseptically homogenizing 25 g or mL (the analytical unit) into 225 mL of the required diluent. If a sample size other than 25 g or mL is used, maintain the 1:10 sample to dilution ratio, such as 11 g or mL into 99 mL.

**NOTE:**

1. Check the pH of the food suspension. If the pH is outside the range of 5.5-7.6, adjust the pH to 7.0 with sterile NaOH or HCl.
2. Prepare succeeding decimal dilutions as required, using a separate sterile pipette for making each transfer.
3. Shake all dilutions immediately prior to making transfers to ensure uniform distribution of the microorganisms present.

4. **Plating**
   1. Agitate each dilution tube to resuspend material that may have settled out during preparation.
   2. Pipette 1 mL or 0.1 mL of the required dilutions to appropriately marked duplicate Petri plates.
   3. In the case of products that tend to adhere to the bottom of the plates, add the inoculum to 1.0 mL of sterile diluent previously placed in the Petri plate.
   4. Pour 12-15 mL of tempered agar into each plate, and mix by rotating and tilting.
5. Allow to solidify. Plates should be poured not more than 15 min after preparation of dilutions.

5. **Incubation**
   1. Incubate plates in the inverted position for 24 h ± 4 h at 35±2°C.
   2. Avoid crowding or excessive stacking of plates to permit rapid equilibration of plates with incubator temperature.

3. **Counting Colonies**
   1. Count colonies promptly after the incubation period.
   2. If possible, select plates with 30-300 colonies (including pinpoint colonies). If counts do not fall within this range select plates that fall nearest to the 30-300 range.

For counting of colonies in cfu/gm or cfu/ ml the formula is as follows:

\[
N = \frac{\sum c}{(n_1+0.1n_2)d}
\]

Where ;

- \(n\) = the number of counts
- \(n_1\) = the number of plates counted in the first dilution
- \(n_2\) = the number of plates counted in the second dilution
- \(d\) = the dilution from which the first counts were obtained (for example, 10-1)
Round the results obtained with above formula to two significant figures.

4. **Reporting**: Total Coliform Count : cfu/g

**A.21 Enumeration of Yeasts And Moulds (CFU/gm)**

**Materials and special equipment**

The following media and reagents are commercially available and are to be prepared and sterilized according to the manufacturer's instructions.

These agars are suitable for yeast and mould count in food products:

(1) Chloramphenicol Yeast extract Glucose Agar (CYGA)
(2) Potato dextrose agar with chloramphenicol (PDA-C)
(3) 20% sucrose (diluent additive for osmophiles)
(4) Malt extract agar containing 50% (w/w) sucrose

Others:

(1) Peptone water (0.1%) (PW)
(2) 1N HCl and 1N NaOH
(3) Gram stain solutions
(4) Stomacher, blender or equivalent
(5) pH meter or paper capable of distinguishing to 0.3 to 0.5 pH units within a range of 5.0 to 8.0
(6) Light microscope
(7) Colony counting device (optional)

(8) Incubator (darkened) capable of maintaining 22 to 25°C,

**Procedure**

Each sample unit shall be analyzed individually. The test shall be carried out in accordance with the following instructions:

1. **Handling of Sample Units**
   1. During storage and transport, the following shall apply: with the exception of shelfstable products, keep the sample units refrigerated (0-5°C). Sample units of frozen products shall be kept frozen.
   2. Thaw frozen samples in a refrigerator or under time and temperature conditions which prevent microbial growth or death.
   3. Analyze the sample units as soon as possible after receipt at the laboratory.

2. **Preparation of Medium**
   Prepare the appropriate media for the analysis being carried out.

**NOTE:**

1. Clean surface of working area with a suitable disinfectant.
2. Mark clearly the duplicate petri plates identifying sample, sample unit, dilution and date of inoculation.
3. **Preparation of Dilutions**

1. Prepare 0.1% peptone water as diluent. An appropriate solute, such as 20% sucrose, should be added to the diluent when enumerating osmophiles in foods such as syrups and fruit juice concentrates. In addition, a 2% solution of sodium citrate, pre-warmed to 45°C, can be used as diluent for high-fat foods such as cheese, butter.

2. Prepare a 1:10 dilution of the food by aseptically blending 25 g or mL (the analytical unit) into 225 mL of the required diluent, as indicated in Table I. If a sample size other than 25 g or mL is used, maintain the 1:10 sample to dilution ratio, such as 11 g or mL into 99 mL.

**NOTE:**

1. Stomach, blend or shake the food sample.
2. Blend or stomach for the minimum time required to produce a homogeneous suspension.
3. Verify the pH of the suspension. If the pH is not between 5.5 and 7.5, adjust the pH to 7.0 with a sterile solution of 1N NaOH or 1N HCl.
4. If the 1:10 dilution is prepared in a dilution bottle, it should be mixed by shaking the tube using vortex mixer.
5. Prepare succeeding decimal dilutions as required, using a separate sterile pipette for making each transfer.
6. Because mould propagules may settle out within a few minutes, it is important to shake all dilutions immediately prior to making
transfers to ensure uniform distribution of the microorganisms present.

4. **Plating**
   1. Agitate each dilution bottle to resuspend material that may have settled out during preparation.
   2. Moulds should be enumerated by a surface spread-plate technique rather than with pour plates. This technique provides maximal exposure of the cells to atmospheric oxygen and avoids heat stress from molten agar. Agar spread plates should be dried overnight before being inoculated. Spread 0.1 mL onto duplicate plates.

5. **Incubation**
   Incubate plates undisturbed in an upright position at 22 to 25°C for 3-5 days. Incubate plates in the dark. Normally, count colonies on plates after 5 days. Examine on the third day and if mould colonies are numerous, count them and then count again on the fifth day, if possible.

**NOTE:**
Handle the plates as little as possible when counting on third day so spores will not be dislodged, which may result in secondary growth.
6. Counting Colonies and Examining Growth

Count colonies, distinguishing, if required, yeast colonies from mould colonies, according to their colonial morphology. Microscopic examination with crystal violet stained smears may be necessary to distinguish yeast colonies from some bacterial colonies that may look like yeast.

If possible, select plates with 15-150 colonies. Determine the identity of pin-point colonies microscopically. If counts do not fall within this range, select plates that fall nearest to the 15-150 range. If the mycoflora consists primarily of moulds, the lower population range is selected; if primarily yeast colonies, the upper limit is counted.

1. Count colonies promptly after the incubation period.
2. If possible, select plates with 15-150 colonies (including pinpoint colonies). If counts do not fall within this range select plates that fall nearest to the 15-150 range.

For counting of colonies in cfu/gm or cfu/ ml the formula is as follows:

\[ N = \frac{\sum c}{(n1+0.1n2)d} \]

Where:

- \( n \) = the number of counts
- \( n1 \) = the number of plates counted in the first dilution
n2= the number of plates counted in the second dilution

d= the dilution from which the first counts were obtained (for example, 10-1)

Round the results obtained with above formula to two significant figures.

