

LAB. MANUAL 8



**MANUAL OF METHODS
OF
ANALYSIS OF FOODS**

FOOD ADDITIVES



**FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
MINISTRY OF HEALTH AND FAMILY WELFARE
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MANUAL FOR ANALYSIS OF FOOD ADDITIVES

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MANUAL FOR ANALYSIS OF FOOD ADDITIVES

1.0 Definition

The Codex Alimentarius Commission has defined “Food Additive” as under:-

Food Additive means any substance not normally consumed as a food by itself and not normally used as a typical ingredient of the food ,whether or not it has nutritive value, the intentional addition of which to food for a technological (including organoleptic) purpose in the manufacture, processing, preparation, treatment , packing, packaging transport or holding of such food results, or may be reasonably expected to result (directly or indirectly) in it or its bye products becoming a component or otherwise affecting the characteristics of such foods. The term does not include contaminants or substances added to food for maintaining or improving its nutritive value.

Food additives are intentionally added to food and must be safe for a lifetime of consumption based on current toxicological evaluation. The definition of food additive does not include contaminants. Thus pesticide residues, metallic contamination, Mycotoxins etc are excluded.

Food additives are used for the purpose of maintaining or improving the keeping quality, texture, consistency, appearance and other technological requirements. Food additives do not include use of vitamins, minerals, herbs, yeast, hops, starter cultures, malt extract etc. Food additives are classified on the basis of their functional use and are grouped as:

- Colours
- Preservatives
- Acidity Regulators
- Antioxidants
- Anticaking agents
- Antifoaming Agents
- Artificial sweeteners

- Enzymes
- Emulsifiers
- Emulsifying agents
- Flavours
- Flavour enhancers
- Modified Starches
- Stabilizers
- Thickening and Gelling agents.
- Foaming Agents
- Raising Agents
- Humectants
- Bulking Agent
- Colour retention Agents
- Firming Agent etc.

1.1 Preservatives:

Preservatives are the compounds used to prevent and retard the microbial spoilage of food. Section 3.1.4 of FSS (Food Product Standards and Food Additives) Regulations, 2011 defines preservative as “a substance which when added to food is capable of inhibiting, retarding or arresting the process of fermentation, acidification or other decomposition of food” They are classified into Class I and Class II preservatives.

Class I preservatives are

1. Common salt
2. Sugar
3. Dextrose
4. Glucose
5. Spices
6. Vinegar or acetic acid
7. Honey
8. Edible vegetable oils

Addition of class I Preservative in any food is not restricted

Class II preservatives are

1. Benzoic acid including salts thereof
2. Sulphurous acid including salts thereof
3. Nitrates or Nitrites and/or Sodium and Potassium in respect of foods like ham, Pickled meat
4. Sorbic acid and its sodium,
5. Potassium and calcium salts
6. Propionates of Calcium or sodium,
7. Sodium, Potassium and Calcium salts of Lactic acid.
8. Nisin
9. Methyl or Propyl parahydroxy Benzoates
10. Sodium Diacetate.

1.2 Benzoic Acid:

1.2.1 Qualitative Methods

(A) Ferric Chloride Test:

Acidify the food product with hydrochloric acid (1+3) and extract with diethyl ether. Evaporate the solvent on a hot water bath removing last traces of solvent under a current of air. Dissolve the residue in few ml of hot water and add few drops of 0.5% ferric chloride solution. Salmon colour precipitate of ferric benzoate indicates the presence of benzoic acid.

(B) Modified Mohler's Test:

To the aqueous solution of the residue obtained as given under method 'A' add one or two drops of 10% sodium hydroxide solution and evaporate to dryness. To the residue add 5-10 drops of sulphuric acid and a small crystal of potassium nitrate. Heat for 10 min in a glycerol bath at 120 – 130 ° C. Cool, add 1 ml of water and make distinctly ammonical. Boil

the solution to decompose any ammonium nitrite (NH_4NO_2) formed. Cool and add a drop of fresh colourless ammonium sulphide $[(\text{NH}_4)_2\text{S}]$ solution. The sulphide solution can be made by passing hydrogen sulphide in 0.88 ammonia. Do not let the layers mix. Red brown ring indicates benzoic acid. On mixing, colour diffuses throughout the liquid and on heating finally changes to greenish yellow. This change differentiates benzoic acid from salicylic acid cinnamic acid. Salicylic acid and cinnamic acid form coloured compounds which are destroyed on heating.

(Ref :- A.O.A.C 17th edn , 2000 Official method 910.02 (b) and (c) Benzoic acid in Foods / Pearsons Composition and Analysis of Foods 9th edn, 1991, page 83 / Manual Methods of Analysis for Adulterants and Contaminants in Foods. I.C M.R 1990, page 34)

1.2.2 Quantitative Methods:

(A) Titrimetric Method:

Principle:

Benzoic acid is separated from a known quantity of the sample by saturating with sodium chloride and then acidifying with dilute hydrochloric acid and extracting with chloroform. The chloroform layer is made mineral acid free and the solvent is removed by evaporation. The residue is dissolved in neutral alcohol and the amount of benzoic acid is determined by titration against standard alkali.

Reagents:

1. Chloroform -distilled
2. Hydrochloric acid (1+3)
3. Sodium hydroxide (10%)
4. Standard sodium hydroxide solution (0.05N)
5. Saturated sodium chloride solution.

Preparation of Sample:

(A) Beverages and liquid products:

Mix the sample thoroughly and transfer 100 gm of the sample into a 250 volumetric flask, using saturated sodium chloride solution. Make alkaline to litmus paper with 10% sodium hydroxide solution and make upto volume with saturated sodium chloride solution. Shake thoroughly and let it stand for 2 hrs. Filter the sample and use the filtrate for determination.

(B) Sauces and Ketchups:

Add 15 gm salt to 150 gm of weighed sample and transfer into volumetric flask. Rinse with saturated sodium chloride solution, Add 15 gm pulverized sodium chloride and then add 10 ml of 10% sodium hydroxide solution and make upto 500 ml volume with sodium chloride solution. Let it stand for 2 hrs with occasional shaking. Filter and use the filtrate for determination.

(C) Jams, Jellies, Preservatives and Marmalades:

Mix 150 gm of sample with 300 ml saturated sodium chloride solution. Add 15 gm pulverised sodium chloride. Add 10 ml of 10% sodium hydroxide solution. Transfer to 500 ml volumetric flask and dilute to volume with saturated sodium chloride solution. Let it stand for 2 hrs with frequent shaking, filter and use the filtrate for determination.

Determination:

Pipette 100 ml to 200 ml of the filtrate into a 250 ml separatory funnel. Neutralize to litmus paper using hydrochloric acid (1+3) and add 5 ml excess. Extract carefully with 40, 30, 30 and 20 ml portions of chloroform. Avoid formation of emulsion by shaking gently with rotatory motion. If emulsion forms, break it by stirring chloroform solution with a glass rod after each extraction, but do not drain any of the emulsion with chloroform layer. Transfer the combined chloroform extract in to a separatory funnel and wash it free from mineral acid by shaking gently and rinsing with water. Drain off the water phase. Dry the chloroform layer over anhydrous sodium sulphate and distil off the solvent.

Remove the last traces of the solvent under a current of air at room temperature. Dry the residue overnight or until no residue of acetic acid is detected if the product is a ketchup. Dissolve residue in 30-50 ml of alcohol neutralised to phenolphthalein and titrate with 0.05 N sodium hydroxide.

Calculate the benzoic acid contents as follows:

$$\text{Benzoic acid (ppm)} = \frac{122 \times \text{Titre} \times \text{Dilution} \times 1000 \times \text{ml of 0.05N sodium hydroxide}}{\text{Weight of sample} \times \text{aliquot taken (100 or 200ml of filtrate)}}$$

(Ref: - A.O.A.C 17th edn, 2000, Official Method 963.19 Benzoic acid in Foods Titrimetric Method)

(B) Spectrophotometric method:

Principle:

Benzoic acid is extracted from prepared sample using diethyl ether and the absorbance of the ether layer is measured at 272 nm, 267.5 nm and 276.5 nm in the UV region. From the corrected absorbance and the calibration graph obtained using standard benzoic acid solution, the amount of benzoic acid is determined.

Reagents:

1. Diethyl ether distilled
2. Hydrochloric acid (1+3)
3. Saturated sodium chloride solution
4. Ammonium hydroxide (0.1%)
5. Standard benzoic acid.

Procedure:

(a) Preparation of standard curve: Prepare solution of benzoic acid in ether containing 50 mgs/l. Determine absorbance of this solution in tightly stoppered cell in Beckman DU or recording spectrophotometer between 265 and 280 nm at 1 nm intervals. Plot absorbance against wavelength and record wavelength of minimum at approximately 267.5 nm as point B. Other minimum at approximately 276.5 nm as point D and highest maximum at approximately 272 nm as point C.

Prepare solution of benzoic acid in ether containing 20, 40, 60, 80, 100 and 120 mg/l. Determine absorbance of these solutions in a spectrophotometer at points B, C and D. For each concentration average absorbance at Band D subtract from absorbance at C.

Plot difference against concentration to get the standard curve.

(b) Preparation of sample: Mix sample thoroughly. Transfer 10 gm or 10 ml to separator and dilute to 200 ml with saturated sodium chloride solution. Make solution definitely acidic to litmus with hydrochloric acid and mix well.

(c) Determination: Extract prepared solutions with 70, 50, 40, and 30 ml portions of diethyl ether, shaking well to ensure complete extraction (break emulsions by standing, stirring or centrifuging). Drain and discard aqueous phase. Wash combined ether extracts with 40 and 30 ml portions hydrochloric acid (1+1000) and discard hydrochloric acid washings (if extraction requires no purification, proceed to next para). Extract ether solution with 50, 40, 30, and 20 ml portions of 0.1% ammonium hydroxide and discard ether. Neutralize combined ammonium hydroxide extracts with hydrochloric acid and add 1 ml excess. Extract the acidified solution with 70, 50, 40 and 30 ml ether.

Dilute combined ether extracts to 200 ml with ether and determine absorbance in stoppered cell in spectrophotometer at wavelengths B, C and D, diluting with ether if necessary to obtain optimum concentration of 20-120 mg/l. Average the absorbance's at B and D, subtract this value from absorbance at C. Determine the concentration of benzoic acid from standard curve correcting for dilutions.

Benzoic acid \approx 1.18 = sodium benzoate.

(Ref :- A.O.A.C 17th edn, 2000, Official method 960. 38 Benzoic acid in nonsolid food and beverages – Spectrophotometric Method / Manual Methods of Analysis for Adulterants and Contaminants in Foods I.C.M.R 1990, page 36).

(C) HPLC Method:

Principle: Benzoic acid is extracted and separated by liquid chromatography (LC) on C18 column, detected by ultra violet absorbance at 230 nm, and quantitated by standard calibration plot.

Apparatus:

- a) Liquid chromatograph equipped with pump, injector, and integrator or data system, and UV detector. Operating conditions: flow rate, 1.0 mL/min isocratic; column temperature, ambient; detector, 230 nm, 0.05 absorbance unit full scale (AUFS); and injection volume, 20 μ L.
- b) LC column. - C18, 4.6 \times 250mm length, 5 μ m.
- c) Ultrasonic bath.

Reagents:

- a) Solvents.—Acetonitrile and water (LC grade).
- b) Sodium Benzoate Standard.
- c) Potassium phosphate monobasic buffer. — Prepare 0.05M potassium dihydrogen orthophosphate; adjust the pH 3.0 with ortho phosphoric acid.
- d) LC mobile phase. —Acetonitrile–phosphate buffer (40 + 60). Combine 400 mL acetonitrile with 600 mL 0.05M potassium dihydrogen orthophosphate. De-gas in ultrasonic bath for 2 min and filter through 0.45 μ polyvinylidene fluoride filter.

Procedure:

(a) Standard preparation: Weigh accurately 25.0 mg of sodium benzoate std & transfer it into 100 ml volumetric flask. Dissolve it in water by sonication & make upto the volume.

This corresponds to 250 ppm of sodium benzoate. Dilute 1, 2, 4, 6, 8 and 10 ml of this standard solution to 50 ml with buffer, this corresponds to 5, 10, 20, 30, 40 and 50 ppm of sodium benzoate respectively. Filter these standards and inject. Plot a graph with concentration (ppm) against area and calculate the slope.

(b) Preparation of Test Solution: Weigh accurately 25.0 mg of sample & transfer it into 100 ml volumetric flask. Dissolve it in water by sonication & make upto the volume. Dilute 5 ml of this solution to 50 ml with buffer.(In case of thick samples weigh the sample in beaker dissolve using sonicator and then transfer to 100ml volumetric flask).Filter the extract through 0.45µ syringe filter. Use this filtered solution for the HPLC analysis. Inject the sample 20µl in HPLC.

(c) Chromatographic Condition:

Detector	:	UV – Visible
Wavelength	:	230nm
Flow rate	:	1 ml/min
Mobile Phase	:	Acetonitrile: Phosphate Buffer (40:60)
Injection volume	:	20 µl
Diluent	:	Water
Column temperature:		Ambient
Run time	:	20 min
Retention time	:	5 – 6 min

(d) Calculation: Inject separately 20 µL of standard solution, record the chromatograms. Develop calibration plot for standards and plot the regression equation for standard benzoate solution. Inject sample solution record the chromatograms and measure the peak responses and calculate the quantity of sodium benzoate.

$\text{Sodium Benzoate (ppm)} = \frac{A}{\text{Slope}} \times \frac{C}{W}$
--

Where

A= Peak area of sodium benzoate

C= Dilution factor

W= Weight of sample in gm.

(Ref: 37.1.62A AOAC Official Method 994.11 Benzoic Acid in Orange Juice Liquid Chromatographic Method)

1.2.3 Estimation of Benzoic acid in the presence of saccharin in Ready- to-serve Beverages:

Principle:

Benzoic acid and saccharin are extracted together from the acidified beverage using diethyl ether and the mixture is titrated with standard sodium hydroxide solution. Saccharin is estimated separately by colorimetric method and the titre equivalent to saccharin content in the sample is deducted from the total titre to calculate benzoic acid content of the sample.

Reagents:

- i. Diethyl ether (distilled)
- ii. Hydrochloric acid (1+3)
- iii. Anhydrous Sodium sulphate
- iv. Standard sodium hydroxide (0.05 N)

Procedure:

Mix the beverage sample and weigh 25 gm, and transfer in to a 250 ml separatory funnel. Add 10 ml of hydrochloric acid (1:3) and extract the contents of the funnel with 50, 40, 30 and 30 ml portions of diethyl ether. Wash combined ether extract with about 15 ml of water by gentle swirling to remove any traces of mineral acid and discard aqueous phase. Pass ether layer through anhydrous sodium sulphate and remove solvent on a water bath and the last traces by blowing air. Dissolve residue in neutralised alcohol and titrate against 0.05 N sodium hydroxide solution using phenolphthalein as indicator. The titre (A) gives the titre equivalent to the mixture of benzoic acid and saccharin.

Determine saccharin content of the sample following the colorimetric procedure given under non-nutritive sweeteners and calculate in parts per million (S). Calculate the titre (B) equivalent to saccharin content of the total sample from the equation:

$$B = \frac{W \times S \times 10^{-6}}{0.05 \times 0.00916} \text{ ml}$$

Where,
 W = Weight of the sample taken for estimation
 S = Saccharin content of the sample (in ppm)
 N = Normality of sodium hydroxide used for titration.