Alternatively,

Wet mounts and gram stains of several diverse types of cells per sample should be examined to confirm that bacteria are not present. Yeast cells and asexual mould spores are generally gram-positive, whereas mould mycelia are gram-negative.

7. Recording Results

Calculate the average count (arithmetic mean) of the duplicate plates

Avoid creating erroneous ideas of precision and accuracy when computing counts

8. Reporting: Yeast and Mould Count : cfu/g

9. Precautions

1. Some yeasts and moulds can be infectious or can cause allergic responses, therefore, it is important to be fairly cautious when working with fungi. Ideally, plates should be held in incubators, not in an open room. Plate lids should generally only be
removed for procedures such as the preparation of a slide for microscopic examination.

2. Flamed needles should be cooled before making transfers to avoid dispersal of conidia and other cells. Cultures should never be smelled.

**A.22 Detection of *E. Coli* per gm**

**Materials and equipment**

The following media and reagents are commercially available and are to be prepared and sterilized according to the manufacturer's instructions.

1. Tergitol – 7 Agar (T-7)
2. McConkey Agar (MC)
3. Eosien Methylene Blue (EMB)Agar
4. Nutrient Broth
5. Peptone water diluent (0.1%)(PW)/ N-Saline
6. pH meter or paper capable of distinguishing to 0.3 to 0.5 pH units within a range of 5.0 to 8.0
7. Stomacher, blender or equivalent for sample preparation/homogenization.
8. Incubator capable of maintaining the growth temperature required for the specific type of aerobic bacteria being enumerated i.e. at 35°C.
9. Colony counting device (optional)
Procedure
The test shall be carried out in accordance with the following instructions:

(1) Preparation of Media
   1. Prepare McConkey, EMB, T-7 agar and dispense in appropriate quantities. Sterilize.
   2. Clean surface of working area with a suitable disinfectant.
   3. Clearly mark the duplicate Petri plates.

(2) Preparation of Dilutions
   1. Prepare sterile 0.1% peptone water diluent.
   2. To ensure a truly representative analytical unit, agitate liquid or free flowing materials until the contents are homogeneous. If the sample unit is a solid, obtain the analytical unit by taking a portion from several locations within the sample unit.
   3. Prepare a 1:10 dilution of the food by aseptically homogenizing 25 g or mL (the analytical unit) into 225 mL of the required diluent. If a sample size other than 25 g or mL is used, maintain the 1:10 sample to dilution ratio, such as 11 g or mL into 99 mL.

NOTE:
   1. Check the pH of the food suspension. If the pH is outside the range of 5.5-7.6, adjust the pH to 7.0 with sterile NaOH or HCl.
2. Prepare succeeding decimal dilutions as required, using a separate sterile pipette for making each transfer.

3. Shake all dilutions immediately prior to making transfers to ensure uniform distribution of the microorganisms present.

(3) Pre-Enrichment

Take 10 ml of diluted sample in 90 ml NB and incubate for 24 hrs. at 35±2°C

(4) Selective Enrichment

1. Transfer a loopful of the inoculum from pre-enrichment culture in to T-7 agar and incubate for 24 hrs at 35±2°C.

2. Pick golden yellow colonies from the T-7 agar and streak on to MC and EMB agar and incubate the plates for 24 hrs at 35±2°C.

3. Observe for characteristic colonies if Pink red colonies and green metallic sheen colonies on MC and EMB agar respectively.

(5) Biochemical Identification Tests

The biochemical tests conducted and interpreted as the reaction of the biochemical reagents as below;

1. Gram stain

2. Indole test

3. Voges Proskauer test
4. Citrate Utilization 
5. Growth on Macconkey broth at 44 ±0.50C 
6. Carbohydrate Utilization for acid and gas 
7. H$_2$S Production test 
8. Motility test 
9. Urease test 

(6) Reporting: E. coli : cfu/g; Present or Absent / g 

A.23 Detection of S.aureus per g 

Materials and equipment 
The following media and reagents are commercially available and are to be prepared and sterilized according to the manufacturer's instructions. 
(1) Baird Parker Agar 
(2) Nutrient Agar 
(3) Peptone water diluent (0.1%)(PW)/ N-Saline 
(4) pH meter or paper capable of distinguishing to 0.3 to 0.5 pH units within a range of 5.0 to 8.0 
(5) Stomacher, blender or equivalent for sample preparation/homogenization. 
(6) Incubator capable of maintaining the growth temperature required for the specific type of aerobic bacteria being enumerated i.e. at 35°C.
**Procedure**

The test shall be carried out in accordance with the following instructions:

(1) *Preparation of Media*

1. Prepare Baird Parker (BP) agar and dispense in appropriate quantities. Sterilize.
2. Clean surface of working area with a suitable disinfectant.
3. Clearly mark the duplicate Petri plates.

(2) *Pre-Enrichment*

1. Prepare a 1:10 dilution of the food by aseptically homogenizing 25 g or mL (the analytical unit) into 225 mL of the required Diluent. If a sample size other than 25 g or mL is used, maintain the 1:10 sample to dilution ratio, such as 11 g or mL into 99 mL.
2. Take 10 ml of above diluted sample in 90 ml 0.1% Peptone and incubate for 24 hrs. at 37±2°C

(3) *Selective Enrichment*

1. Transfer a loopful of the inoculum from pre-enrichment culture in to BP agar and incubate for 30 hrs at 37±2°C.
2. Observe for characteristics shiny black colonies with grey margin on the BP agar.

(4) Biochemical Identification Tests

The biochemical tests conducted and interpreted as the reaction of the biochemical reagents as below;

1. Gram Staining
2. Coagulase Test

(5) Reporting:

*Staphylococcus aureus* Present or absent / gm
B CHEWING GUM AND BUBBLE GUM

As per FSSA, Chewing and bubble gum shall be prepared from chewing gum base or bubble gum base, natural or synthetic, non-toxic; cane sugar and liquid glucose (corn syrup).

B.1 Preparation of sample:

Cut into small bits/ pieces around 50-75 g and mix well. Store in an airtight container.


B.2 Determination of Moisture - Vacuum Oven method

Apparatus

(1) Aluminum dish – 75mm diameter and about 25mm height with close fitting cover.
(2) Dessicator
(3) Vacuum oven
**Procedure**

1. Accurately weigh about 5 g of sample in a dish previously dried and weighed. Distribute the material as evenly as practicable over the bottom of the dish by gentle sidewise movements.

2. Place dish in vacuum oven, remove cover of dish and dry the material for 6-8 hours at 70ºC under 25 mm Hg pressure. During heating admit slow current of air into oven.