Calculate Benzoic acid content of the sample (ppm) from the equation,

$$\frac{(A - B) \times \text{sodium hydroxide} \times 122 \times 10^3}{W}$$

Sodium hydroxide = Normality of sodium hydroxide used for titration
 W = Weight of the sample taken
 A = Titre corresponding to total sample
 B = Titre corresponding to saccharin content

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods I.C.M.R 1990, page 36)

1.3 BENZOIC AND SORBIC ACID

1.3.1 Qualitative Method:

Principle:

These preservatives are separated by steam distillation, extracted into ether from the acid solution and the ethereal extract is examined by TLC.

Reagents:

- (i) 50% hydrochloric acid (v/v).
Diethyl ether (peroxide free): The absorbance characteristics of this must be checked and if necessary the ether redistilled. Petroleum ether or cyclohexane may be used in place of ether as long as the absorbance in the range 220 - 290 is acceptably low e.g. by use of spectroscopically pure grade.
- (ii) TLC developing solvent-Ethanol: Ammonia (9:1)
- (iii) Standard solution: 1% benzoic acid and Sorbic acid in ethyl acetate.
- (iv) Peroxide- ferric chloride spray reagent: Mix equal volume of freshly prepared 2% ferric chloride and 0.5% hydrogen peroxide solution.
- (v) Thiobarbituric acid spray reagent: 0.2% solution in water of 2- thiobarbituric acid.
- (vi) Solid magnesium sulphate heptahydrate.
- (vii) 1M sulphuric Acid.
- (viii) 1M sodium hydroxide.
- (ix) TLC plates and silica gel G.

Procedure:

Place a weighed portion, usually 25 -50 gm of sample in a one L steam distillation flask, add 100 gm of magnesium sulphate and 100 ml 1M sulphuric acid. Steam and distill as rapidly as possible, collecting about 450 ml in 10 min steam-distillate in the flask containing 10 ml 1 M sodium hydroxide solution. Heating the flask containing the sample may result in a coloured or impure distillate. Add 15 ml 1 M sulphuric acid to the distillate and dilute to 500 ml. Extract and aliquot (100 ml) with three or four 25 ml portions of diethyl ether or other solvent. Combine the extracts and wash them with a few ml of water Dry the combined solvent layer over anhydrous sodium sulphate and reduce to 1 ml at the lowest temperature possible. Use of a rotary evaporator is preferable Spot 20 µl or less on the silica gel G TLC plate along with standard solution. Develop for about 10 cm using developing solvent. Air dry the plate and spray with peroxide-ferric chloride reagent. Benzoic acid shows as a mauve coloured spot (R_f 0.5) and Sorbic acid may be distinguished as a yellow coloured spot slightly below it. Further spraying with TBA solution and heating at 100°C for 5 min, Sorbic acid appears as a pink spot a little below benzoic acid (R_f 0.45)

(Ref: - F.A.O Manuals of Food Quality Control 14/2 1980, page10 /Pearsons Composition and Analysis of Foods 9th edn 1991, page89)

1.3.2 Sorbic Acid - Quantitative Method:

(A) UV Spectrophotometric Method: (Applicable to fresh dairy products, cottage cheese, ricotta and mozzarella cheese, sour cream and yoghurt)

Principle:

Sorbic acid is extracted from the sample using the solvent mixture of diethyl ether and petroleum ether (1: 1) and absorbance of the extract is measured at 250 nm. Sorbic acid in another aliquot is destroyed with permanganate and absence of the peak at 250 nm is taken as confirmation of the presence of sorbic acid in the sample.

Apparatus

- (i) Spectrophotometer

Reagents:

- (i) Metaphosphoric acid Solution: Dissolve 5 gm in 250 ml water and dilute to 1 L with alcohol.
- (ii) Mixed ethers (40-60): Petroleum ether and anhydrous diethyl ether (1+1).
- (iii) Potassium permanganate solution: Dissolve 15 gm in 100 ml water.
- (iv) Sorbic acid standard solution (1 mg/ml): Dissolve 100 mg and make up to 100 ml with mixed ethers.
- (v) Working standard solution: Dilute 5 ml to 100 ml with the solvent.
- (vi) Reference solution: Shake 10 ml of mixed ethers with 100 ml of phosphoric acid solution and dry the supernatant ether layer with anhydrous sodium sulphate.

Procedure:

Homogenise the sample (cheese and related products) by cutting into small pieces using a food chopper or by shredding it over a sieve. With creamed Cottage and similar cheeses place 300 – 600 gm of sample at 15°C in a 1 L cup of a high speed blender and blend for the minimum time (2 – 5 min) required to obtain a homogeneous mixture. Accurately weigh about 10 gm of the prepared sample, in a high speed blender, add enough phosphoric acid to yield a total of 100 ml of liquid in the mixture. Blend for one minute and immediately filter through Whatman No.3 paper. Transfer 10 ml of filtrate to a 250 ml separator containing 100 ml of mixed ethers and shake for one minute. Discard the aqueous layer and dry the ether extract over 5 gm of anhydrous sodium sulphate and read the absorbance at 250 nm against reference solution. Determine the concentration of sorbic acid from the standard curve prepared as follows

Add 1, 2, 4 and 6 ml of working standard solution to 100 ml volumetric flask and dilute to volume with mixed ethers. Determine the absorbance at 250 nm against mixed ethers. Plot absorbance (A) against mg sorbic acid / 100ml determine the sorbic acid content of the

sample from its absorbance by making use of standard curve.

The final result may be expressed in ppm.

$$\% \text{ sorbic acid} = (\text{mg sorbic acid} / \text{gm sample}) \times (1 / 1000) \times 100$$

Confirm the presence of sorbic acid as follows:

Add 2 ml potassium permanganate solution to the remaining ether solution and shake for one minute. Filter the ether layer through Whatman No.1 paper, dry over anhydrous sodium sulphate and take absorbance readings between 220 and 300 nm. Absence of peak at 250 nm confirms the presence of sorbic acid this confirmation is advisable in addition to TLC or other qualitative tests.

(Ref: - A.O.A.C 17th edn, 2000 Official method 974.10 Sorbic Acid in Dairy Products Spectrophotometric Method / F.A.O Manuals of Food Quality Control, 14 / 2 1980, Page 13)

(Ref :- F.A.O Manuals of Food Quality Control 1986, 14 / 7, Page 60 /Pearsons Composition and Analysis of Foods 9th edn , 1991, Page 89)

1.4 p - Hydroxy Benzoates (Parabens):

(A) Detection of p-hydroxy benzoic acid and its esters by TLC:

Principle:

The sample is acidified and extracted with diethyl ether. The concentrated ethereal extract is subjected to TLC. Using U.V or Denige's reagent for visualization

Apparatus

- (i) TLC Apparatus.

(ii) Viewing cabinet with short wave (254 nm) UV light.

Reagents:

(i) Silica gel G

(ii) Developing solvent: Toluene: methanol: acetic acid (90: 16: 8).

(iii) 2% solutions in diethyl ether (methyl, ethyl or propyl hydroxy benzoates and the free acid).

(iv) Denige's reagent: Mix 5 gm of yellow mercuric oxide with 40 ml water, cool in ice-salt and very slowly add freezing cold sulphuric acid (20 ml) with stirring. Add another 40 ml of water.

(v) Sulphuric acid – 10% by volume.

(vi) Sodium nitrite - 2% freshly prepared.

(vii) Sodium sulphate - anhydrous.

(viii) Solvent ether.

Procedure:

Add 5 ml of 10% sulphuric acid to 10 gm of sample and grind with sodium sulphate in a mortar until the sample is dry. Add about as much sodium sulphate again. Grind the sample with small successive quantities of ether and decant the ether. Filter the ether extract, evaporate at a low temperature and dissolve the residue in methanol (1 ml).

Spot 20 µl along with standard and about the same amount of the 2 % standards on the TLC plate and develop the chromatogram with developing solvent. View the plate under UV (254 nm). Para-hydroxy benzoates show black spots. Interfering substances may be present,

so caution should be used in interpreting the results Mark any quenched area lightly with a pin and spray lightly with Denige's reagent. P-hydroxy benzoate gives a white spot, visible by its different reflectivity from the background. Heat at 100°C for 5 min and spray lightly with 2% sodium nitrite solution.

Appearance of red spots indicate the presence of p-hydroxy benzoic acid esters.

Interpretation

If the object of the test is to confirm that the amounts of any p – hydroxy benzoates present are below the prescribed limit, the quantities of the standard spotted can be chosen to correspond to that maximum. Sample spots of lower intensity are taken to indicate compliance.

(Ref:- F.A.O. Manuals of Food Quality Control 1980, 14 / 2 Page 12 / F.A.O Manuals of Food Quality Control 1986, 14 / 7 Page 58)

(B) Qualitative test for para -hydroxy benzoic acid:

The test is applied on neutral ammonium salt of para - hydroxy benzoic acid. Extract 4-hydroxy (para) benzoic acid from the acidified food with ether and remove the solvent. Dissolve residue in few drops of dilute ammonium hydroxide solution in a test tube. Add a few drops of Millon's reagent (dissolve 3 ml mercury in 27 ml cold fuming nitric acid and dilute with an equal volume of water). Presence of 4-hydroxy benzoic acid is revealed by rose-red colour. Many aromatic substances with a hydroxyl group attached to the benzene ring give red colour. (eg. salicylic acid gives orange red colour with Millon's reagents). The test cannot be considered specific for 4 – hydroxyl benzoic acid. Salicylic acid can however be distinguished by intense violet colour given with ferric chloride

(Ref: - Pearsons Composition and Analysis of Foods 9th edn, 1991Page 85)

1.4.1 Quantitative Method:

Principle:

The 4-hydroxy benzoic acid esters present in the sample are hydrolysed using alkali and is extracted with diethyl ether after acidification of the sample. After re-extraction with sodium hydroxide from ether, colour is developed with Denige's reagent and the absorption is read at 518 nm.

Reagents:

- (i) Dilute sulphuric acid: Dilute 100 ml conc. sulphuric acid to 300 ml with water.
- (ii) Denige's reagent: Dissolve 5 gm of mercuric oxide in 20 ml of conc. sulphuric acid and dilute to 100 ml with water.
- (iii) Potassium ferrocyanide (15%): Prepare in water.
- (iv) Zinc sulphate (30%): Prepare in water.
- (v) Sodium hydroxide (5%): Prepare in water.
- (vi) Diethyl ether.
- (vii) Sodium nitrite (2%): Freshly prepared in water.

Procedure:

To 2 gm of the sample add 60 ml water at 50°C and adjust the pH to 7.5 with sodium hydroxide (5% solution). Heat at 50°C for 30 min with occasional stirring. Add 2 ml of potassium ferrocyanide and mix carefully. Add 2 ml zinc sulphate, mix and dilute to 100 ml with water and set aside for 30 min Filter, take 50 ml filtrate and add 1 ml of dilute sulphuric acid. Extract with 3 x 50 ml portions of diethyl ether. Wash the combine ether extracts with

water (3 × 5 ml/30 sec), add a drop of phenolphthalein and shake with 3 ml of 0.25M sodium hydroxide solution. Wash with 3 ml of water and combine the alkaline extracts, remove any traces of ether on hot water bath and make upto volume (10 ml).

Take 5 ml of solution and add 5 ml of Denige's reagent. Heat in a boiling water bath for 5 min Cool, add 5 drops of 2% aqueous sodium nitrite solution and allow to stand for 45 min Measure the absorbance of pink colour at 518 nm.

Dissolve 50, 100, 200, 400 and 600 mg of ester in 3 ml quantities of 0.25N sodium hydroxide, make upto 5 ml with water and carry out the above method starting from addition of 5 ml Denige's reagent to prepare a calibration graph and determine concentration

(Ref: - Pearsons Composition and Analysis of Foods 9th edn, 1991, Page 86).

Estimation of Benzoic acid, sorbic acid and parabens from food samples:

HPLC-UV method:

Principle:

Benzoic acid, Sorbic acid and Parabens are separated from a known quantity of the sample by saturating with sodium chloride and then acidifying with dilute hydrochloric acid and extracting with chloroform. The chloroform layer is evaporated to and the residue is dissolved in neutral alcohol and the amount of benzoic acid is determined by HPLC-UV method.

Reagents:

- a. Anhydrous ethanol AR grade
- b. Methanol HPLC grade
- c. Glacial acetic acid - HPLC grade
- d. Deionized water (DI) - $\geq 10 \text{ M}\Omega$
- e. Ammonium acetate - HPLC grade
- f. Chloroform - HPLC grade
- g. Acetonitrile - HPLC grade

- h. Sodium Chloride – AR grade
- i. Hydrochloric acid – AR grade
- j. Anhydrous sodium sulfate – AR grade

Preparation of samples:**Beverages and liquid products:**

Mix the sample thoroughly and transfer 100 gm of the sample into a 250 mL volumetric flask, using saturated sodium chloride solution. Make alkaline to litmus paper with 10% sodium hydroxide solution and make up to volume with saturated sodium chloride solution. Shake thoroughly and let it stand for 2 hrs. Filter the sample and use the filtrate for determination.

Sauces and Ketchups:

Add 15 gm salt to 150 gm of weighed sample and transfer into volumetric flask. Rinse with saturated sodium chloride solution, Add 15 gm pulverized sodium chloride and then add 10 ml of 10% sodium hydroxide solution and make up to volume with sodium chloride solution. Let it stand for 2 hrs with occasional shaking. Filter and use the filtrate for determination.

Jams, Jellies, Preservatives and Marmalades:**A. Preparation of sample**

Mix 150 gm of sample with 300 ml saturated sodium chloride solution. Add 15 gm pulverised sodium chloride. Add 10 ml of 10% sodium hydroxide solution. Transfer to 500 ml volumetric flask and dilute to volume with saturated sodium chloride solution. Let it stand for 2 hrs with frequent shaking, filter and use the filtrate for determination.

Methodology:**B. Preparation of stock solutions:**

Stock standards (4.0 mg/mL benzoic acid, sorbic acid, methyl, ethyl, propyl, and butyl parabens):- Weigh 400.0 mg each of benzoic acid, sorbic acid, methyl, ethyl, propyl, and butyl parabens into a 100 mL volumetric flask. Add approximately 50 mL 70% ethanol to dissolve, and dilute to volume with 70% ethanol. Dilute the stock solutions to 0, 10, 20, 40, 60, 80 and 100 µg/mL in 70% ethanol for preparation of calibration curves.

Determination:

A. Preparation of sample

Pipette 100 ml to 200 ml of the filtrate into a 250 ml separatory funnel. Neutralize to litmus paper using hydrochloric acid (1+3) and add 5 ml excess. Extract carefully with 40, 30, 30 and 20 ml portions of chloroform. Avoid formation of emulsion by shaking gently with rotatory motion. If emulsion forms, break it by stirring chloroform solution with a glass rod after each extraction, but do not drain any of the emulsion with chloroform layer. Transfer the combined chloroform extract in to a separatory funnel and wash it free from mineral acid by shaking gently and rinsing with water. Drain off the water phase. Dry the chloroform layer over anhydrous sodium sulphate and distil off the solvent. Remove the last traces of the solvent under a current of nitrogen at room temperature. Dissolve residue in 100 ml of alcohol.

HPLC – UV method:

Column: Column - 15cm ×4.6mm ID, C-18, 5 µm particle size

Mobile phase A: 1.5% acetic acid + 1.5% ammonium acetate in DI water

Mobile phase B: 100% methanol

Gradient: (Flow rate: 1.0 mL/min)

Detections – 254 nm UV (by using UV/PDA detectors)

Injection – 10/20 µL of sample

The samples shall be injected in the sequence

a. Blank

- b. Standards
- c. Blank
- d. Reconstituted extract from the samples
- e. Quality control standards (20 µg/mL and 60 µg/mL standards samples can be used for quality control)

Chromatogram: The components are eluted in the sequence, Benzoic acid, Sorbic acid, methyl, ethyl, and propyl and butyl parabens.