3. Cover dish, transfer to dessicator and weigh soon after room temperature is attained.

4. Redry for one hour and repeat the process till the difference between the two successive weighing is less than 2 mg. Report percent loss in weight as moisture %.

**Calculations**

\[
\text{Moisture content, } \% \text{ by mass} = \frac{(W3 - W2)}{(W3 - W2)} \times 100
\]

Where

- \( W1 \) = Weight of prepared sample taken for test in g
- \( W2 \) = Weight of empty moisture dish in g
- \( W3 \) = Weight of (dish + dried sample) in g

[Ref: - IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]
**B.3 Ash insoluble in dilute HCl**

**Reagents**

(1) Dilute hydrochloric acid - Approx 5 N (445ml in 1 litre)

**Procedure**

1. Weigh accurately about 10 g of the prepared sample in a tared, clean and dry platinum basin of 100 ml capacity.
2. Carbonize the material in the dish with the flame of a burner. Complete the ignition by keeping in a muffle furnace at 550 + 25˚C until gray ash results. Cool in a desiccator.
3. To the ash, add 25ml of the dilute hydrochloric acid, cover with a watch glass and heat on a small flame of a burner to near boiling.
4. Allow it to cool and filter the contents of dish through Whatman filter paper No. 42 or its equivalent. Wash the filter paper with hot water until the washings are free from chlorides. (To check this, add few drops of 2M Nitric acid and 0.1 M Silver nitrate solution to the filtrate obtained. No precipitate or milky turbidity should occur in the solution, if it is chloride-free.)
5. Return the filter paper and the residue to the dish. Keep it in an air oven maintained at 105 +2˚C for about three hours. Ignite in the muffle furnace at 550 +25˚C for one hour. Cool the dish in a desiccator and weigh.
6. Heat again for 30 minutes in the muffle furnace, cool and weigh.
7. Repeat this process of heating for 30 minutes, cooling and weighing till the difference between two successive weighing is less than one milligram. Note the lowest mass.

**Calculation**

\[
\text{Acid insoluble ash, percent by mass} = \frac{M_1 \times 100}{M_2}
\]

Where

- \( M_1 \) = mass in g of the acid insoluble ash
- \( M_2 \) = mass in g of the prepared sample taken for the test

**B.4 Determination of Sulphated Ash**

**Reagents**

1. Sulphuric Acid - 10 percent (m/m)

**Procedure**

1. Accurately weigh about 5 g of the prepared sample into a 9-cm diameter platinum basin.
2. Add 5 ml of sulphuric acid to the material in the dish.
3. Gently heat the dish on a hot plate until the material is well carbonized and then increase the heat until the evolution of sulphuric acid fumes ceases.

4. Ash the carbonized matter in a muffle furnace at 550 ± 25°C.

5. Cool the ash and moisten it with 2-3 ml of sulphuric acid. Heat strongly on a hot plate until sulphuric acid fumes ceases to be evolved and finally ash in the muffle furnace at 550 ± 25°C for two hours.

6. Cool in a desiccator and weigh.

7. Heat again in a muffle furnace for 30 minutes at 550 ± 25°C. Cool in a desiccator and weigh.

8. Repeat the process of heating in the muffle furnace for 30 minutes, cooling and weighing till the difference between two successive weighing is less than 1 mg. Record the lowest mass.

**Calculation**

\[
\text{Sulphated ash, % by mass} = \frac{M_1}{M_2} \times 100
\]

Where

\[M_1 = \text{mass in g of the sulphated ash}\]

\[M_2 = \text{mass in g of the prepared sample taken for the test.}\]

[Ref: - IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]
B.5 Test for presence of added synthetic colour

As per Clause A.6

B.6 Determination of Titanium Di-oxide in Chewing Gum and Bubble Gum

Reagents

(1) Titanium di-oxide standard solution: (0.1 mg/ml) - Weigh accurately 50 mg titanium di-oxide in a 250 ml beaker. Add 15 g anhydrous sodium sulphate and 50 ml conc. Sulphuric acid. Add 1 or 2 glass beads, cover with watch glass and heat on a hot plate to boil and dissolve. Cool and add 100 ml distilled water accurately with stirring. (If the solution is cloudy warm on a steam bath to clarify) Cool and transfer to 500-ml volumetric flask containing 200-ml water. Make up to volume.

(2) Sulphuric Acid – 1:9 volume

(3) Hydrogen peroxide – 30% grade

(4) Sodium Sulphate – Analar grade

Procedure:

1. Accurately weigh 2-3 grams of prepared sample in a 100-ml silica dish.
2. Char the material on a burner and ash in muffle furnace at 800°C for 3-4 hours.

3. Cool, add 2-g anhydrous sodium sulphate and 10 ml conc. H₂SO₄, cover with watch glass and bring to boiling on a hot plate and dissolve.

4. Cool thoroughly and rinse the watch glass carefully with 30-ml water. Transfer to 100-ml volumetric flask. (If solution is cloudy heat on steam bath to clarify) cool and dilute to volume with water.

5. Transfer 3-ml aliquot of the sample solution to 5 ml volumetric flask or graduated cylinder. Dilute to volume with 10% H₂SO₄. Add 0.2 ml 30% H₂SO₄. Mix well.

6. Measure the absorbance at 408 nm against a prepared blank. Determine the concentration of TiO₂ in sample using a standard curve.

**Preparation of Standard Curve:**

Transfer 0, 1, 2, 3 and 4 ml of TiO₂ standard solution (0.1 mg/ml) to 5-ml volumetric flask or graduated cylinder. Dilute to volume with 1+ 9 H₂SO₄. Mix well. Measure the absorbance of the colour at 408 nm in a spectrophotometer and prepare a standard curve.

**Calculation:**

\[
\text{TiO}_2 \text{ (in g %)} = \frac{\text{Dilution x mg TiO}_2 \times \text{Dilution x Absorbance of sample} \times 100}{\text{Wt. of sample x Dilution x Absorbance of standard} \times 1000}
\]
B.7 Determination of Gum-Base Content

Reagents and Apparatus:

(1) Chloroform, analar grade
(2) Soxhlet extraction apparatus
(3) Whatman thimble

Procedure:

1. Weigh 4-5 g of sample (W) in a Whatman thimble and extract the gum in a continuous extraction apparatus (Soxhlet extractor) with chloroform for 8 hours.
2. Distill off or evaporate the chloroform extract on a steam bath and transfer the flask to an oven maintained at 100°C and dry it for 4-5 hours.
3. Cool in a desiccator and weigh (W2).
4. Chloroform extract must be dried by keeping the flasks for 30 minutes and weighed, till constant mass is achieved.