Calculations:

Calculate concentration of each preservative in sample as follows:

Using peak areas or peak heights and concentrations of standards, construct linear standard curve for each compound based on formula $y = mx + C$, where 'x' is concentration (ppm), y is peak area or height, m is slope, and c is the intercept. Calculate recovery of fortified sample and sample results.

Reference:

Ali, M. Sher. J. Assoc. Off. Anal. Chem., 1985, 68. 488-492.

Note: Relevant ISO methods can also be used as alternative methods.

US ISO 22855:2008 - Fruit and vegetable products — Determination of benzoic acid and sorbic acid concentrations — High-performance liquid chromatography method First Edition 2009-mm-dd

ISO 9231:2008 (HPLC-UV) method for determination of benzoic acid and sorbic acid contents.

1.5 Sulphur Dioxide:

Sulphur dioxide is a widely accepted preservative for many food products such as beverages, squashes, grape resins, dehydrated food products, caramel etc. It is also used for bleaching of sugars and often occurs as a residual component in sugar samples.

1.5.1 Qualitative test:

(1) Add small amount of sulphur free zinc and several ml hydrochloric acid to approximately 25 gm sample (with addition of water, if necessary) in 200 ml Erlenmeyer flask. Hydrogen sulphide generated may be detected with lead acetate paper. Traces of metallic sulphides occasionally present in vegetables give same reaction as sulphites under conditions of above test.

Verify positive results obtained by this method by Monier-Williams quantitative method.

(Ref: - A.O.A.C 17th edn, 2000 Official Method 975.32 Sulphurous Acid in Food Qualitative Test)

(2) Qualitative Test

Reagents

(1) Iodine - Barium chloride - Dissolve 3 gm Iodine in water containing 3 gm Potassium Iodide. Add 2 gm Barium Chloride dissolved in water and dilute to 100 ml

Apparatus

(1) Conical flask with a small bubbler in the form of a small thistle funnel bent twice in the stem so that gases evolved pass through the reagent placed in the funnel.

Procedure

Place 5 gm sample in the flask, add 0.1 gm copper acetate, a piece of marble and 10 ml of conc hydrochloric acid and fit on the bubbler. Allow the acid to act on the marble for 10 min and then heat to boiling. The iodine is decolorized and in the presence of sulphur dioxide a precipitate of barium sulphate settles in the tube. The formation of turbidity is inconclusive as it may be due to other substances such as volatile oils.

(Ref :- Pearsons Composition and Analysis of Foods 9th edn , 1991, Page 71 / F.A.O Manuals of Food Quality Control 1980, 14 / 2 Page3)

1.5.2. Quantitative Method:

(A) Modified-Monier-Williams Method

Apparatus:

- a) Conical flasks
- b) Beakers & pipette
- c) Distillation Apparatus:

(Note: In this method, back pressure inside apparatus is limited to unavoidable pressure due to height of 3% hydrogen peroxide solution above tip of bubbler (F). Keep back pressure as low as possible to avoid loss of sulphur dioxide through leaks. Use thin film of stop cock grease on sealing surfaces of all joints except joint between separatory funnel and flask. Clamp together each joint to ensure complete seal throughout analysis.)

Assemble apparatus which includes:

- 1) Inlet adapter (A)
- 2) Separatory funnel (B)
- 3) Round-bottom flask (C)
- 4) Gas inlet tube (D)
- 5) Allihn condenser (E)
- 6) Bubbler (F)
- 7) Vessel (G)

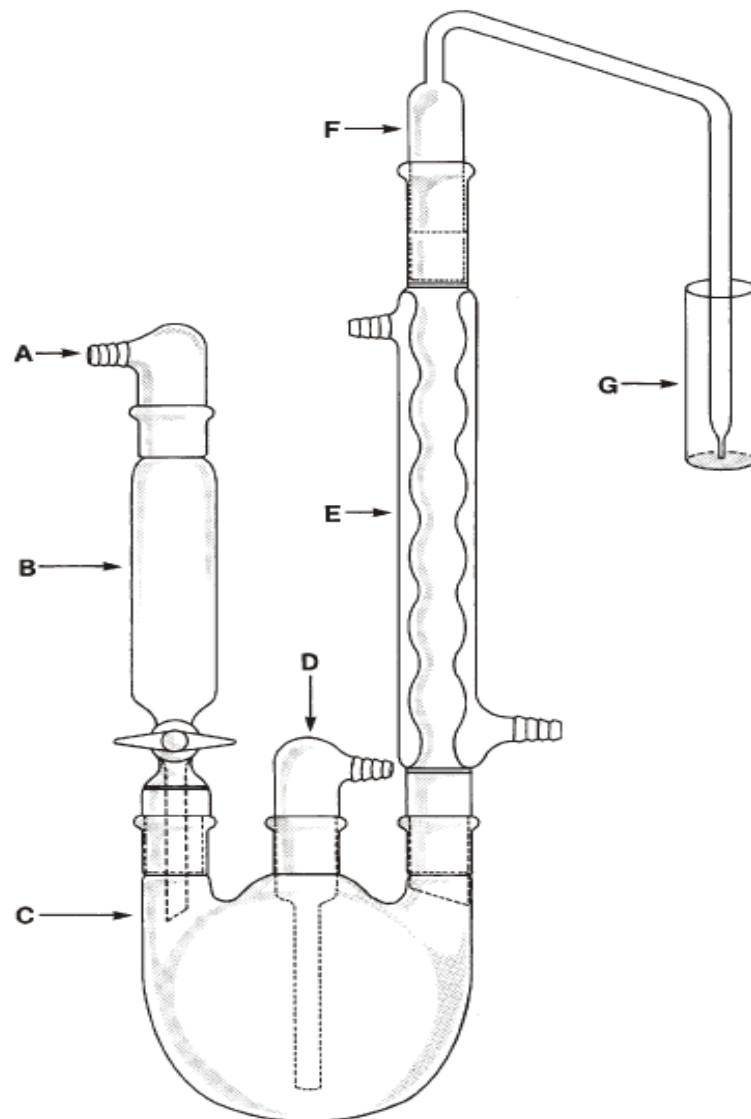


Figure 1: Apparatus for optimized Monier-Williams Method:

Reagents:

(i) Hydrogen peroxide solution (3%): Dilute 100 ml of 30% hydrogen peroxide to about 700 ml in a 1000 ml graduated cylinder. Take 100 ml portion of diluted solution and titrate in a 250 ml conical flask with 0.01 N sodium hydroxide to pH 4.1 using pH meter. Calculate the amount of sodium hydroxide required neutralizing the main solution, adding this amount, stirring and checking the pH. To standardise the hydrogen peroxide, pipette 10 ml of the solution into a 100 ml volumetric flask and make up to volume. Pipette 5 ml of this diluted solution into a 500 ml flask, add about 300 ml of water and 10 ml of 6N sulphuric acid and titrate with 0.1 N potassium permanganate to a permanent pink colour.

1 ml of 0.1N Potassium permanganate = 0.0017 gm hydrogen peroxide.

- (ii) Bromophenol blue solution (0.1%): Dissolve 0.1 gm in 100 ml water.
- (iii) Phenolphthalein indicator (0.1%): Dissolve 0.1 gm in 50 ml ethyl alcohol and dilute to 100 ml with water.
- (iv) Sodium carbonate- saturated solution: Dissolve enough sodium carbonate to prepare a saturated solution. Add several drops of phenolphthalein. Discard this solution when it becomes decolourised.

Procedure:

Circulate cold water through condenser. Add from a graduated cylinder 20 ml of 3% hydrogen peroxide to the conical flask and 5 ml to the trap. Assemble and connect to condenser. Weigh 50 gm of blended or well mixed portion of the sample, transfer into 500 ml flask through gas inlet tube, using 300 ml of water. Replace gas inlet tube immediately, making sure all connections are well greased and tight. Remove the gas inlet tube, and slowly add 20 ml of conc. hydrochloric acid. Replace the tube and see if bubbles nitrogen enters the receiving flasks. If not, check joint for leaks. Adjust nitrogen (99.9% pure) to give a flow of 15-20 bubbles per minute through the tube. Heat the solution to boil. Dried fruits and vegetables require boiling for one hour. For all other products 30 min boiling is enough. After the solution boils for the required time, wash the hydrogen peroxide solution from the trap into the conical flask. Rinse the trap with water. Add 3 drops of bromophenol blue indicator and titrate with 0.05N sodium hydroxide solution to a pale sky-blue end point using 5 ml micro burette. Run a blank titration on 20 ml, of hydrogen peroxide and correct results accordingly.

1 ml of 0.01N sodium hydroxide = 32.03 mg of sulphur dioxide

	32.03 × Titer × Normality of
	sodium hydroxide ×1000
µg/gm (ppm) of sulphur dioxide =	-----
	Sample Weight

(Ref: 47.3.43; AOAC Official Method 990.28 Sulphites in Foods; Optimized Monier-Williams Method)

Note: Relevant ISO methods can also be used as alternative methods.

ISO 5522:1981 Fruits, vegetables and derived products — Determination of total sulphur dioxide content.

(B) Colorimetric Method: (Applicable to dry fruits)**Principle:**

Sulphur dioxide bleaches p-rosaniline and by addition of formaldehyde, sulphur dioxide forms an additional product releasing free rosaniline which is pink in colour, the amount of free rosaniline released is equivalent to the sulphur dioxide present.

Reagents:

- (i) Formaldehyde solution (0.015%): Prepare by diluting 40% formaldehyde in two steps 10 to 1000 and 75 to 2000.
- (ii) Acid bleached p-rosaniline hydrochloride: Place 100 mgs p-rosaniline-hydrochloric acid and 200 ml water in 1 L volumetric flask. Add 150 ml hydrochloric acid (1+1) and dilute to volume. Let stand 12 hrs before use.
- (iii) Sodium tetra-chloro mercurate: Place 23.4 gm sodium chloride and 54.3 gm mercuric chloride in a 2 L volumetric flask. Dissolve in approximately 1000 ml water, dilute to volume.
- (iv) Sulphur dioxide standard solution: Dissolve approximately 170 gm sodium bisulphate in water and dilute to 1 L. Standardize with 0.01N iodine solution before use (approximately 100 µg sulphur dioxide/ml.)

Preparation of Standard Curve:

Add 5 ml mercurate reagent to series of 100 ml volumetric flasks. Then add 0, 1, 2, 3 ml of sulphur dioxide standard solution. Dilute to volume with water and mix.

Transfer 5 ml portions to 200 mm test tubes containing 5 ml rosaniline reagent. Add 10 ml 0.015% formaldehyde solution, mix and hold for 30 min at 22°C. Read the absorbance against zero standard (blank) and plot standard curve.

Determination:

Weigh 10 ± 0.02 gm ground dried fruit and transfer to blender with 290 ml water. Cover and blend for 2 min. Withdraw 10 gm aliquot from bottom of blender with 10 ml calibrated free running pipette and transfer to 100 ml volumetric flask containing 5 ml 0.5N sodium hydroxide (use 2 ml for apples and 1 ml for golden raisins). Swirl and mix approximately for 15-30 seconds. Add 4 ml 0.5N sulphuric acid (2 ml for apples and 1 ml for golden raisins) and 20 ml mercurate reagent and dilute to volume. For blank, omit 10 ml fruit extract.

Transfer 2 ml sample solution to 200 mm test tube containing 5 ml rosaniline reagent. Add 10 ml of 0.015% formaldehyde solution and mix and hold it for 30 min at 22°C. Read absorbance at 550 nm against blank. Refer to standard curve and convert results to ppm sulphur dioxide.

(If same colorimetric tube or cell is used for successive sample, clean between use with hydrochloric acid (1+1) and water).

(Ref :- A,O,A,C 17th edn, 2000 Official Method 963.20 Sulphurous acid in Dried Fruit – Colorimetric Method / Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990, Page 43)

1.6 NITRATE AND NITRITE IN FOODS

Sodium and potassium salts of nitrate and nitrite are added mainly to preserve meat and meat products such as cured meat and meat pickles.

Determination of Nitrate and Nitrite in Foods**Principle:**

The sample is clarified with alumina cream and the amount of nitrate present determined by allowing it to diazotise arsenilic acid and coupling the diazonium salt with n-1-naphthylethylene diamine. The colour so formed is extracted into n-butanol and the

absorbance is measured at 545 nm. An aliquot of the sample is mixed with spongy cadmium in order to reduce any nitrate present and the nitrite so produced is determined in the same way. The amount of nitrate present is then calculated by subtracting the nitrite from the total.

Reagents:

- (i) Water: This may be distilled or de-ionised but a blank must be carried out to check that it is of satisfactory quality for the preparation of the spongy cadmium.
- (ii) Alumina Cream: Prepare a saturated solution of potassium aluminium sulphate and add ammonia slowly with stirring until pH is 7.0.
- (iii) N-naphthylethylene diamine: dihydrochloride 0.1% in distilled water
- (iv) Arsenic acid monohydrate: 0.1% in 5M hydrochloric acid.
- (v) Buffer pH, 9.6: Prepare 0.7M ammonium chloride (37.45 gm/l) in distilled water and add 0.88 ammonia until the pH is 9.6.
- (vi) Spongy cadmium: Place zinc rods in 20 % aqueous cadmium sulphate solution and leave for 3 or 4 hrs. Separate the precipitated cadmium, wash twice with distilled water and then macerate with water for 2-3 min. Activate by shaking with 2M hydrochloric acid and then wash at least 5 times with distilled water, keep the cadmium under distilled water and prepare freshly for each batch of determination.
- (vii) Standard nitrite solution: Weigh out 0.4783 gm of sodium nitrite and dilute to one L with water. Dilute this 10 times to get 10 mgs /1 of nitrite nitrogen.
- (viii) n- butanol

Procedure:

Mix the sample thoroughly by macerating or homogenizing and weigh 5 gm into a 150

ml beaker. Add 50 ml water and heat to 80°C stirring gently.

Maintain at 80°C for 10 min add 20 ml alumina cream and transfer gently to a 100 ml volumetric flask. Cool and dilute to volume with water. Mix and filter through Whatman No.4 filter paper rejecting the first 10 ml of filtrate.

The filter paper must be previously washed with at least 100 ml of hot water to remove the small amounts of nitrate that it may have contained

(a) Determination of nitrite:

Pipette 10 ml of filtrate into a 50 ml volumetric flask, add 2 ml of arsenic acid solution and mix. Leave for 5 min, and then add 2 ml of naphthylethylenediamine solution. Mix and leave for 10 min. If the solution is clear, dilute to 50 ml with water and read the absorbance at 538 nm using a 1 cm cell. If the solution is cloudy, transfer to a 100 ml separator, saturate with salt and extract with n-butanol using 20, 15 and then 5 ml. Pass the butanol extracts through a small cotton pledget in a funnel into a dry 50 ml calibrated flask and dilute to volume with n-butanol. Read the absorbance at 545 nm in a 1 cm cell.

(b) Determination of nitrate:

Pipette 10 ml of filtrate into a small stoppered conical flask. Add 5 ml of buffer solution and one gram of wet cadmium. Stopper the flask and shake for 5 min filter the solution through a washed filter paper into a 50 ml volumetric flask rinsing the cadmium and the filter paper with 5ml water. Determine the nitrite in the filtrate as given above starting at “add 2 ml of arsenic acid solution”.

(c) Preparation of standard curve:

Pipette into a series of 50 ml volumetric flasks dilute standard solution of sodium nitrite containing 2-15 µg of nitrite nitrogen and develop the colour as given in the procedure for nitrite. Read the absorbance and plot standard curve. Repeat the experiment and extract the colour with n-butanol and read the absorbance at 545 nm and also plot a standard curve

for this solvent. From the graph calculate the nitrite content before and after reduction and calculate the nitrate content by subtraction.

(Ref: - F.A.O Manuals of Food Quality Control 14 / 2, 1980, Page 22)

NOTE: For the purpose of sensitive Quantitation at lowest levels an EPA 300 A or ion-pair electrode test for the quantification of nitrate and nitrite may also be used.

1.7 PROPIONIC ACID AND ACETIC ACID

Qualitative Test:

Principle:

Volatile acids such as acetic, propionic, butyric and valeric acids are steam distilled and the distillate is concentrated after neutralization. Separation of these acids is achieved by Paper Chromatography and visualized by spraying with methyl red and bromothymol blue.

Reagents:

Developing solvent- Acetone: tert-butanol: n-butanol: ammonia (2+1+1+1) Prepare fresh.

Spray reagents: Add 200 mgs of each of methyl red and bromothymol blue to a mixture of 100 ml of formalin and 400 ml of alcohol. Adjust pH to 5.2 with 0.1N sodium hydroxide.

Standard acid solution: Pipette 1 ml each of acetic, propionic, butyric and valeric acids into 100 ml volumetric flasks separately and dilute to volume with water. Pipette 1 ml of each stock solution into 25 ml beakers and 1 ml each into another beaker (mixture), neutralise with 0.1 N sodium hydroxide using cresol red indicator and evaporate to dryness without charring. Dissolve in 0.5 ml water. Use these solutions for chromatography.

Procedure:

Steam-distill 20 gm of well mixed sample and collect 200 ml distillate. Immediately neutralise the distillate with 0.1N sodium hydroxide using cresol red indicator and evaporate just to dryness and dissolve in 0.5 ml water. Spot 1-2 μ l along with standards on Whatman No.1 paper and allow them to air dry. Clip paper to glass rod and suspend in a tank with 50 ml mobile phase in a trough (since mobile phase is heavy, use 3 clips to hold the paper to glass rod to prevent sagging).

Develop the chromatogram approximately to 2.5 cm from top of the paper, remove and let air dry. Spray with the spray reagent (spray should be uniform). Faint yellow spots indicate presence of acids, heavier blue spots are due to sodium ion. To intensify spots, expose paper to ammonia fumes. Entire paper immediately turns to green and acids gradually appear as red spots. Since colour of acids is not stable, mark spot with pencil as soon as they are completely developed.

Identify them in sample from the Rf values of standard acids.

(Ref :- Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990, Page 45).

An alternate Gas chromatographic method for analysis of acetic acid and propionic acid can also be followed:

Principle:

The volatile organic acids such as acetic acid, propionic acid, butyric acid and valeric acid are steam distilled and the distillate is used for GC analysis. The results can be confirmed after the analysis of their respective standards.

Chemicals:

Standard Acetic acid, propionic acid, butyric acid, valeric acid – AR grade
Deionized water.

Procedure:

Steam-distill 20 gm of well mixed sample and collect 200 ml distillate. Distillate can either be directly used for on-column injections or can be dissolved in any appropriate solvents.

GC-Method:

- a) Column: FFAP column 30 m length × 0.53 mm id × 0.5 μ film thickness
- b) Carrier gas: Nitrogen/Argon
- c) Detector: FID/MS
- d) Injection temperature: 250°C
- e) Oven temperature: Initial 80°C – Final 200°C
- f) Detector temperature: 250-300°C
- g) Injection volume: 1-2 μL

Reference:

J. Beihoffer and C. Ferguson, J. Chromatogr. Sci., 1994, 32, 102-106.

Or

AOAC Official Method 950.35 [Ref. JAOAC 33, 677(1950); 34, 284(1951); 36, 769(1953)]
Acetic and Propionic Acids in Bread/cakes -Chromatographic Method may also be referred as alternative method.

1.8 DIETHYL CARBONATE IN WINES**Principle:**

Diethyl carbonate is extracted with carbon disulfide and subjected to Gas Chromatography.

Reagents:

- (i) Carbon disulfide: Treat 200 ml carbon disulfide with 20 ml fuming nitric acid, then wash with 20 ml portions of water until it is neutral to pH paper.
- (ii) Diethyl carbonate solution (0.5 mg/ml): Dissolve 50 mgs of diethyl carbonate in 100 ml alcohol.

Apparatus/Conditions:

A DB 130 mts × 0.25 mm id capillary column is recommended provided conditions are standardized.

Test tube, separatory funnel, Gas Chromatograph with Flame ionization detector, 10 µl syringe, column (6' 1/8") stainless steel, packed with 15% trimethylol pantrypalargonate on 60-100 mesh celite 545 (10-20% carbowax 10 M on 60 mesh fibrebrick C 22 may be used but yields poorer-separation);

GLC conditions: Temperature: Column (80°C), injection port 180°C detector (200°C), gas flow rate (ml/min), Nitrogen-carrier gas (35), Hydrogen (35), air (400), recorder 1-25 mV, and diethyl carbonates. Retention time is approx 15 min.

Procedure:

Measure 100 ml sample and transfer to 250 ml separatory funnel. Add 1 ml of alcohol and 20 ml carbon disulfide and shake for about 1 min. Let layers separate, transfer portion of lower layer to small test tube and centrifuge 2-3 min at 2000 rpm to clarify. Slowly inject 5 µl (5 sec) clear solutions from 10 µl syringe. Designate peak area as "A". Likewise, add 1 ml standard to 100 ml wine and proceed for its determination, beginning "transfer to 250 ml separator". Designate the peak areas as "A'".

C = mg/ml standard.

$$\text{mg of diethyl carbonate/L} = \frac{C \times A \times 10}{(A' - A)}$$

(Ref: - A.O.A.C 17th edn, 2000 Official Method 972.14 Diethylcarbonate in Wines – Gas Chromatographic Method)

1.9 SALICYLIC ACID

Salicylic acid is an unpermitted preservative.

Preparation of sample

- a. Non alcoholic beverages: - May be extracted directly. If troublesome emulsion forms during extraction, pipette 100 ml into a 250 ml volumetric flask and add 5 gm of sod. Chloride and shake until dissolved. Dilute to volume with alcohol, shake vigorously, let stand 10 min, shaking occasionally, filter and take filtrate for test.
- b. Alcoholic liquids: - Make 200 ml of test sample alkaline to litmus paper with about 10 % sodium hydroxide solution and evaporate on steam bath to about $\frac{1}{2}$ its original volume. Dilute to original volume with water and filter if necessary.
- c. Solid or semisolid substances: - Grind and mix thoroughly. Transfer 50 – 200 gm according to the consistency of the sample to 500 ml volumetric flask, add water to make about 400 ml and shake until mixture becomes uniform, add 2- 5 gm calcium chloride and shake until dissolved. Make distinctly alkaline to litmus paper with about 10 % sodium hydroxide solution, dilute to volume with water, shake thoroughly, and let stand 2 hrs shaking frequently and filter.

(Ref: - A.O.A.C 17th edn, 2000 Official Method 975. 29 Salicylic acid in Food and Beverages, Preparation of sample)

Qualitative Methods:

(A) **Ferric chloride test:** Salicylic acid is extracted from the acidified food with diethyl ether and the solvent evaporated. The residue obtained on evaporation of the ether is dissolved in hot water. On treating this with 1 % neutral ferric chloride solution salicylic acid gives magenta colour.

(B) **Jorissen test:** Dissolve residue from ether extract as obtained in method 'A' in little hot water. Cool 10 ml solution in test tube Add 4 or 5 drops of 10% potassium nitrite solution, 4 or 5 drops of 50% acetic acid and one drop of 1% copper sulphate solutions. Mix thoroughly. Boil

for a few min and cool. Development of Bordeaux red colour indicates presence of salicylic acid. Benzoic acid in large excess gives a buff coloured precipitate.

(Ref :- A.O.A.C. 17th edn 2000 Official Method 975.30 Salicylic acid in Food and Beverages , Qualitative tests / Manual Methods of Analysis for Adulterants and Contaminants in Foods I.C.M.R 1990. Page 46).

Estimation of salicylic acid by HPLC-UV Method:

Principle:

Salicylic acid is extracted from known quantity of foods analysed by HPLC-UV method

Reagents:

- a) Anhydrous ethanol AR grade
- b) Methanol HPLC grade
- c) Deionized water (DI) $\geq 10 \text{ M}\Omega$
- d) Sodium Chloride – AR grade
- e) Phosphoric acid – AR grade

Preparation of sample

- (a) **Non alcoholic beverages:** - May be extracted directly. If troublesome emulsion forms during extraction, pipette 100 ml into a 250 ml volumetric flask and add 5 gm of sod. Chloride and shake until dissolved. Dilute to volume with alcohol, shake vigorously, let stand 10 min, shaking occasionally, filter and take filtrate for test.
- (b) **Alcoholic liquids:** - Make 200 ml of test sample alkaline to litmus paper with about 10 % sodium hydroxide solution and evaporate on steam bath to about $\frac{1}{2}$ its original volume. Dilute to original volume with water and filter if necessary.
- (c) **Solid or semisolid substances:** - Grind and mix thoroughly. Transfer 50 – 200 gm according to the consistency of the sample to 500 ml volumetric flask, add water to make about 400 ml and shake until mixture becomes uniform, add 2- 5 gm calcium

chloride and shake until dissolved. Make distinctly alkaline to litmus paper with about 10 % sodium hydroxide solution, dilute to volume with water, shake thoroughly, let stand 2 hrs shaking frequently and filter.

(Ref :- A.O.A.C 17th edn, 2000 Official Method 975. 29 Salicylic acid in Food and Beverages, Preparation of sample)

Chromatographic conditions/requirements:

- a) Column: Column - 15cm x 4.6mm ID, C-18, 5 µm particle size
- b) Mobile phase : Water/methanol/phosphoric acid (60/40/0.2%)
- c) Elution: 1.0 mL/min isocratic for 20 min
- d) Detection: 254 nm (UV-detector)

Preparation of standard solution:

400 mg of salicylic acid is dissolved in 50 mL 70% ethanol and made up to 100 mL volume with 70% ethanol. Standard dilutions shall be made as and when necessary from this stock solution.

Injection – 10/20 µL of sample

The samples shall be injected in the sequence

- a. Blank
- b. Standards
- c. Blank
- d. Reconstituted extract from the samples
- e. Quality control standards (20 µg/mL and 60 µg/mL standards samples can be used for quality control)

Chromatogram:

The components are eluted in the sequence, Benzoic acid, sorbic acid, methyl, ethyl, propyl and butyl parabens.

Calculations:

Calculate concentration of each preservative in sample as follows:

Using peak areas or peak heights and concentrations of standards, construct linear standard curve for each compound based on formula $y = mx + C$, where 'x' is concentration (ppm), y is peak area or height, m is slope, and c is the intercept. Calculate recovery of fortified sample and sample results.

Reference:

D.P. Venema et al., J. Agric. Food Chem., 1996, 44, 1762-1767

2.0 BORATES IN FOOD:**Qualitative test:****Principle:**

Borates give red colour with curcumin (the colouring matter found in turmeric).

Reagents:

- (i) Calcium oxide
- (ii) 5N hydrochloric acid
- (iii) 1 % ethanolic solution of curcumin (or turmeric extract)
- (iv) Oxalic acid, saturated solution.

Procedure:

Mix the sample with lime (about 10 parts to one) or sodium carbonate and if necessary

moisten with water. Dry in the oven, then ash at a dull red heat. A completely white ash is not necessary. Cool, add water and 5 N hydrochloric acid till the solution is acidic. Filter into a porcelain dish, add 4 drops of oxalic acid solution and 1 ml of curcumin solution and evaporate on a water bath.

If borates are present, the residue turns into bright red colour and changes to dark green when exposed to ammonia fumes.

(Ref: - F.A.O Manuals of Food Quality Control 1980, 14 / 2 Page27 /Pearsons Composition and Analysis of Foods 9th edn, 1991 Page 82)

OR

AOAC Official Method 970.33 Boric Acid and Borates in Food Qualitative Test, First Action 1970.

3.0 NON -NUTRITIVE SWEETENERS

3.1 Saccharin:

3.1.1 Qualitative methods:

Preparation of the test sample:

(a) Non-alcoholic beverages:

Add 3 ml hydrochloric acid to about 25 ml of the sample in a separator. If vanillin is present remove it by extraction with several portions of petroleum ether. Discard petroleum ether. Extract with 50, 25 and 25 ml portions of diethyl ether + petroleum ether (1+1) and wash combined extracts with 5 ml water and remove the solvent by evaporation.

(b) Semi-solid preparations:

Transfer 25 gms of sample to 100 ml volumetric flask with small amount of water and add enough boiling water to make about 75 ml, let mixture stand one hour shaking

occasionally. Then add 3 ml acetic acid, mix thoroughly, add slight excess (5 ml) of 20% neutral lead acetate solution, dilute to volume, mix with cold water and let it stand for 20 min and filter. Transfer 50 ml filtrate to separator and proceed as in (a).

(Ref: - A.O.A.C 17th edn, 2000 Official Method 941.10 Saccharin in Food / Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990, Page 47)

Detection:

(a) By conversion to salicylic acid (applicable in the absence of salicylic acid):

Dissolve residue in about 10 ml hot water, add 2 ml sulphuric acid (1+3). Heat to boiling, add a slight excess of 5% Potassium permanganate solution drop wise and partly cool the solution. Dissolve about 1 gm of sodium hydroxide in it and filter into silver crucible (silver crucible \with lids are available). Evaporate to dryness and heat for 20 min at 210- 215°C. Dissolve the residue in hot water, acidify with hydrochloric acid and add a few drops of neutral ferric chloride solution (0.5%). Violet colour indicates salicylic acid which is formed from saccharin. By this method all so called false saccharin and any salicylic acid naturally present (also added salicylic acid when not present in too large amount) are destroyed and 5 mg / L saccharin is detected.

(Ref: - A.O.A.C 17th edn, 2000 Official Method 941.10 (B) Saccharin in Food / Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990, Page 48)

(b) Phenol sulphuric acid test:

To the residue obtained after removing solvent, add 5 ml of phenol sulphuric acid reagent (pure colourless crystals dissolved in equal weight of sulphuric acid) and heat for 2 hrs at 135-140°C. Dissolve in small amount of hot water and make it alkaline with 10% sodium hydroxide. Magenta or reddish-purple colour develops if saccharin is present.

(Ref :- A.O.A.C 17th edn, 2000 Official Method 941.10 (c) Saccharin in Food / Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990, Page 48)

(c) Resorcinol sulphuric acid test:

To the residue add 5 drops of resorcinol-sulphuric acid (1:1) and heat on a low flame until the product turns red. Dissolve in 10 ml of water and make it alkaline using 10% sodium hydroxide solution and add few drops of iodine solution. A green fluorescence is developed if saccharin is present.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods I.C.M. R, 1990
Page 48)

3.1.2 Quantitative Methods:

(A) General method for non-alcoholic beverages:

Principle: Saccharin is extracted from a known quantity of acidified sample with diethyl ether. The solvent is removed and the residue is digested with hydrochloric acid and made to a known volume. An aliquot is treated with Nessler's reagent and the absorbance of the coloured product is measured at 425 nm.

Reagents:

- (i) Hydrochloric acid
- (ii) Diethyl ether
- (iii) Nessler's reagent: Dissolve 100 gms of Mercury (II) iodide and 70 gms of potassium iodide in small amount of water, add this solution slowly, with constant stirring to the cooled solution of 160 gms of sodium hydroxide, in 500 ml water and dilute to 1 L.
- (iv) Distilled water (ammonia free)
- (v) Standard solution: Dissolve 0.2921 gm of Ammonium chloride in 1 L of ammonia free distilled water (equivalent to 1 gm of saccharin in 1 L water). Dilute to get 200 µg/ml of saccharin equivalent.