Calculation:

\[
\text{Gum base Content} \% = \frac{W2 - W1}{100 \times W}
\]
Where

\[ W_2 = \text{Weight of flask and extracted gum, in g} \]
\[ W_1 = \text{Weight of empty flask, in g} \]
\[ W = \text{Weight of sample taken for test, in g} \]


**B.8 Determination of Reducing sugars and Sucrose**

**Reagents**

1. Fehling A: Dissolve 69.28 g copper sulphate (CuSO$_4$.5H$_2$O) in distilled water. Dilute to 1000 ml. Filter and store in amber coloured bottle.
2. Fehling B: Dissolve 346 g Rochelle salt (potassium sodium tartrate) (K Na C$_4$H$_4$O$_6$. 4H$_2$O) and 100 g NaOH in distilled water. Dilute to 1000 ml. Filter and store in amber coloured bottle.
3. Neutral Lead Acetate: Prepare 20% neutral lead acetate solution. (This reagent is used to clarify sugar solutions)
4. Potassium Oxalate Solution: Prepare 10% Potassium oxalate (K$_2$C$_2$O$_4$.H$_2$O) solution. This reagent is used to remove the excess lead used in clarification.
5. Methylene Blue Indicator: Prepare 1% of methylene blue solution in distilled water.
**Determination of Reducing Sugars:**

1. Weigh accurately 25 g of sample and transfer to 250 ml volumetric flask.
2. Add 10 ml of neutral lead acetate solution and dilute to volume with water and filter. Transfer an aliquot of 25 ml of the clarified filtrate to 500 ml volume flask containing about 100 ml water.
3. Add potassium oxalate in small amounts until there is no further precipitation. Make up to volume.
4. Mix the solution well and filter through Whatman No. 1 filter paper.
5. Transfer the filtrate to a 50 ml burette.

**Preliminary Titration:**

Pipet 5 ml each of Fehling A and B into 250 ml conical flask. Mix and add about 10 ml water and a few boiling chips or glass beads. Dispense solution. Heat the flask to boiling. Add 3 drops of methylene blue indicator. Continue the addition of solution dropwise until the blue colour disappears to a brick-red end point. (The concentration of the sample solution should be such that the titre value is between 15 and 50 ml). Note down the titre value.
**Final Titration:**

Pipet 5 ml each of Fehling A and B. Add sample solution about 2 ml less than titre value of the preliminary titration. Heat the flask to boiling with in 3 minutes and complete the titration. Perform the titration duplicate and take the average. Calculate the reducing sugar % as shown below:

\[
\text{Reducing Sugars } \% = \frac{\text{Dilution x Factor of Fehling (in g) x 100}}{\text{Weight of sample x Titre value}}
\]

(as Invert Sugar)

**Determination of Total Reducing Sugars:**

Pipette an aliquot of 50 ml of the clarified, de-leded filtrate to a 100 ml volumetric flask. Add 5 ml of conc. HCl and allow standing at room temperature for 24 hours. Neutralize with conc. NaOH solution followed by 0.1N NaOH. Make up to volume and transfer to 50 ml burette having an offset tip and perform the titration on Fehling solution similar to the procedure described in the determination of reducing sugars.

\[
\text{Reducing Sugars } \% = \frac{\text{Dilution x Factor of Fehling (in g) x 100}}{\text{Weight of sample x Titre value}}
\]

**Determination of Factor (for Invert Sugar) of Fehling Solution:**

Accurately weigh around 4.75 g of analar grade sucrose. Transfer to 500 ml volume flask with 50 ml distilled water. Add 5 ml conc. HCl and allow to stand for 24 hours. Neutralize with NaOH solution and make up to volume. Mix well and transfer 50 ml to a 100 ml volumetric flask and makeup to volume. Transfer to a burette having an offset tip.

Perform the titration of Fehling solution following the similar procedure as above:

\[
\text{Reducing Sugars %} = \frac{\text{Factor Titre x Weight of sucrose (in g)}}{500} \\
\text{(as Invert Sugar)}
\]

**B.9 Determination of Sucrose**

**Calculation**

\[
\text{Sucrose %} = \left( \frac{\text{Total reducing sugars}}{\text{invert sugar %} - \text{reducing sugars %}} \right) \times 0.95
\]

B.10 Determination of Total protein
As per Clause A.7

B.11 Determination of fat
As per Clause A.8

B.12 Determination of Pb, Cu & Zn
As per Clause A.12

B.13 Determination of filth
As per Clause A.14

B.14 Determination of TPC
As per Clause A.19

B.15 Determination of Coliform
As per clause A.20

B.16 Determination of Yeast & Mould
As per Clause A.21
B.17 Determination of E.Coli

As per Clause A.22

B.18 Determination of S. aureus

As per Clause A.23
C. CHOCOLATE

As per FSSA, chocolate is a homogenous product obtained by an adequate process of manufacture from a mixture of one or more the ingredients, namely cocoa beans, cocoa nib, cocoa mass, cocoa press cake and cocoa dust, including fat reduced cocoa powder with or without addition of sugars, cocoa butter, milk solids including milk fat.

Types of chocolate

Milk chocolate: Milk chocolate is the homogenous product obtained by an adequate process of manufacture from one or more of cocoa nib, cocoa mass, cocoa press cake, cocoa powder with sugar and milk solids including milk fat & cocoa butter.

Milk covering chocolates: Milk covering chocolate is same as milk chocolate and is suitable for covering purposes.

Plain chocolate: Plain chocolate is the homogenous product obtained by an adequate process of manufacture by one or more cocoa nib, cocoa mass, cocoa press cake, cocoa powder including milk fat & cocoa butter.

Plain covering chocolates: Plain covering chocolate is same as plain chocolate and is suitable for covering purposes.
Filled Chocolate: Filled chocolate is a product having an external coating of chocolate with a center clearly distinct through its composition from the external coating. Filled chocolate does not include flour, confectionery, pastry and biscuit products.

Composite chocolate: Composite chocolate is a product containing at least 60% m/m of chocolate and edible wholesome substances such as fruits, nuts etc.

Blended chocolate: Blended chocolate is a blend of milk and plain chocolates in varying proportions.

C.1 Preparation of Sample

- Melt the product in a beaker at a temperature of 45-50°C. Pour the melted sample on a marble slab and mix thoroughly with a spatula till the product is solidified and transfer to a stoppered glass bottle. Store in a cool place.

- Chill the material until hard and then grate or shear to a fine granular condition Mix thoroughly and transfer to a stoppered glass bottle. Store in a cool place.