Procedure:

Add 2 ml of hydrochloric acid to 50 gm of accurately weighed sample in separator funnel and mix. Extract with 350 ml of diethyl ether. Filter the ether extract through cotton into a clean 250 ml conical flask and evaporate the solvent.

Add 6 ml hydrochloric acid and 5 ml ammonia free water and evaporate on a hot water

bath to about 1 ml. Again add 6 ml hydrochloric acid and 5 ml water and evaporate to 1 ml. Dilute the solution to 50 ml with ammonia free water. To 2 ml of this solution in a 25 ml volumetric flask add 1 ml of Nessler's reagent and make up to volume. Similarly take 0.5, 1, 2, 3 and 4 ml portions of standard solutions (200 µg/ml) into 25 ml volumetric flasks and develop the colour with Nessler's reagent. Read the absorbance of the product at 425 nm against reagent blank similarly prepared. Compute the saccharin content of the sample from the calibration graph.

(Ref:- A.O.A.C 17th edn, 2000 Official Method 934.04 Saccharin in Non Alcoholic Beverages / Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990, Page 48)

B) Phenol -sulphuric acid Colorimetric Method:

Principle: Saccharin is extracted from the acidified sample with chloroform and the solvent evaporated. The residue obtained is treated with phenol sulphuric acid and heated at 175°C for 2 hrs. After making alkaline with sodium hydroxide the absorbance is read a 558 nm.

Reagents:

- (i) Chloroform
- (ii) Ether
- (iii) Chloroform +Benzene (95+5)
- (iv) Methanol
- (v) Phenol (colourless crystals)
- (vi) Sulphuric Acid
- (vii) Saccharin standard.

Preparation of sample:**(a) Soft drinks (carbonated beverages and low calorie drinks):**

De-carbonate the beverage by repeated shaking and pouring from one beaker to another. Transfer 10 ml sample to 125 ml separatory funnel with Teflon stopcock. Add 15 ml water and 0.5 ml 1N sodium hydroxide. Extract with 50 ml chloroform benzene mixture shaking 1 min. Let layers separate and discard the solvent layer (benzoic acid and benzoates do not interfere).

(b) Fruit Juices:

Transfer 50 ml sample to 100 ml volumetric flask. Add slight excess of 5% neutral lead acetate solution (<10 ml). Dilute to volume with water, let it stand for 1 hour and filter. Take aliquot of filtrate containing 1-3 mg saccharin and proceed as in determination of saccharin beginning "add 5 ml hydrochloric acid (1+4) and extract with chloroform -benzene mixture".

(c) Food sweetener tablets and concentrated liquids:

Grind 10-20 tablets to uniform powder. Accurately weigh 0.5 gm powder or measure 10 ml liquid concentrate sample into 500 ml volumetric flask and dilute to volume with water. Take 10-15 ml aliquot for analysis. If liquid concentrate contains parabens as preservatives, acidify by adding 5 ml hydrochloric acid (1+4) to aliquot and extract with 20 ml carbon tetrachloride. Discard carbon tetrachloride and proceed as in determination beginning, "Extract aqueous phase by shaking 1 min each time".

(d) Jellies and preserves:

Blend sample and weigh 25 gm into 50 ml beaker. Heat on water bath to make sample fluid. Transfer to 250 ml volumetric flask using 25 ml hot water to rinse beaker. Dilute to volume with methanol and mix thoroughly. Let it stand for 1 min and filter. Transfer an aliquot containing 1-3 mg saccharin to 50 ml beaker. Evaporate to 1/2 volume on water bath

to remove alcohol and transfer with about 25 ml hot water to 125 ml separatory funnel. Proceed as in determination of saccharin beginning, "add 5 ml hydrochloric acid (1+4) and extract with chloroform -benzene mixture".

(e) Low calorie high protein powder, granules and liquids:

Grind granules in mortar to uniform powder. Transfer 10-20 gm powder accurately weighed into 250 ml volumetric flask with 150 ml hot water.

Mix thoroughly to dissolve. Add slight excess of 5% neutral Pb (OAC)₂ solution (30 ml). Dilute to volume with cold water. Mix and let it stand 1 hr and filter. For liquids use 50 gms sample and proceed as in determination beginning "add 5 ml hydrochloric acid (1+4) and extract with ether-benzene".

(f) Chocolate bars:

Shred samples and weigh 25 gm into beaker. Add 150 ml hot water and mix with magnetic stirrer to disperse or emulsify. Add slight excess of 5% neutral Pb (OAC)₂ solution (30 ml). Transfer to 250 ml volumetric flask with water and dilute to volume. Mix, let stand 1 hr and filter. Using 50 ml aliquot proceed as in determination beginning "add 5 ml hydrochloric acid (1+4) and extract with ether -benzene".

Determination:

Transfer aliquot as specified, of prepared sample and standard (1-3 mg saccharin) solution to the separating funnels. Add 5 ml hydrochloric acid (1+4) and Extract by shaking for one min each time with 50, 30 and 20 ml chloroform -benzene solvent mixture (95+5) or with ether: benzene (95+5) as specified in sample preparation. Filter the combined solvent extract through funnel fitted with pledget of glass wool and containing 10 gm anhydrous sodium sulphate into 100 ml volumetric flask. Dilute to volume with the same solvent mixture used above and mix. Transfer 20 ml aliquot to 50 ml Erlenmeyer flask. Evaporate the solvent to dryness in shallow water bath and complete drying in oven at 100°C for 20 min Pipette 1 to 5 ml hot melted phenol to Erlenmeyer flask and swirl until the residue is dissolved. Add with

caution 1.2 ml sulphuric acid by pipette and swirl. Prepare blank by pipetting 2.5 ml hot melted phenol and 1.5 ml sulphuric acid into 50 ml Erlenmeyer flask.

Stopper the flask with tight cap covered with aluminium foil and heat for 2 hrs at 175°C in an oven. Cool and add approximately 30 ml hot water to the flask and mix. Add 10 ml 20% sodium hydroxide solution and mix. Transfer quantitatively to 100 ml volumetric flask and dilute to volume with water.

Read the absorbance of the solution in spectrophotometer at 558 nm. Determine the concentration by comparing with a calibration curve.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods I.C.M.R 1990 Page 49)

3.2 DULCIN:

3.2.1 Qualitative methods:

Preparation of sample: Extract 100 ml of sample (made alkaline with 10% sodium hydroxide solution, or alkaline aqueous extract prepared with 3 × 50 ml portions of diethyl ether. Divide ether extract equally into three porcelain dishes, let the solvent evaporate at room temperature and dry the residue.

Detection:

- (a) Deniges-Tourrou Test: Moisten dry residue with nitric acid and add one drop of water. Presence of dulcin is indicated by orange red coloured precipitate.

Note: Vanillin, if present, interferes in the method.

- (b) Modified laparola-Mariani Test: Expose the residue to hydrochloric acid gas for 5 min and add one drop of anisaldehyde. Presence of dulcin is indicated by blood red colour.

(Ref:- A.O.A.C 17th edn, 2000 Official Method 957.11 Dulcin in Food / Manual Methods of Analysis for adulterants and Contaminants in Foods I.C.M.R 1990 , Page 50)

- (c) Dimethylamino benzaldehyde method: To the residue add one drop of dimethyl aminobenzaldehyde (1 gm dissolved in 10 ml hydrochloric acid and made up to 100 ml). A brick red colour indicates presence of dulcin.

(Ref: - Manual Methods of Analysis for adulterants and Contaminants in Foods I.C.M.R 1990, Page 50)

3.2.2 Quantitative Method:

UV-Spectrophotometric Method:

Principle:

Dulcin is extracted from the prepared sample under alkaline conditions with diethyl ether. The residue after removal of solvent is taken in ethyl acetate.

The absorbance of the solution is read at 294 nm.

Reagents:

- (i) Diethyl ether
- (ii) Ethyl acetate
- (iii) 10% Sodium hydroxide solution

Procedure:

Transfer 50 gm of sample into a 250 ml separatory funnel and make it alkaline with 10% sodium hydroxide solution. Extract with 4 × 100 ml portions of diethyl ether, shaking 2 min each time. Wash the combined extracts with 10 ml of water and discard the water layer. Evaporate the solvent and dry the residue at 110°C for 30 min. Dissolve the residue in 50 ml ethyl acetate and transfer into a 100 ml volumetric flask and make up to volume. Make further dilutions if necessary. Read the absorbance in a spectrophotometer at 294 nm against redistilled ethyl acetate. Prepare a standard graph taking standard dulcin in ethyl acetate and compute the amount of dulcin in the sample.

(Ref:- A.O.A.C 17th edn, 2000 Official Method 957.11(D) Dulcin in Food, Quantitative Method / Manual Methods of Analysis for adulterants and Contaminants in Foods I.C.M.R 1990 , Page 51)

3.3 CYCLAMATE**3.3.1 Qualitative Methods****(A) Sodium nitrite Test****Procedure:**

Add 2 gm of barium chloride to 100 ml of sample or aqueous extract prepared by grinding sample, adding water to mix uniformly. Add 2-5 gm calcium chloride and shake to dissolve. Make alkaline with 10 % sodium hydroxide, shake, let stand for 2 hrs and filter. Acidify filtrate with 10 ml of hydrochloric acid and add 0.2 gm of sodium nitrite. Warm the contents on a hot plate. A white precipitate of Barium Sulphate is obtained in the presence of cyclamate.

Note: - Sulphur dioxide interferes with test. Verify its absence by qualitative test.

(Ref:- A.O.A.C 17th edn, 2000, Official Method 957.09 Cyclohexylsulphamate (Cyclamate) salts in non alcoholic beverages / Pearson's Composition and Analysis of Foods

9th edn, 1991, Page 270 / Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990 Page 51)

(B) Thin Layer chromatography Method for Beverages

Principle

The beverage is extracted with ethyl acetate, the concentrated extract subjected to TLC on silica gel and the spots visualized. Saccharin, cyclamate, 5 - nitro- 2 propoxyaniline (P - 4000) and dulcin are detected.

Apparatus

- (i) TLC Apparatus
- (ii) UV lamp (short wave - 254nm)

Reagents

Prepare solutions fresh on day of use

- 1) Developing solvent - n- butanol - alcohol - NH₄OH - H₂O (40 + 4 + 1 + 9, by volume)
- 2) Chromogenic agents -
 - i. Bromine in carbon tetrachloride, 5 % by volume.
 - ii. 0.25 % fluorescein in dimethyl formamide -alcohol (1+ 1).
 - iii. 2 % N - 1 - Naphthyl- ethylenediamine- 2 hydrochloric acid in alcohol.
- 3) Standard Mixture - 50 mg cal cyclamate, 10 mg sod Saccharin, 4 mg dulcin and 4 mg P - 4000 in 10 ml dilute alcohol (1+ 1) 5 μ l =25 μ g cyclamate, 5 μ g saccharin, 2 μ g dulcin , and 2 μ g P - 4000. Warm solution to dissolve dulcin if necessary. Avoid contact with P 4000.
- 4) Silica Gel

Preparation of Test sample

Decarbonise beverage by repeated shaking and pouring. To 50 ml of test portion in 125 ml separator, cautiously add 10 ml of sulphuric acid (1+1). Cool, extract with 2 × 50 ml portions of petroleum ether (shake gently but thoroughly) and discard petroleum ether. To aqueous layer, cautiously add 5 ml 50 % sodium hydroxide solution, cool, and extract with two portions of ethyl acetate (use 60 ml for cola products to prevent emulsion). Filter ethyl acetate extracts through ethyl acetate- washed cotton into beaker with pouring lip. Evaporate to 5- 10 ml on steam bath using air current and transfer to graduated tube (do not let solution evaporate to dryness before transfer, compounds may be difficult to redissolve). Evaporate solution in graduated tube to dryness on steam bath with air current. Dilute to 2.5 ml with NH₄OH – H₂O- alcohol (5 +5 + 10) and mix thoroughly. Any insoluble residue in tube will not interfere with determination

Preparation of Plates and Tank

Slurry 30 gm silica gel H with 75-80 ml water and apply as 0.25 layer to five 20 × 20 cm plates Dry plates 1 hr at room temperature. Do not dry in oven, Do not store in dessicator cabinet. Score layer 5 mm from each side edge and remove 5 mm band of adsorbent from bottom edge of layer.. Use plates within 36 hrs after preparation

Line developing tank with absorbent paper. Pour 25 ml developing solvent into tank, wetting paper. Put developing solvent upto 1 cm in the tank. Place lid on tank, let stand ½ hr to saturate tank atmosphere.

Detection:

Mark TLC plate at edges only, 2.5 cm from bottom to designate spotting line. Mark dotted line 10 cm above spotting line. Spot total of 5 µl each of standard mixture and test portion (level 1). Dilute test portion to 5 ml with ammonia – water – alcohol (5+ 5 + 10) and spot 5 µl (level 2). Place spots 2 cm apart and 2 cm from edges. Spot 1 µl at a time and use warm air blower to dry spot between applications to confine spot diameter. Use same

technique to spot test portion and standard (Total volume spotted should be 5 μ l. Use mixed standard rather than superimposed single standards Place the plate in the tank and develop to 10 cm line (about 1 hr). Dry the plate in a fume cupboard until the layer is no longer translucent (about 10 min) View under short wave (254 nm) UV. Outline any fluorescent saccharin spot at R_f about 0.5 (Spot may be crescent shaped if a large amount of cyclamate is present). In the fume cupboard spray chromogenic agent 1 and 2 lightly to moderately in immediate succession until the cyclamate standard appears as pink spot at R_f about 0.3 – 0.4. P – 4000 is a brown pink spot at R_f about 0.85. Spray chromogenic agent 3 on a plate until the background pink fades to light yellow. The contrast of cyclamate and P 4000 improves and at R_f about 0.7 dulcin appears. The dulcin spot may be brown – pink or blue depending on the condition of the spray reagents and the concentration of the sweetener. The plate may be resprayed with chromogenic agent (3) to restore contrast if the pink background reappears.

(Ref: - A.O.A.C 17th edn 2000, Official Method 969.27. Non Nutritive sweeteners in Non Alcoholic Beverages / F.A.O Manuals of Food Quality Control 1980 14 / 2 Page 109)

3.3.2 Quantitative Method:

Principle:

Cyclamate is hydrolysed by acid under pressure to cyclohexylamine, which is extracted with chloroform and treated with ethanolic p-benzoquinone to form a coloured product which absorbs at 493 nm.

Apparatus:

Beaker; pipette; separatory funnel;

Autoclave: Operated at 15 psi (121-125 $^{\circ}$ C);

Homogenizer.

Reagents:

- (i) Cyclamate (Na or Ca salt): Dry sod or cal cyclamate 4 hrs at 100°C.
- (ii) Prepare 1 mg/ml solution by accurately weighing and dissolving in distilled water (Standard solution).
- (iii) P-benzoquinone: Prepare 0.3% in absolute alcohol. Prepare fresh before use

Procedure:

Homogenise the sample using the homogenizer and weigh accurately a known quantity of the sample (containing 15-30 mg of cyclamate) into a beaker. Take another beaker, pipette 10 ml of standard solution. Dilute both the sample and standard to 40 ml with water. Add 13 ml of 6N hydrochloric acid and finally dilute to 60 ml with water. Place each beaker inside a 400 ml beaker, cover larger beaker with a watch glass and autoclave for 7 hrs at 15 psi (121-125°C). Alternatively hydrolysis can be achieved by adding 5 ml of conc. hydrochloric acid and 5 ml of 30% hydrogen peroxide to the sample solution and keeping the flask in a boiling water bath for 2 hrs. Transfer the contents into 250 ml separatory funnel, adjust the pH 12.0 using 10% sodium hydroxide, add a few drops more and extract with 3 x 25 ml chloroform. Wash the combined chloroform extracts to make them free of alkali, dry it over anhydrous sodium sulphate and make up to 100 ml in a volumetric flask with chloroform. Pipette an aliquot of sample and standard solution into 50 ml volumetric flask in 60°C water bath for 2 hrs protected from direct light. Cool, dilute to volume and read the absorbance at 493 nm in a spectrophotometer and calculate the cyclamate content of the sample

(Ref:- A.O.A.C 17th edn, 2000, Official Method 969.28 sodium cyclamate and calcium cyclamate in canned fruit, Colorimetric method/ Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990 Page 51).

3.3.3 Quantitative analysis:**HPLC-UV Method**

The HPLC-UV method can be used for quantitative analysis of cyclamates only after derivatisation with o-phthallaldehyde using reverse phase separation.

Indirect UV detection is possible for cyclamate using HPLC or capillary electrophoresis.

Detection limits are 5–10 mg kg⁻¹ for capillary electrophoresis and 28 mg l⁻¹ for HPLC

A reversed phase HPLC-UV method for simultaneous detection of aspartame, saccharine and cyclamate in soft drinks without using derivatization.

Purified water HPLC grade; phosphoric acid, disodium phosphate, sodium hydroxide

HPLC method

The mobile phase composition, gradient and debit are shown:

Table 1. HPLC pump setup

Time (min)	22 mM phosphate buffer pH 2.5	Acetonitrile	Flow rate (ml min ⁻¹)
0	93	7	1.400
8	93	7	1.400
8.1	85	15	1.400
18	85	15	1.400
18.1	93	7	1.400

Because saccharine and cyclamate get eluted very close to each other, it is necessary to acidulate the solution in order to ensure a better retention on the column and a better separation. The best value will be found at pH 2.5 ensuring a good separation at the baseline of the constituents.

Sample volume - 100 µl, and total analysis time 24 min “Best chromatogram” at 196 nm.

Sample preparation: dilute the soft drinks in 1:5 ratio, filter on a nylon syringe filter (0.45 µm) followed by injection of 100 µl.

(Ref. M.D. croitoru et al. Acta Alimentaria, Vol. 40 (4), pp. 459–465 (2011) Direct HPLC-UV determination of cyclamate, saccharine and aspartame from soft drinks)

3.4 ASPARTAME:

3.4.1 Qualitative Method: (Detection and determination of aspartame by Thin Layer Chromatography).

Principle:

Aspartame is extracted from dry beverage powders using methanol: acetic acid (80: 20) and isolated by TLC. Detection and estimation is made by comparison with the standard.

Reagents:

- (i) methanol : acetic acid (80 : 20)
- (ii) Aspartame stock solution: Dissolve 50 mg of standard aspartame in about 80 ml of solution (i) and make upto 100 ml in a volumetric flask.
- (iii) Working standard solution: Dilute to the mark 1 ml of stock solution in a 10 ml volumetric flask with solution (i) Repeat 1 ml increments with 8 other flasks until 9 standards containing from 1 to 9 ml of solution (ii) are obtained. The standards so obtained will contain 0.05 to 0.45 μg of aspartame/ μl (0.05,0.10,0.15,0.20,0.25,0.30,0.35,0.40 & 0.45 μg)
- (iv) Starch solution: Dissolve 600 mg of soluble starch in 120 ml of water, boil for 10 min and filter through medium filter paper (Whatman No.2 or equivalent).
- (v) Potassium iodide/starch solution: Dissolve 500 mg of potassium iodide in 100 ml of starch solution.
- (vi) Developing solvent: methanol: glacial acetic acid: water: chloroform (60: 4: 12: 128).
- (vii) Tert.-butyl hypochlorite chamber: Add 5 ml of tert-butylhypochlorite to a small

glass vessel which can be capped and which contains a glass wool wick reaching its top. This vessel is then uncapped in the tert- butyl hypochlorite chamber – 15 min before use.

(viii) Silica gel G.

Preparation of sample:

- a) Dry powders: Extract aspartame from dry powders by shaking for 20 min with 100 ml of solution (i). The quantity of the sample to be taken depends on its theoretical aspartame content. Assuming 100% extraction, 5 gm of a product containing 0.4% aspartame, extracted into 100 ml would produce 2 µg/µl of supernatant. Since 2 µl are to be spotted, the resulting chromatogram should match the 4 µg standard spot which is achieved by spotting 2 µl of 2 µg/ µl standard.

- b) Liquids: Treat liquids as in (a) except the extract is made up to 100 ml with solution (I).

Procedure:

Spot 2 µl of the sample solution and standard using a micro-pipette and use a cool air stream for drying. Place the plate in the development chamber and allow the solvent to ascend about 15 cm. Remove the plate from the chamber and allow it to air-dry for 15 min Place the plate in the tert- butylhypochlorite chamber for 15 min and allow it to air dry in fume hood for 30 min Spray the plate with the potassium iodide-starch solution and estimate the aspartame content of the sample by comparison with the standard spots.

Note: Tert-butylphyochlorite is toxic when absorbed through the shaken or inhaled. Use gloves while handling it.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods , I.C.M.R 1990 Page 52)

3.4.2 Quantitative Method:

(A) U.V. Spectropotometric Method:**Principle:**

Aspartame is extracted from tablets with aqueous methanol and the absorbance of the filtered solution is measured at 258 nm.

Reagents:

- a) Solvent mixture: Mix 350 ml of water with 150 ml of methanol and allow to equilibrate at room temperature.
- b) Standard aspartame solution: Transfer 72 mg of aspartame (previously dried at 105°C for 2 hrs) into a 100 ml volumetric flask, add 50 ml of solvent mixture and shake till it dissolved and make upto the volume with solvent mixture.

Preparation of sample:

Transfer accurately weighed powdered tablet material equivalent to the average weight of 4 tablets into a volumetric flask. Add 50 ml of solvent mixture and shake for 30 min on a flask shaker and make upto volume with solvent mixture. Filter through Whatman No. 1 filter paper discarding the first 20 ml filtrate and collect the filtrate in a stoppered flask.

Procedure:

Measure the absorbance of the standard solution and test solution at 258 nm against the solvent mixture. Calculate the aspartame content of the tablet from the absorbance of sample and standard.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990 Page 53)

3.4.3 Quantitative analysis:

HPLC-UV Method

The HPLC-UV method can be used for quantitative analysis of aspartame only after derivatisation with o-phthallaldehyde using reverse phase separation.

Indirect UV detection is possible for cyclamate using HPLC or capillary electrophoresis.

Detection limits are 5–10 mg kg⁻¹ for capillary electrophoresis and 28 mg l⁻¹ for HPLC.

A reversed phase HPLC-UV method for simultaneous detection of aspartame, saccharine and cyclamate in soft drinks without using derivatization.

Purified water HPLC grade; phosphoric acid, disodium phosphate, sodium hydroxide.

HPLC method

The mobile phase composition, gradient and debit are shown:

Table 1. HPLC pump setup

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Because saccharine and cyclamate get eluted very close to each other, it is necessary to acidulate the solution in order to ensure a better retention on the column and a better separation. The best value will be found at pH 2.5 ensuring a good separation at the baseline of the constituents.

Sample volume - 100 µl, and total analysis time 24 min “Best chromatogram” at 196 nm.

Sample preparation: dilute the soft drinks in 1:5 ratio, filter on a nylon syringe filter (0.45 µm) followed by injection of 100 µl.

(Ref. M.D. croitoru et al. Acta Alimentaria, Vol. 40 (4), pp. 459–465 (2011) Direct HPLC-UV determination of cyclamate, saccharine and aspartame from soft drinks)

3.5 ACESULFAME K:-

3.5.1 Qualitative Method (Thin-layer Chromatographic detection of acesulfame Saccharin and cyclamate):

Apparatus:

- a) UV lamp (360 nm);
- b) Ion-exchange resin: Amberlite LA-2.

Reagents:

- (i) Polyamide
- (ii) 2,7-dichlorofluorescein
- (iii) Bromine
- (iv) Formic acid
- (v) Ammonia 5%
- (vi) Xylol
- (vii) Propanol
- (viii) Methanol
- (ix) Developing solution: Xylol: n-propanol: formic acid:: 5:5:1

Procedure:

Extract the sweetener from acidified food product with water or take acidified aqueous extract and pass through the ion-exchanger and wash with water. Elute the sweeteners with dilute ammonia solution. Evaporate the ammonical solution under vacuum to dryness and take up the residue in 1 ml of 50% methanol (alternatively extract these sweeteners from acidified sample, pH 0.6, with ethyl acetate and use concentrated ethyl acetate for TLC).

Apply 2-10 µl of sample solution along with standards on TLC plates coated with polyamide. Develop the plate to about 15 cm height with a developing solvent consisting of xylol: n- propanol: formic acid (5:5:1). Dry the plates in a current of air and spray with 0.2% solution of dichlorofluorescein and after being dried, examine under UV light. To identify the spots in day light, place the plate in chamber containing bromine and then expose to ammonia vapour. Spots appear on a reddish background.

3.5.2 Quantitative method: (Analysis of Acesulfame by High Pressure Liquid Chromatography)

Apparatus:

- (i) Beaker, pipette, flasks,
- (ii) HPLC instrument of any suitable make with a UV detector at 227 nm, column; Lichrosorb-RP 18 (10 µm).

Reagents:

- (i) Mobile phase: methanol : water (10 : 90) : Adjust this mixture to 0.01M using tetrabutylammonium sulphate,
- (ii) Standard solution of acesulfame: 0.1 mg/ml in distilled water.

Operating Conditions:

- (1) Pressure: 160 bar
- (2) Flow rate: 40 ml/hr

- (3) Temperature (ambient)
- (4) Sample Volume: 10-20 μl .

Preparation of sample:

- (a) Liquid samples such as juices: filter through 0.45 μm filter (Millipore Inc.) and inject 10-20 μl .
- (b) Solid samples: Stir 10 gm of sample vigorously with 100 ml distilled water for 30 min and centrifuge. Pass an aliquot of this solution through 0.45 μm filter, discard the first few drops of filtrate and collect the filtrate and chromatograph.

Procedure:

Inject standard solution ranging from 5-20 μl and record the peaks.

Calculate the peak area and draw a calibration graph using μg of substance vs peak area. Inject samples solution ranging from 10-20 μl and record the peak area for sample. Calculate the acesulfame content of the sample from its peak area and the calibration graph.

3.5.3 Determination of Acesulphame - K, Aspartame and Saccharin by High Performance Liquid Chromatography

3.5.3.1 Principle

Extraction of sample with water or eluent, if necessary, clarification on solid phase extraction column or with Carrez reagent, chromatography at an HPLC reversed phase column and spectrophotometrical determination at a wavelength of 220 nm

3.5.3.2 Reagents

1. Acetonitrile HPLC grade
2. Methanol HPLC grade
3. Pot. dihydrogen phosphate
4. Di potassium hydrogen phosphate
5. Tetra butyl ammonium hydrogen sulphate
6. Phosphoric acid 85% (w/w)
7. Phosphoric acid 5% (w/w). Carefully pipette 6 ml of Phosphoric acid at (6) above into a 100 ml volumetric flask which already contains 80 ml water. Dilute to mark with water.
8. Hydrochloric acid 25 % (w/w)
9. Carrez Solution 1 – Dissolve 15 gm Potassium hexacyanoferrate ($K_4[Fe(CN)_6] \cdot 3H_2O$) in water and dilute to 100 ml
10. Carrez Solution 2 – Dissolve 30 gm Zinc Sulphate ($ZnSO_4 \cdot 7H_2O$) in water and dilute to 100 ml

11. Phosphate Buffer solution II – (KH_2PO_4 0.0125 mol / L, pH = 3.5. Dissolve 1.70 gm potassium dihydrogen phosphate in 800 ml of water in a 1000 ml beaker. Adjust to pH 3.5 with phosphoric acid. Transfer the solutions to a 1000 ml vol. flask and dilute to mark with water.
12. Phosphate Buffer solution III – pH 6.5 Dissolve 5.46 gm of potassium dihydrogen phosphate in 500 ml water in a 1000 ml beaker. Adjust to pH 6.5 with dry di potassium hydrogen phosphate. Add to the solution 3.4 gm of tetra butyl ammonium hydrogen sulphate and stir to dissolve. Adjust the pH to 6.5 by addition of more di potassium hydrogen phosphate. Add 250 ml of methanol and adjust the pH to 4.0 by drop wise addition of hydrochloric acid (8). Transfer this solution into a 1000 volumetric flask and dilute to the mark with water.
13. Mobile Phase – Phosphate buffer and either acetonitrile or methanol. Filter the phosphate buffer used for the mobile phase and either acetonitrile or methanol separately through suitable membrane filters, of pore size $0.45 \mu\text{m}$ and de gas for 5 min in an ultrasonic bath. Add carefully measured the required amounts of phosphate buffer and acetonitrile as given in A,5 and mix. Prepare the mobile phase freshly on the day of use
14. Control solution - containing acesulphame – K, Sodium saccharin and aspartame (and optionally diketopiperazine, aspartylphenyl alanine, phenylalanine, caffeine, benzoic acid, theobromine, hydroxyl methyl furfural, and vanillin)
15. In a 100 ml volumetric flask, weigh to the nearest 0.1 mg, 30 mg of acesulphame – K, 20 mg of sodium saccharin, 220 mg of aspartame (and optionally 60 mg caffeine, 100 mg benzoic acid, 100 mg vanillin, 10 mg diketopiperazine, 10 mg of phenylalanine, 10 mg of aspartylphenylalanine, 20 mg of hydroxyl methyl furfural and 70 mg of theobromine). Dissolve and dilute to mark with water. Pipette 20 ml of the solution into a 100 ml vol. flask and dilute to mark with water.
15. Stock solution – Weigh to the nearest 0.1 mg, 100 mg of Acesulphame – K, 100 mg of sodium saccharin and 100 mg of aspartame in the same 100 ml volumetric flask. Dissolve and dilute to mark with water.

- 16 Standard Solution 1 – Pipette 10 ml of the stock solution (15) into a 100 ml vol. flask and dilute to mark with water.
17. Standard solution 2 – Pipette 5 ml of the stock solution (15) into a 100 ml vol flask and dilute to mark with water.
18. Standard Solution 3 – Pipette 1 ml of the stock solution (15) into a 100 ml vol flask and dilute to mark with water.

3.5.3.3 Apparatus

1. Analytical Balance
2. High speed blender or homogenizer
3. Volumetric flasks – 100, 250, 500 and 1000 ml capacity.
4. Beaker 1000 ml
5. Pipettes – 1, 5, 10 , 20, 25, 100 ml
6. Micropipette 1000 μ L
7. Graduated cylinder – 1000 ml
8. Funnel
9. Fluted filter papers, medium fast qualitative
10. Water bath
11. Ultrasonic bath

12. Centrifuge

13. Degassing system

14. Membrane filters – pore size 0.45 µm or smaller with filter holders and suitable syringe.

15. Solid phase extraction column

16. High Performance Liquid Chromatograph – equipped with UV detector (capable of operating at a wavelength of 220 nm, preferably a diode array detector) and equipped with recorder or integrator which allows measurement of peak heights and peak areas)

17. Column , Reverse phase – a RP C 18 stationary phase of 5 µm, a length of 250 mm, internal dia 4 mm, a guard column, RP C 18 (optional but strongly recommended for all solid sample materials.

Performance criteria for suitable analytical columns are the baseline resolution of the respective analyte.