- Alternatively melt in a suitable container by placing container in water bath at about 50 °C. Stir frequently until test portion melts and reaches temperature of 45 – 50 °C, remove from bath, stir thoroughly and while still hot remove test portion for analysis using glass or metallic tube provided with close fitting plunger to expel test portion from tube or disposable plastic syringe.
C.2 Determination of Moisture - Vacuum Oven method

**Apparatus**

1. Aluminum dish – 75mm diameter and about 25mm height with close fitting cover.
2. Dessicator
3. Vacuum oven

**Procedure**

1. Accurately weigh about 10 g of sample in a dish previously dried and weighed.
2. Distribute the material as evenly as practicable over the bottom of the dish by gentle sidewise movements.
3. Place dish in vacuum oven, remove cover of dish and dry the material for six hours at 80 ± 1 °C at a pressure not exceeding 5 mm of Hg. During heating admit slow current of air into oven.
4. Cover dish, transfer to dessicator and weigh soon after room temperature is attained.
5. Redry for one hour and repeat the process till the difference between the two successive weighing is less than 2 mg. Report percent loss in weight as moisture %.

**Calculations:**

\[
\text{Moisture content, } \% \text{ by mass} = \frac{(W3 - W2)}{W1} \times 100
\]

Where:

- \(W1\) = Weight of prepared sample taken for test in g
- \(W2\) = Weight of empty moisture dish in g
- \(W3\) = Weight of (dish + dried sample) in g

[Ref: - IS: 6287-1985 (Reaffirmed 2010) Methods of Sampling and Analysis for Sugar Confectionery]

**C.3 Determination of Fat**

**Apparatus**

(1) Buchner funnel – of 9 cm size.

(2) Soxhelet Apparatus - with 250 ml flat bottom extraction flask
Reagents

(1) Hydrochloric acid - sp.gr. 1.16

(2) Filter aid - a suitable brand

(3) Petroleum ether - redistilled below 60°C

(3) Sodium sulphate - anhydrous

Procedure

1. Weigh accurately about 10 to 20 g of the prepared sample into a 400-ml beaker and add 30 ml of water and 25 ml of hydrochloric acid.
2. Heat for 30 minutes on a steam bath, with frequent stirring.
3. Add 5 g of filter aid and 50 ml of ice-cold water and chill for 30 minutes in ice-cold water.
4. Fit a heavy piece of linen into the buchner funnel and moisten with water.
5. Apply gently suction and pour over it a suspension of 3 g of filter aid in 30 ml of water. Filter the hydrolyzed mixture by gentle suction, rinsing the beaker three times with ice-cold water, taking care to leave a layer of liquid on the filter.
6. Finally wash three times with ice-cold water and suck dry.
7. Transfer the filter-cake from the funnel to the original beaker, using a small piece of filter paper to transfer any material adhering to the funnel.
8. Wash the funnel with petroleum ether into the beaker and evaporate the ether on a steam bath.

9. Break up the cake with a glass rod and allow it to remain on the steam bath until the contents are so dry as to enable pulverizing easily. Place in an oven at 100 ± 2°C for one hour.

10. Add 15 g of powdered anhydrous sodium sulphate and mix well. Transfer the mixture to the fat extraction thimble of the soxhlet apparatus. Wash the beaker with 50 ml of petroleum ether and transfer the washings to the thimble. Extract the fat with petroleum ether so that at least 300 ml has been circulated.

11. Transfer the extract to a tared dish and evaporate the petroleum ether on a steam bath.

12. Dry the fat till the difference in weight between successive weighing is not more than 1 mg.

**Calculation**

\[
\text{Total Fat % by mass} = \frac{10000 \times w}{W \times (100 - M)}
\]

Where,

\(w\) = weight in g of fat

\(W\) = weight in g of prepared sample taken for the test

\(M\) = moisture, percent by weight, in the prepared sample
Note: - In case of plain covering chocolate, fat can be extracted directly in a soxhlet apparatus


Also see A.O.A.C 17 th edn, 2000 Official Method 963.15 Fat in Cocoa Products – Soxhlet Extraction Method

C.4 Determination of Non Fat Milk Solids

Reagents

(1) Petroleum ether

(2) Sodium Oxalate solution – Approximately one percent (w/v)

(3) Glacial acetic acid

(4) Tannic acid solution- approximately 10 percent (w/v)

(5) Concentrated sulphuric acid- sp. Gr. 1.84

(6) Catalyst mixture- 1.0 g of selenium and 5.0 g of mercuric oxide intimately mixed together.

(7) Alkali solution- prepared by dissolving 300 g of sodium hydroxide and 10 g of sodium thiosulphate in 500 ml of water.
(8) Standard Sulphuric Acid-Approximately 0.1 N

(9) Methyl Red Indicator solution- Dissolve one gram of methyl red in 200 ml of rectified spirit (95 percent by volume).

(10) Standard Sodium Hydroxide Solution- approximately 0.1 N

**Procedure**

1. Weigh accurately about 10 g of the prepared sample and extract the fat by shaking and centrifuging with two consecutive portions each of 100 ml of petroleum ether. Remove the last traces of ether from the extracted residue in an air oven.

2. Shake the de-fatted residue with 100 ml of water for 4 minutes and then add 100 ml of sodium oxalate solution. Stopper and shake vigorously for 3 minutes.

3. Allow this mixture to stand for 10 minutes, shake again for 2 minutes and then centrifuge for 15 minutes.

4. Pipette 100 ml of the clear supernatant liquid into 250-ml beaker and add one milliliters of glacial acetic acid, stir gently, stand for a few minutes and then add 4 ml of freshly prepared tannic acid solution and stir.

5. Allow the precipitate to settle and filter through a Whatman filter paper No. 42 overlaid with paper pulp, in a 7cm Buchner funnel, wash twice with the sodium oxalate solution containing one percent (w/v) of the glacial acetic acid and two percent (w/v) of tannic acid solution.
6. Digest the precipitate in a Kjeldahl flask with 20 ml of sulphuric acid, 15 g of sodium sulphate and 1 g of the catalyst, for 30 minutes after the mixture has become clear.

7. Cool the contents of the flask. Transfer quantitatively to a round-bottom flask, with water, the total quantity used being about 200 ml. Add with shaking a few pieces of pumice stone to prevent bumping.

8. Add 50 ml of alkali solution carefully over the side of the flask so that it does not mix at once with the acid solution but forms a layer below the acid.

9. Assemble the apparatus, taking care that the tip of the condenser extends below the surface of the sulphuric acid contained in the beaker.

10. Mix the contents of the flask by shaking and distill until all ammonia has distilled over into the standard sulphuric acid.

11. Detach the flask from the condenser and shut off the burner. Rinse the condenser thoroughly with water into the beaker. Wash the tip carefully so that all traces of condensate are transferred to the beaker.

12. When all the washings have drained into the beaker, add two or three drops of the methyl red indicator solution and titrate with standard sodium hydroxide solution. Carry out a blank using all reagents in the same quantities but without the sample to be tested.
**Calculation**

Non-fat milk solids % by mass = \[ \frac{3126.2 \times (B-A) \times N}{W} \]

(on moisture free basis)

Where,

- \( B \) = volume in ml of standard sodium hydroxide solution used to neutralize the acid in the blank determination
- \( A \) = volume in ml of standard sodium hydroxide solution used to neutralize the excess of acid in the test with the sample
- \( N \) = normality of standard sodium hydroxide solution
- \( W \) = weight in g of the sample taken for the test


Also See A.O.A.C 17th edn, 2000 Official Method 939.02 Protein (Milk) in Milk Chocolate - Kjeldahl Method

Note: - Milk solids can also be determined from the orotic acid content which remains unchanged by heating during manufacture

[Ref:- Pearson’s Composition and Analysis of Foods 9th edn page384 – see method at clause 15.5 of Methods of Analysis for Cereal and Cereal Products]
C.5 Test for Rancidity

Reagent

Phloroglucin dihydrate: 0.1% in diethyl ether

Procedure

1. Take 10g of prepared sample.
2. Add 10ml of 0.1% Phloroglucin dihydrate solution.
3. Appearance of pink colour indicates presence of rancidity.

[Ref :- IS:7679-1978]

C.6 Determination of Ash Insoluble in dilute HCl

Reagents

(1) Dilute hydrochloric acid - Approx 5 N (445 ml in 1 litre).

Procedure

1. Weigh accurately about 10 g of the prepared sample in a tared, clean and dry platinum basin of 100ml capacity.
2. Carbonize the material in the dish with the flame of a burner.
3. Complete the ignition by keeping in a muffle furnace at $550 \pm 10^\circ C$ until gray ash results.
4. Cool in a desiccator.
5. To the ash, add 25ml of the dilute hydrochloric acid, cover with a watch glass and heat on a small flame of a burner to near boiling.
6. Allow it to cool and filter the contents of dish through Whatman filter paper No. 42 or its equivalent. Wash the filter paper with hot water until the washings are free from chlorides. (To check this, add few drops of 2M Nitric acid and 0.1 M Silver nitrate solution to the filtrate obtained. No precipitate or milky turbidity should occur in the solution, if it is chloride-free.)
7. Return the filter paper and the residue to the dish. Keep it in an air oven maintained at $105 \pm 2^\circ C$ for about three hours. Ignite in the muffle furnace at $550 \pm 10^\circ C$ for one hour.
8. Cool the dish in a desiccator and weigh. Heat again for 30 minutes in the muffle furnace, cool and weigh.
9. Repeat this process of heating for 30 minutes, cooling and weighing till the difference between two successive weighing is less than one milligram. Note the lowest mass.

**Calculation**

Acid insoluble ash, percent by mass $= \frac{100 \times M1}{M2}$

Where,

$M1$ = mass in g of the acid insoluble ash
M2 = mass in g of the prepared sample taken for the test


C.7 Determination of Milk Fat (in Milk Chocolate, White Chocolate)

Procedure

1. Extract sufficient quantity of fat (generally 7 g) from 25-30 g of sample using Soxhlet extraction method.
2. Weigh 5 g of fat and determine R.M Value.
3. Extrapolate the milk fat content from the observed R.M value taking the standard value of 28 for pure milk fat.

Determination of RM Value of extracted fat

Requirements

Apparatus

(1) Flat-Bottom Boiling Flask— The flask (A)) shall be made of resistance glass.
(2) Still-Head — The still-head (B) shall be made of glass tubing of wall thickness 1.25 ± 0.25 mm. A rubber stopper, fitted below the bulb of the longer arm of the still-head, and used for connecting it to the flask, shall have its lower surface 10 mm above the center of the side-hole of the still-head.

(3) Condenser — The condenser (C ) shall be made of glass.

(4) Receiver — The receiver (D) shall be a flask, with two graduation marks on the neck.

(5) Asbestos Board — An asbestos board (E), 120 mm diameter, 6 mm in thickness, with a circular hole about 65 mm in diameter, shall be used to support the flask over the burner.

(6) Bunsen Burner

(7) Reichert- Meissl Distillation Apparatus

Reagents

(1) Glycerine

(2) Conc. NaOH Solution : 50 % (w/w). Dissolve NaOH in an equal weight of water and store the solution in a bottle protected from carbon dioxide. Use the clear portion free from deposit.

(3) Pumice Stone Grains —1.4 to 2.0 mm in diameter.

(4) Dilute H2SO4 Solution -1 N.

(5) Standard NaOH solution-0.1 N.
(6) Phenolphthalein Indicator — Dissolve 0.1 g of phenolphthalein in 100 ml of 60 % rectified spirit.

(7) Ethyl Alcohol — 90%, v/v neutral to phenolphthalein.

Procedure

1. Weigh accurately 5.00 ± 0.01 g of the filtered oil or fat into the boiling flask.
2. Add 20 g of glycerol and 2 ml of conc. NaOH solution from a burette to which access of carbon dioxide is prevented and whose orifice is wetted before running in the liquid, the first few drops from the burette being rejected.
3. Heat the flask and its contents with continuous shaking on a gauze over the naked flame until the fat, including any drops adhering to the upper parts of the flask, has been saponified and the liquid becomes perfectly clear. Avoid overheating during this saponification.
4. Cover the flask with a watch glass, and allow the flask to cool a little. Add 90 ml of boiling distilled water, which has been vigorously boiled for about 15 min. After thorough mixing, the solution should remain clear. If the solution is not clear (indicating incomplete saponification) or is darker than light yellow (indicating overheating), repeat the saponification with a fresh sample of the oil or fat. If the sample is old, the solution may sometimes be dark and not clear.
5. Add 0.6 to 0.7 g of pumice stone grains and 50 ml of dilute sulphuric acid, and immediately connect the flask with the distilling apparatus. Place the flask on the asbestos board.

6. After the fatty acids have melted and separated into a clear liquid layer on gentle warming, heat the flask without altering the flame so that 110 ml of liquid distils over in the course of 19-21 min.

7. The distillation is considered to begin when the first drop forms in the still head.

8. Keep the water flowing in the condenser at a sufficient speed to maintain the temperature of the outgoing water from the condenser between 15°C and 20°C.

9. Collect the distillate in a graduated flask.

10. As soon as 110 ml have distilled over, stop heating the boiling flask and replace the graduated flask by a measuring cylinder of about 25 ml capacity to catch washings.

11. Close the graduated flask with the stopper, and, without making the contents, place it in a water-bath at 15°C for 10 min, making sure that the 100-ml graduation mark is below the level of the water. Swirl round the contents of the flask from time to time.

12. Dry the outside of the flask and then mix the distillate by closing the flask and inverting it four or five times, but do not shake.

13. Filter through a dry Whatman No. 4 filter paper. Reject the first 2-3 ml of the filtrate and collect the rest in a dry flask.

14. Pipette 100 ml of the filtrate in a titration flask, add 0.1 ml of phenolphthalein indicator solution and titrate with standard 0.1N NaOH solution until the liquid becomes slightly pink.
15. Run a blank test without the fat but using the same quantities of reagents and following the same procedure.