3.5.3.4 Procedure

Preparation of sample test solution

- (1) Clear liquid products (lemonades, cola, and beverages) Dilute 20 ml of the liquid in a 100 ml volumetric flask with water. Filter the solution through a membrane filter of pore size 0.2 µm before injection.
- (2) Cloudy liquid samples (juices, flavoured milk drinks) Dilute 20 ml sample with 50 ml water in a 100 ml volumetric flask. Add 2 ml Carrez solution 1, mix and 2 ml of Carrez solution 2, dilute to mark with water and filter through a fluted filter paper. Pass the filtrate through a membrane filter of pore size 0.45µm before injection.

To make allowance for the volume of any precipitate, if the fat free insoluble matter in the initial sample mass exceeds approx 3 gm, it is advisable to centrifuge the clarified

solution for 10 min before filtering it quantitatively into a 100 ml volumetric flask. Wash the settled matter twice with water and centrifuge again, collect each of the supernatant in the 100 ml vol flask and then dilute the solution to mark with water.

- (3) Jams, preserves, marmalade and related products. Weigh to the nearest 1 mg, 20 gm of homogenized sample in a 100 ml vol. flask, add about 60 ml water and place the flask in an ultrasonic bath at 40°C for 20 min the temperature should not exceed 40°C since aspartame can get degraded. Cool to room temp. Add 2 ml Carrez solution 1, mix followed by 2 ml Carrez solution 2. Shake vigorously and allow to stand for 10 min Dilute to mark with water. Filter the solution through a fluted filter paper. Pass the filtrate through a membrane filter of pore size 0.45 µm before injection. To make allowance for any precipitate, if the fat free insoluble matter in the initial mass exceeds 3 gm, it is advisable to centrifuge the clarified sample solution for 10 min at 1400 rpm before filtering it quantitatively into 100 ml vol flask. Wash with water and centrifuge again as in case of cloudy liquid samples

- (4) Semisolid and solid products -

Weigh 10 – 20 gm of thoroughly homogenized sample in a 100 ml vol flask. Add about 50 ml water and place the vol flask in an ultra sonic bath at 40°C for 20 min Cool to room temperature, add 2 ml Carrez solution 1 , mix and add 2 ml of Carrez solution 2, dilute to mark with water and filter through a fluted filter paper. In case of very complex matrices, additional purification using the solid phase extraction column may be necessary to protect the separating column, since colouring, flavouring and fat can not be separated by Carrez solution. In this case add 2 ml of clarified filtrate to the cartridge, previously activated with 3 ml of methanol and 20 ml water and elute with about 20 ml of mobile phase. Pass the filtrate through a membrane filter of pore size 0.45 µm before injection. To make allowance for the volume of any precipitate follow procedure mentioned above.

- (5) Custard Powder

Weigh 10 gm sample in a 500 volumetric flask. Add about 400 ml of water and proceed as described above. Add 6 ml of Carrez solution 1 and 2 for clarification

3.5.3.5 Identification

Identify the intense sweeteners by comparing the retention times of the analyte concerned in the sample solution with that of the standard substance or by simultaneous injection of the standard solution and the sample solution.

3.5.3.6 Determination

Integrate the peak areas or determine the peak heights and compare the results with the corresponding values for the standard substance with the nearest peak area / height or use a calibration graph. Check the linearity of the calibration graph.

3.5.3.7 Chromatographic conditions

Type – reversed phase (RP) Stationary phase and column lengths – spherical particles of 3 μm , for column lengths of 100mm, upto 10 μm for column lengths of 300 mm internal diameter – 4.0 mm Guard column –recommended (optional) – bondapak C 18 or partisil ODS3, or superspher60 RP select B

Flow rate – 0.8 ml /min to 1 ml / min

Injection volume – 10 μl upto 20 μl

Detection – Photometrical (UV) at a wavelength of

217 nm for aspartame

227 nm for Acesulphame – K

265 nm for saccharin

220 nm for all intense sweeteners if the detector does not allow a wavelength switch in one run.

Mobile phase – The following proportions are satisfactory

Solution A – phosphate buffer = 0.02mol / l pH = 4.3

Solution B =phosphate buffer = 0.0125 mol / l pH = 3.5

Solution C phosphate buffer = pH 6.5

Solution D – acetonitrile

Solution E – methanol

- a) Solution A + solution D (90+10 v/v)
- b) Solution B + solution D (80 = 20 v/v)
- c) Solution B + solution D (85 + 15 v/v)
- d) Solution B + solution D (90 + 10 v/v)
- e) Solution B + solution D (95 + 5 v/v)
- f) Solution B + solution D (98 + 2 v/ v)
- g) Solution C + solution E (90 +10 v/v)

3.5.3.8 Calculation

Calculate the mass fraction w expressed in mg / kg or mass concentration p in mg / L of the intense sweetener as under

$$w \text{ or } p = \frac{A_1 \times V_1 \times m_1 \times F_1}{A_2 \times V_2 \times m_0} \times 1000$$

Where,

A_1 = peak area of the intense sweetener concerned obtained with sample test solution

A_2 = peak area of the intense sweetener concerned obtained with the standard test solution

V_1 = total volume of sample test solution in ml

V_2 = total volume of the standard test solution in ml

m_1 = mass of the intense sweetener concerned in standard test solution

m_0 = initial sample mass in gms or ml

F_1 = dilution factor for the purification method used (e.g. Column Clarification =10, Carrez clarification = 1)

(Ref: - European Standard EN 12865)

3.5.4 Liquid Chromatographic Determination of Caffeine, Benzoate and Saccharin in Soda Beverages:

Principle:

Saccharin, benzoate and caffeine are simultaneously quantified in soda beverages by liquid chromatography on μ -Bondapak-C18 column using acetic acid (20%) buffered to pH 3.0 with saturated sodium acetate and modified by adding 0.2% isopropanol. The concentration of the additives in the sample is determined by measuring the peak heights using a UV absorbance detector monitored at 254 nm.

Apparatus:

Beaker, pipette, flasks,

A Liquid Chromatograph equipped with a solvent delivery system and injector or equivalent, UV detector at 254 nm, chart recorder/integrator and μ -Bondapak C18 column 300 \times 4 (i.d) mm, flow rate 2 ml/min Detector sensitivity adjustable from 0.02– 0.05 AUFS.

Reagents:

- (i) Mobile Phase: 20% acetic acid (v/v) buffered to pH 3.0 with saturated sodium acetate solution modify with 0-2% isopropanol to obtain base line resolution and retention times of standards from mixed standard solution in approximately 10 min De-gas prior to use.
- (ii) Standard solutions: Prepare individual standard solutions from standard compound to get following concentrations-sodium saccharin: 0.5 mg/ml, caffeine: 0.05 mg/ml and sodium benzoate: 0.5 mg/ml. Use these solutions to determine sensitivity for detector response and retention times of individual standards.
- (iii) Mixed standard solution: Prepare solution containing 0.5 mg/ml sodium, saccharin 0.05 mg/ ml caffeine and 0.5 mg/ml of sodium benzoate. Use this solution to optimise LC conditions for complete resolution and to quantify.

Preparation of Sample:

- (a) **Carbonated beverages:** Decarbonate by agitation or ultrasonic treatment. If free of

particulate matter, inject directly.

- (b) **Beverages containing particulate matter:** Filter through millipore filter (0.45 µm) discarding first few ml filtrate. If large amount of particulate matter is present, centrifuge prior to filtration. Inject filtered solution directly.

Determination:

Inject known volume (10 µl) of mixed standard solution in duplicate. Peak heights should agree within ± 2.5%. Inject known volume of prepared sample in duplicate. Measure peak heights of standards and sample components.

$$\% \text{ compound} = C1 \times (H/H1) \times V1/V \times 0.1$$

Where,

C1= concentration of standard in mg/ml

H and H1= average peak heights of sample and standard respectively

V and V1= volume injected in µl of sample and standard respectively.

(Ref:- Manual Methods of Analysis for Adulterants and Contaminants in Foods , I.C.M.R 1990 Page 55)

3.5.5 HPLC method for the determination of Caffeine, Benzoate and saccharin.

1.0 This Test method can be used for the determination of Preservatives and sweeteners i.e. Benzoic acid, Caffeine, Aspartame, Acesulfame-K & Sorbate in Soft drinks by HPLC.

2.0 Equipment and Reagents

2.1 Equipment

- i. HPLC with UV Detector
- ii. Calibrated Micro pipettes- 20 to 200µL and 100 to 1000µL capacity ranges

iii. Ultrasonic bath

2.2 Glass Ware

1. 200 μ L glass inserts
2. 50 ml glass beakers
3. Syringe Filters 0.22 μ size
4. Syringes 2mL polypropylene
5. HPLC Vials
6. 10 ml Calibrated Volumetric Flasks
7. 5ml Poly propylene Ria Vials

2.3 Chemicals

- (1) Ethanol, HPLC Grade
- (2) Pure water
- (3) Ammonium Phosphate Mono basic, GR Grade
- (4) Methanol, HPLC Grade

2.4 Reference Standards

All the standards used have purity of $\geq 96\%$. The details of estimated analyte are given below.

Sl. No	Name of Standard
1	Acesulfame
2	Aspartame
3	Benzoic acid
4	Caffeine
5	Sorbic acid

3.0 Procedure

3.1 Preparation of Reagents

3.1.1 Preparation of Mobile phase: Dissolve 6.9gm of ammonium phosphate mono basic in 500ml Distilled water. Sonicate it for 5 min then replace 180ml of this buffer with same amount of ethanol. Sonicate it for 5 min

and then vacuum filter it through 0.22 μ filter paper to remove particles and dissolved gases

3.1.2 Preparation of Preservatives Standard Stock Solution: Weigh accurately equivalent to 100mg of each standard into a separate 10mL volumetric flask and dissolve in methanol: Water (1:1). Make up the volume with the same. Label it with name of the standard, concentration and preparation date. Store the solution in a refrigerator at 2-8°C.

3.2 Preparation of Preservative working standard solution

Prepare working standard at conc. 1000mg/L by transferring ~1mL of each stock solution (~10000mg/L) into a 10mL volumetric flask and makeup with methanol and store the solution in a refrigerator at 2-8°C. Prepare the solution once in three months.

3.3 Preparation of standard dilutions for calibration curves

Prepare the following dilutions ranging from 10mg/L to 200mg/L with 1:1 methanol: H₂O, “as shown below”, using working standard solution and further dilutions.

Stock Conc. (mg/L)	Volume of Stock(mL)	Volume of Diluent(mL)	Final Volume (mL)	Final Conc. (mg/L)
1000	0.200	0.800	1	200
1000	0.100	0.900	1	100
100	0.100	0.900	1	10

3.4 Preparation of calibration curve

Prepare the following concentrations ranging from 1 to 300 mg/L and label them as CC1 to CC7.

Stock Conc. (mg/L)	Volume of Stock(mL)	Volume of Diluent (mL)	Final Volume (mL)	Final Conc. (mg/L)	Name
1000	0.300	0.700	1	300	CC-7
1000	0.200	0.800	1	200	CC-6
1000	0.100	0.900	1	100	CC-5
1000	0.050	0.950	1	50	CC-4
200	0.100	0.900	1	20	CC-3
100	0.100	0.900	1	10	CC-2
10	0.100	0.900	1	1	CC-1

3.5 Preservation

Samples should be stored at 2-8°C.

3.6 Sample Preparation:

Take 20ml of sample (all soft drinks) into a 50ml glass beaker and degas it by placing in an ultrasonic bath. Then filter the degassed sample through 0.22µ filter and then inject into HPLC system

3.7 Batch Organization

For each new batch of samples, follow the following order

Mobile phase blank, CC1 to CC7, max of 10 samples followed by one fortified control sample and so on.

3.8 Injection

Inject the prepared samples to HPLC for the analysis. Ensure following conditions mentioned in section 3.9 for the determination of preservatives and sweeteners in injected samples.

3.9 Chromatographic Conditions for HPLC

A summary of the HPLC conditions are as follows:

Column : Hibar C18, 4.6 × 250mm, 5µ

Mobile Phase : 18:82 Ethanol: Water containing 60mM
 NH_2HPO_4

Injection Volume : 15 μL

Absorbance Wavelength : 214 nm

4.0 DATA PROCESSING

The chromatograms were acquired using the computer-based software supplied by manufacturer of the instrument. The concentration of the unknown was calculated from the calibration curve using below equation.

$$y = mx + c$$

Where, y = Analyte areas

x = concentration of Analyte

m = slope of the calibration curve

c = y-axis intercept value

4.1 CALCULATIONS

Concentration of analyte (mg/L) =

$$\frac{\text{Concentration obtained by HPLC in mg/L} \times \text{Final volume}}{\text{Volume of sample in the solution}}$$

4.2 REPORTING

Report the values above >50ppm for Acesulfame, Aspartame, Benzoic acid, Sorbic acid and >1ppm for Caffeine.

(Ref: - Determination of Acesulfame-K, aspartame, saccharin, benzoic acid and caffeine using High performance liquid chromatographic method --EN 12856:1999.)

4.0 FOOD COLOURS

The colouring matter in food may be (a) natural and (b) Synthetic colours. They may also be classified as (a) water soluble and (b) oil soluble. They have to be separated from food

before identification can be done. Natural colours consist of chlorophyll, carotenes, cantaxanthene, riboflavin, annatto, saffron, turmeric, curcumin, caramel etc. Synthetic colours are of importance as they are widely used in different foods. They are classified as acidic and basic dyes. Only 8 coal-tar food colours are permitted to be used in certain food products under the provisions of FSS (Food Product Standards & Food additives) Regulations, 2011. They include three red shades namely Carmoisine, Ponceau 4 R, Erythrosine, two Yellow shades namely Sunset Yellow FCF and Tartrazine, two blue shades i.e. Brilliant Blue FCF and Indigo Carmine and one green shade i.e. Fast Green FCF. However certain unpermitted colours such Metanil yellow, Rhodamine B, Orange G, Blue VRS, Auramine and certain unidentified water and oil soluble colours (such as Sudan red colours) often appears as adulterants in foods.

4.1 Identification of Natural Colours:

Caramel: Caramel is detected by Fiehe's reaction. Extract the sample solution with 50 ml ether and evaporate it in a porcelain dish. To the residue add 3 drops of 1% solution of resorcinol in hydrochloric acid. The presence of caramel is indicated by appearance of rose colour.

Cochineal: Shake amyl alcohol solution of the material with dilute ammonia. A purple colour is produced in the presence of cochineal.

Turmeric (curcumin): Evaporate an alcoholic extract of the material almost to dryness on the water bath with a piece of filter paper. Moisten the dried paper with a few drops of weak solution of boric acid to which some drops of hydrochloric acid have been added. Dry the paper again. If turmeric is present, the dry paper will be cherry red in colour which changes to bluish green by a drop of sodium hydroxide or ammonium hydroxide.

Annatto: Shake the melted fat or oil with 2% sodium hydroxide solution and pour the aqueous extract on moistened filter paper. The filter paper will show a straw colour which will remain with a gentle wash with water. Dry the paper and add a drop of 40% stannous chloride solution and dry carefully. If the colour turns purple, the presence of annatto is confirmed.

Chlorophyll: Extract the sample with ether and treat the ether extract with 10% potassium hydroxide in methanol. Colour becomes brown, quickly returning to green, confirms the

presence of chlorophyll.

Betanin: Extract the aqueous suspension with amyl alcohol. It remains in aqueous phase. Dye it with a piece of tannin mordanted cotton, a terracotta shade is produced in presence of betanin.