**Calculations:**

\[
\text{Milk Fat, \% by mass} = (\text{RV} - 0.2) \times F
\]

(on dry Basis) 26

Where,

\text{RV} = \text{Reichert value obtained for extracted fat}

\text{F} = \text{Total Fat \% in the sample}

0.2 = \text{Reichert value of cocoa butter}

26 = \text{Reichert value of milk fat}

C.8 Determination of Cocoa Solids

Procedure

1. Treat 50 g milk chocolate with three 100 ml portions of ether in centrifuge bottle, centrifuging and decanting after each addition.
2. Dry residue in bottle and crush to powder with flat end glass rod. Shake with 100 ml 1% Sodium oxalate $\text{Na}_2\text{C}_2\text{O}_4$ (w/v) and let stand 30 minutes.
3. Centrifuge and decant, wash in bottle with three 100 ml portions of water at room temperature shaking well each time until no cocoa material adheres to the bottle. Centrifuge 10 – 15 minutes after each washing and decant.
4. Wash residue in the same fashion with two 100 ml portions of alcohol and one 100 ml portion of ether. With the aid of small portions of ether, transfer residue resulting from ether, alcohol and aqueous extract to tared aluminium dish provided with tight fit cover. Use small amount of acetone and policeman to transfer any material that sticks to bottom.
5. Evaporate liquid carefully on steam bath and dry residue in oven at 100 °C.
6. Cool dish in dessicator and weigh till constant mass is achieved.

To obtain moisture free and fat free cocoa mass multiply the weight of residue with factor 1.43.
To obtain weight of chocolate liquor multiply the weight of residue with factor 2.2 (This factor is based on fat content of 54% in chocolate liquor).

[Ref: - A.O.A.C 18th edn, 2005 Official Method 931.05 Cocoa solids of chocolate liquor]

C.9 Determination of Reducing Sugars

Reagents:

(1) Fehling A: Dissolve 69.28-g copper sulphate (CuSO₄.5H₂O) in distilled water. Dilute to 1000 ml. Filter and store in amber coloured bottle.

(2) Fehling B: Dissolve 346 g Rochelle salt (potassium sodium tartrate) (K Na C₄H₄O₆. 4H₂O) and 100 g NaOH in distilled water. Dilute to 1000 ml. Filter and store in amber coloured bottle.

(3) Neutral Lead Acetate: Prepare 20% neutral lead acetate solution. (This reagent is used to clarify sugar solutions)

(4) Potassium Oxalate Solution: Prepare 10% Potassium oxalate (K₂C₂O₄.2H₂O) solution. This reagent is used to remove the excess lead used in clarification.

(5) Methylene Blue Indicator: Prepare 1% of methylene blue solution in distilled water.

Procedure & Calculations:

As per Clause A.9
C.10 Determination of Sucrose
As per Clause A.10

C.11 Determination of Chocolate Component of Filled Chocolate

Procedure
1. Weigh to the nearest 0.1g, 500g of the filled chocolate.
2. Scrape the chocolate coating and separate the filling.
3. Weigh the filling to the nearest 0.1g.

Calculation

\[
\text{Chocolate component, \% by mass} = \frac{(M_1 - M_2) \times 100}{M_1}
\]

Where,

\(M_1\) = mass in g, of the filled chocolate taken for test

\(M_2\) = mass in g, of the filling

C.12 Determination of Edible Wholesome Substances

**Procedure**

1. Weigh to the nearest 1.0g, 500g of the product containing fruits, nuts etc.
2. Break the sample into small pieces and place them in 1 litre glass/metal container.
3. Cover the sample with meted cocoa butter and place container in a warm oven until the added ingredients can be separated upon stirring.
4. Sieve contents through a 20 mesh sieve and allow the liquid to drain completely.
5. Next soak the sieve containing ingredients in trichloroethylene and stir gently for a minute or two.
6. Remove cleaned nuts, fruits etc. onto a tray and let the solvent evaporate. Weigh to the nearest 0.1g.

**Calculation**

Wholesome ingredients, % by mass = \[
\frac{\text{Mass of residue \times 100}}{\text{Sample wt.}}
\]

C.13 Determination of Total protein

As per Clause A.7

C.14 Determination of Pb, Cu & Zn

As per Clause A.12

C.15 Determination of filth

As per Clause A.14

C.16 Determination of TPC

As per Clause A.19

C.17 Determination of Coliform

As per clause A.20

C.18 Determination of Yeast & Mould

As per Clause A.21

C.19 Determination of E.Coli

As per Clause A.22
C.20 Determination of S. aureus

As per Clause A.23
D. ICE LOLLIES or EDIBLE ICES

D.1 Determination of Reducing sugars
As per Clause A.9

D.2 Determination of Sucrose
As per Clause A.10

D.3 Determination of Pb, Cu & Zn
As per Clause A.12

D.4 Determination of TPC
As per Clause A.19

D.5 Determination of Coliform
As per clause A.20

D.6 Determination of Yeast & Mould
As per Clause A.21
D.7 Determination of E. Coli

As per Clause A.22

D.8 Determination of S. aureus

As per Clause A.23

D.9 Isolation of Salmonella per 25 gm

The procedure consists of five distinct stages. The initial handling of the food and the non-selective enrichment stage (preenrichment) vary according to the type of food examined.

i) Non-Selective Enrichment (Preenrichment).
The test sample is initially inoculated into a non-inhibitory liquid medium to favour the repair and growth of stressed or sublethally-injured salmonellae arising from exposure to heat, freezing, desiccation, preservatives, high osmotic pressure or wide temperature fluctuations

ii) Selective Enrichment
Replicate portions of each preenrichment culture are inoculated into two enrichment media to favor the proliferation of salmonellae through a selective repression or inhibition of the growth of competing microorganisms.
iii) Selective Plating
Enrichment cultures are streaked onto selective differential agars for the isolation of salmonellae Purification Presumptive Salmonella isolates are purified on MacConkey agar plates or BSA plates.

iv) Biochemical Screening
Isolates are screened using determinant biochemical reactions.

v) Serological Identification
Polyvalent and/or somatic grouping antisera are used to support the tentative identification of isolates as members of Salmonella spp.

Materials and Equipment
(1) Nutrient Broth (NB).
(2) Brilliant Green Water.
(3) Buffered Peptone Water (BPW).
(4) Brilliant Green Broth (TBG).
(5) Selenite Cystine Broth (SC).
(6) Bismuth Sulfite Agar (BS).
(7) Brilliant Green Sulfa Agar (BGS).
(8) MacConkey Agar,
(9) Rappaport Vassiliadis medium (RV)
(10) Nutrient Agar.
(11) Triple Sugar Iron Agar (TSI).
(12) Lysine Iron Agar (LIA).
(13) Urea Agar (Christensen's).
(14) Commercial biochemical test kits.
(15) Polyvalent and single grouping somatic (O) and flagellar (H) antisera.
(16) N- Saline.
(17) Blender, stomacher or other homogenizing device.
(18) Incubator or water bath capable of maintaining 35±2°C and 44°C.

**Procedure**

(1) *Handling of Sample Units*
Analyze samples as soon as possible. If necessary, store samples under time and temperature conditions that will prevent the growth or death of native microflora.

(2) *Non-selective Enrichment (Pre-enrichment)*
Sample Analysis
The required analytical unit is dispersed into a suitable non-selective enrichment broth Nutrient broth (NB) and buffered peptone water (BPW) are equally reliable and can be used interchangeably as general purpose preenrichment. If the pH of the preenrichment mixture lies outside the range of 6.0 - 7.0, adjust with 1N NaOH or 1N HCl.
NOTE:

If the sample unit consists of a container with little food material, thoroughly rinse the interior of the container with a suitable preenrichment broth medium and incubate the rinse in a sterile flask. This eventuality is more frequently encountered in situations involving consumer complaints or food poisoning investigations. A positive Salmonella and a negative medium control should be set up in parallel with the test samples. Incubate the preenrichment mixture and the positive and negative controls at 35±2°C for 18 - 24 h.

NOTE:

The negative medium control should not show any evidence of growth after incubation whereas the absence of growth in the positive control would invalidate test results.

(3) Selective Enrichment
With a sterile pipette, transfer 1.0 mL of the preenrichment culture into each of 9 mL of selenite cystine (SC) and Rappaport Vassiliadis broths. Incubate SC and RV broths for 24±2 h at 35±2°C and 42±2°C, respectively.
(4) Selective Plating
Streak replicate loopsful of each selective enrichment culture onto BSA and BG agar to obtain well isolated colonies. The enrichment cultures may be streaked onto additional plating media for the isolation of Salmonella. Incubate plates at 35±2°C for 24±2 h. If colonies suggestive of Salmonella have not developed on BSA and BGA plates, incubate for an additional 24±2 h. Examine incubated plates for colonies suggestive of Salmonella. Typical Salmonella usually occur as pink to fuchsia colonies surrounded by red medium on BG agar, and as black colonies on BS agar with or without a metallic sheen, and showing a gradual H2S- dependent blackening of the surrounding medium with increasing incubation time.

(5) Biochemical Screening
With a sterile needle, inoculate suspect colonies into the biochemical media or in commercial diagnostic kits that would yield equivalent results. Incubate the biochemical media for 18-24 h at 35±2°C.

Note:
Erroneous biochemical results may be obtained if tubes are not loosely capped during incubation.

Commercially available diagnostic kits may be used to obtain detailed biochemical profiles of bacterial isolates. If none of the isolates from a
particular analytical unit are suggestive of Salmonella, the analytical unit is considered to be free of salmonellae. If the presence of Salmonella is suspected, proceed with serological testing. If serological testing is not to be performed within 72 h, inoculate suspect isolates into nutrient agar slants and incubate at 35±0.5°C for 24±2 h. Store the agar slants at refrigerator (4 to 10°C) temperature. Nutrient agar slants that have been stored for more than 72 h should not be used for serological testing. Prepare fresh agar slants for this purpose.

(6) Serological Identification Testing with somatic polyvalent antiserum

1. Mark the following sections on an agglutination plate: C+ (positive control), C- (negative control) and T (test culture).
2. Add one drop of physiological saline to each of the areas marked T and C+, and two drops to the area marked C-.
3. Remove sufficient culture material from a triple sugar iron, lysine iron or nutrient agar slant to prepare a heavy suspension in the test area (T) and in the negative control (C-) area. The inoculum should be withdrawn from the slope portion of agar slants.
4. For the positive control, prepare a heavy suspension of a known Salmonella culture in the area marked C+.
5. Prepare somatic polyvalent antiserum as directed by the manufacturer; add one drop to each of the areas marked T and C+.
7. Mix the culture-saline-antiserum suspensions in T and C+ and the saline-culture mixture in C- with a sterile needle or loop. Tilt the slide preparation back and forth for 1 min.

8. Hold the slide against a dark background and observe for agglutination. Salmonella cultures usually agglutinate within 1 min.

9. False positive reactions from microorganisms that are closely related to Salmonella may occur. Such misleading reactions can be resolved through further testing with somatic grouping and flagellar antisera.

10. The serological test for a given culture is invalidated if the negative control shows agglutination (autoagglutination).

(7) Reporting: Salmonella - Present or Absent / 25g

**D.10 Detection of Listeria monocytogenes per gm**

**Materials and equipment**

The following media and reagents are commercially available and are to be prepared and sterilized according to the manufacturer's instructions.

(1) Half Fraser Broth
(2) Fraser Broth
(3) Oxford Agar
(4) Palcam Agar
(5) Nutrient Agar

(6) Peptone water diluent (0.1%)(PW)/ N-Saline

(7) pH meter or paper capable of distinguishing to 0.3 to 0.5 pH units within a range of 5.0 to 8.0

(8) Stomacher, blender or equivalent for sample preparation/homogenization.

(9) Incubator capable of maintaining the growth temperature required for the specific type of aerobic bacteria being enumerated i.e. at 35°C.

Procedure

The test shall be carried out in accordance with the following instructions:

(1) Preparation of Media

1. Prepare Half Fraser, Fraser broth, Oxford and Palcam agar and dispense in appropriate quantities. Sterilize.
2. Clean surface of working area with a suitable disinfectant.
3. Clearly mark the duplicate Petri plates.

4. Pre-Enrichment

1. Prepare a 1:10 dilution of the food by aseptically homogenizing 25 g or mL (the analytical unit) into 225 mL of the required Diluent.
2. Take 10 ml of above diluted sample in 90 ml Enrichment broth and incubate for 24 hrs. at 30±2°C
3. Take 0.1 ml of above in 10 ml of Fraser broth and incubate for 48 hrs. at 37±2°C

5. Selective Enrichment
1. Transfer a loopful of the inoculum from enrichment culture in to Oxford and Palcam agar and incubate for 48 hrs at 37±2°C.
2. Observe for characteristics colonies on the Palcam and Oxford agar.

6. Biochemical Identification Tests
The biochemical tests conducted and interpreted as the reaction of the biochemical reagents as below:

1. Gram Staining
2. Catalase Test
3. Carbohydrate Fermentation Test
4. Nitrate Reduction Test
5. Haemolysis Test
6. Oxidase Test
7. Indole Test
8. Urease Test
9. H2S Test
10. VP Test
11. MR Test

Reporting: Listeria monocytogenes Present or absent / gm

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