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

4.2 ISOLATION, IDENTIFICATION AND ESTIMATION OF SYNTHETIC FOOD COLOURS:

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:

Pipette; beaker; flask;

Reagents:-

(a) 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;

(b) Paper:

Whatman No. 1 chromatographic paper.

(c) Solvents:

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

Procedure:

- (i) 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.
- (ii) 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.
- (iii) Alcoholic liquids (e.g. Wine):** Boil to remove alcohol and acidify if necessary as in (a).
- (iv) Starch based foods (e.g. cakes, custard powder etc):** Grind 10 gms 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.

(v) Candied fruits: Treat as in (i).

(vi) Products with high fat content (e.g. Sausages, meat, fish paste):

Petroleum and extract the colour with hot water (acidify etc. as usual). Note that oil soluble

(vii) De-fat the sample with light 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).

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 min 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 synthetic dye is indicated. 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 woolen 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 synthetic dyes are acidic, hence an indication of the presence of a basic

dye suggests that an unpermitted colour is present

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. (V) 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

Determination of Synthetic food colours in food products:

1. For samples containing single colour

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

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 (40-60°C) 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 x 45 cm) containing aluminium oxide acidified with 1% hydrochloric acid. Elute the adsorbed colour with 1% ammonia.

Evaporate the eluate to dryness on a hot water bath, dissolve the residue with 0.1 N hydrochloric acid, transfer quantitatively to a 100 ml volumetric flask and make up the volume with 0.1N hydrochloric acid. 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

Sl No	Name of Colour	Absorption maxima (nm)
1	Carmosine	516
2	Ponceau 4 R	507
3	Erythrosine	527
4	Green FCF	624
5	Indigo Carmine	609
6	Brilliant Blue FCF	630
7	Tartrazine	427
8	Sunset yellow FCF	482

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 hydrochloric acid. Prepare a blank by cutting an equivalent strip from plain portions of the chromatogram and elute with 0.1 N hydrochloric acid. Make up the eluate to a known volume (100 ml) with 0.1 N hydrochloric acid 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 gms 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 (200 × 200 mm) to thickness of 0.5 mm. Allow the plate to air dry and then dry at 120°C for two hrs.

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 hydrochloric acid. 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 hydrochloric acid. 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)

3. Determination of Food Colours in Beverages by HPLC (an alternate method).

3.2.0 Equipment and Reagents

3.2.1 Equipment

- 1) HPLC with UV Detector
- 2) Calibrated Micro pipettes- 20 to 200 μ L and 100 to 1000 μ L capacity ranges
- 3) Ultrasonic bath

3.2.2 Glass Ware

Calibrated Volumetric Flasks-10mL and 25mL capacity

- 1) 200 μ L glass inserts
- 2) 50 ml Glass Beakers
- 3) Syringe Filters 0.22 μ size
- 4) Syringes 2mL polypropylene
- 5) HPLC Vials
- 6) 10 ml Calibrated Volumetric Flasks
- 7) 5ml Poly propylene Ria Vials

3.2.3 Chemicals

- 1) Acetonitrile, HPLC Grade
- 2) Pure water
- 3) Acetic acid, HPLC Grade
- 4) Methanol, HPLC Grade

3.2.4 Reference Standards

All the standards used have purity of $\geq 96\%$ and if possible, traceable e.g. NIST. The details of estimated analyte are given below.

Sl.No	Name of Standard
1	Tartrazine
2	Indigo carmine
3	Ponceau-4R

4	Sunset Yellow
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3.3.0 Procedure

3.3.1 Preparation of Reagents

3.3.1.1 Preparation of Mobile phase:

Mobile phase A: In a 1L Reagent Bottle take 500 ml of Pure water and then add 1.54gm of ammonium acetate, sonicate for 5 min and then makeup to volume with water. Label it as 20mM ammonium acetate.

Mobile phase B: In a 1L Reagent Bottle take 500 ml of methanol and then add 1ml of formic acid, sonicate for 3 min and then makeup to volume with water. Label it as 0.1% formic acid in methanol.

3.3.1.2 Preparation of Colours Standard Stock Solution: Weigh accurately equivalent to 100mg of standard into a 10mL volumetric flask and dissolve in methanol: Water (1:1). Make up the volume with the same. Label it with name of the standard, concentration and preparation date. Store the solution in a refrigerator at 2-8°C.

3.3.2 Preparation of Colours working standard solution

Prepare working standard at conc. 100mg/L by transferring ~100µL of each stock solution (~10000mg/L) into a 10mL volumetric flask and makeup with methanol and store the solution in a refrigerator at 2-8°C. Prepare the solution once in three months.

3.3.3 Preparation of standard dilutions for calibration curves

Prepare the following dilutions ranging from 10mg/L to 100mg/L with 1:1 methanol: H₂O, “as shown below”, using working standard solution and further dilutions.

Stock Conc. (mg/L)	Volume of Stock(mL)	Volume of Diluent(mL)	Final Volume (mL)	Final Conc. (mg/L)

1000	0.100	0.900	1	100
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3.3.4 Preparation of calibration curve

Prepare the following concentrations ranging from 10 to 100 mg/L and label them as CC1 to CC5.

Stock Conc. (mg/L)	Volume of Stock(mL)	Volume of Diluent(mL)	Final Volume (mL)	Final Conc. (mg/L)	Name
1000	0.100	0.900	1	100	CC-5
1000	0.075	0.925	1	75	CC-4
1000	0.050	0.950	1	50	CC-3
200	0.100	0.900	1	20	CC-2
100	0.100	0.900	1	10	CC-1

3.3.5 Preservation

Samples should be stored at 2-8°C.

3.3.6 Sample Preparation

Take 20ml of sample into a 50ml glass beaker and degas it by placing in an ultrasonic bath. Then filter the degassed sample through 0.22µ filter and then inject into HPLC system.

3.3.7 Batch Organization

For each new batch of samples, follow the following order

Mobile phase blank, CC1 to CC5, max of 10 samples followed by one fortified control sample and so on.

3.3.8 Injection

Inject the prepared samples to HPLC for the analysis, maintaining the following conditions.

Chromatographic Conditions for HPLC

A summary of the HPLC conditions are as follows:

Column: Inertsil ODS, 3.0×75mm, 3μ

Injection Volume : 10μL

Absorbance Wavelength : 484 nm

Gradient conditions:

Time, min	Flow, mL	Solvent Ratio, %
0	0.3	15
1	0.3	15
5	0.3	60
8	0.3	90
9	0.3	10
10	0.3	10

3.4.0 Data Processing

The chromatograms were acquired using the computer-based software supplied by the manufacturer of the equipment. The concentration of the unknown was calculated from the calibration curve by using the below equation.

$$y = mx + c$$

Where,

y = Analyte areas

x = concentration of Analyte

m = slope of the calibration curve

c = y-axis intercept value

5.0 Calculations

$$\text{Concentration} = \frac{\text{Concentration obtained by HPLC in mg/L} \times \text{Final volume mg/L}}{\text{Volume of sample in the solution}}$$

6.0 Reporting

Report the values above >10ppm for Tartrazine, Indigocarmine, Ponceau-4R, and Sunset Yellow.

4.3 OIL SOLUBLE COLOURS:

Detection/identification:

Procedure:

(i) Absorption of colours on silica gel: Dissolve about 5 ml of sample in 25 ml of hexane. Add about 10 gm of silica gel (Column Chromatography Grade) and 2 gm of anhydrous sodium sulphate to the solution and stir the mixture for 5 min on a magnetic stirrer. In case the supernatant liquid layer is still coloured, add a further quantity of silica gel (2-3 gm) and stir once again for 5 min. Oils/fats are removed by stirring repeatedly (4-5 times) with 25 ml of hexane and draining out each time.

(ii) Recovery of colours from the silica gel: Extract the colouring matter from silica gel with 2-3 volumes of diethyl ether (15-20 ml each). Evaporate the total ethereal extract in a porcelain dish on a hot water bath.

(iii) Separation of colours from interfering matters: Dissolve the extract in 0.5 ml of diethyl ether and apply as a band on a preparatory TLC plate (thickness 300 μm). Develop the plate in a chamber containing hexane. Allow the plate to dry in air and scrape the colour bands into a small conical flask. Add about 1 gm of anhydrous sodium sulphate followed by 10 ml diethyl ether, and warm gently for a while on a hot water bath.

Decant the ethereal extract into a porcelain dish. Repeat the extraction for complete recovery of colour and concentrate the total extract to about 0.5 ml.

(iv) Identification of colours: Spot 20-50 μl (depending on the concentration of the colour) of the concentrated extract on a silica gel plate (thickness 300 μm) along with reference oil soluble colours in the solvent (dissolved in diethyl ether). Develop the plates using any one of the following solvent systems.

Solvent Systems

A	Benzene	- Hexane	- Acetone	40 : 45 : 7.5 : 1 (v/v)
B	Benzene	- Hexane	- Acetic Acid	40 : 60 : 1 (v/v)
C	Toluene	- Hexane	- Acetic acid	50 : 50 : 1 (v/v)

Air dry the plates and measure the R_f values of the coloured spots and compare with reference colours.

Confirm the identity of colours by spraying the following spray reagent:

Spraying reagent: 2.5 gm of boric acid and 1.5 ml of chloroacetic acid were dissolved in 100 ml of hydrochloric acid (Sp. gr. 1.19)

Observe the changes in colour as described below:

- (i) Curcumin and turmeric spots change to saffron colour and red colour respectively.

- (ii) Sudan TV (red) change to violet colour.
- (iii) Butter yellow changes to brilliant pink colour.
- (iv) Chlorophyll (buff green) change to shining green.

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

4.4 Determination of oil soluble dyes in Capsicum and Turmeric and their products by High Performance Liquid Chromatography

4.4.1 Apparatus

1. HPLC equipped with gradient pump, PDA detector, autosampler and computer data work station Chromatography column, Symmetry C 18, 250 mm x 4.6 mm, i.d, μm
2. Balance readable to 0.0001 gm
3. 50 ml culture tubes with Teflon lined cap
4. Vortex mixer
5. Wrist action shaker
6. 5 ml Luer- Lok disposable syringe
7. Whatman Nylon 0.45 μ filter or eqvt
8. Fisher brand PrepSep silica SPE columns, 500 mg load, 3 cc or eqvt
9. SPE Vaccum manifold

10. Evaporator / concentrator
11. Volumetric pipettes, various sizes
12. Volumetric flasks , various sizes
13. 25 ml graduated cylinder

4.4.2 Reagents

- 1) Methylene Chloride (HPLC grade)
- 2) Acetonitrile (HPLC grade)
- 3) Acetic acid glacial (HPLC grade)
- 4) Acetone (HPLC grade)
- 5) Methanol (HPLC grade)
- 6) Sodium Chloride (A.R)
- 7) Solution A – Prepare a 200 gm / L sodium chloride solution
- 8) Solution B – prepare a 12.5 % methanol in sodium chloride solution using solution A
- 9) Ethyl ether (HPLC grade)
- 10) Hexane (HPLC grade)
- 11) Sudan -1

12) Sudan –II

13) Sudan – III

14) Sudan –IV

15) Sudan Orange G

16) Sudan Red B

17) Dimethyl Yellow

18) Para Red

19) Cis – bixin

4.4.3 Preparation of calibration standards

1. Weigh a stock standard of Sudan 1-4, Para Red and dimethyl Yellow dyes by accurately weighing 0.025 gm of each dye in a 100 ml volumetric flask. Dissolve the dyes with methelene chloride. This is stock standard A.
2. Prepare a stock standard of Sudan Orange G and Sudan Red B dyes by accurately weighing 0.025 of each dye into a 100 ml volumetric flask. Dissolve the dye with methylene Chloride. This is stock standard B.
3. Prepare a stock standard of cis –bixin by accurately weighing 0.025 gm of the dye into a 100 ml volumetric flask. Dissolve the dye with methylene chloride. This is stock standard C.

Note – cis- bixin will isomerise to its trans configuration in solution. A new stock cis- bixin standard and working standards must be prepared when this occurs

4. Prepare four calibration standards from stock standard A and four calibration standards from stock standard B and C containing the following concentration of the dyes 0.1 µg / ml, 1 µg / ml, 5 µg / ml and 10 µg / ml. Dilute the eight

calibration standard solutions to volume with acetonitrile.

5. Transfer the calibration standards to autosampler vials and inject on the HPLC instrument

Note:

- (1) Correct each standard weight to pure dye content based on the declared purity of the dye
- (2) Store all standards in a freezer when not in use
- (3) Standard should be injected after each 4-6 sample injections
- (4) After the instrument linearity has been established by running the calibration standard series, then a single point standard calibration can be run with the 1.0 µg / ml standard. However the PDA detector must be capable of detecting a 0.10 µg / ml solvent standard.

4.4.4 Acquisition Parameters

- (1) Mobile Phase A – 1.0 % acetic acid
- (2) Mobile Phase B – Acetonitrile
- (3) Mobile Phase C - acetone
- (4) Gradient time programme

Time (Min)	Mobile phase A %	Mobile Phase B %	Mobile Phase C %
0	30	70	0
13.3	5	95	0
20.0	0	100	0

23.3	0	100	0
24.0	0	0	100
28.0	0	0	100
28.7	30	70	0
32.7	30	70	0

a) Flow rate: - 1.5 ml / min

b) Detection wavelength -300 nm to 650 nm

c) Injection volume – 20µL

d) Column temperature -25 °C

4.4.5 Preparation of sample control and spikes

(1) Accurately weigh 4.0 – 8.0 gm of control sample into 5 separate 50 ml culture tubes. To one of the samples, pipette 10 µl of the stock standard A solution (the concentration of each dye in this spike sample will be between approx 0.3 -0.6 mg / kg). To a second sample pipette 100 µl of stock standard A solution (the conc of each dye in the sample will be between 3-6 mg / kg). To a third sample pipette 10 µl of stock standard B and 10 µl of stock standard C solutions (the conc of each dye in this spiked sample will be approx 0.3- 0.6 mg / kg. To a fourth sample pipette 100 µl of stock standard B and 100 µl of stock standard C solution (the conc of each dye in this spiked sample will be between 3-6 mg /kg)

(2) Pipette 20 ml of acetonitrile into each tube, cap and shake on a wrist action shaker for 1 hr. Allow the solids to settle or centrifuge

(3) Filter through 0.45 µ nylon filter into autosampler vials and inject on the HPLC

Note: - Spike recovery should be between 75-125% of the calculated amount of each dye Spikes should be run with each different sample matrix to identify coeluting or interfering peaks from

the sample matrix. For similar sample matrixes, a spiked sample should be run with every 10 sample extracts.

4.4.6 Preparation of sample

Accurately weigh 4.0 – 8.0 gm of sample in a 50 ml culture tube

Pipette 20 ml of acetonitrile into each tube, cap and shake on a wrist action shaker for 1 hr. allow the solids to settle or centrifuge

Filter through a 0.45 µ nylon filter into auto-sampler vials and inject on HPLC instrument

Note: - A sample clean up step may be necessary for concentrated or complex products in order to remove some of the compounds that interfere with the chromatographic peaks of interest. To perform this clean up proceed as below. When testing for Sudan Orange G, sample clean up must be performed to eliminate matrix interferences which coelute with the Sudan Orange G peaks in capsicum samples.

4.4.7 Sample and spike sample clean up

Pipette 5 ml of acetonitrile into a 50 ml centrifuge tube. Pipette 5 ml of hexane into centrifuge tube. Use a 25 ml graduated cylinder to add 25 ml of solution B to the centrifuge tube. Shake gently for 30 seconds, then centrifuge for 3 min Prepare a silica SPE column for each sample.

Initially prewash the silica SPE with one column volume of ethyl ether followed by two column volumes of hexane. Discard the eluted solvent wash Note: - Prewash each silica SPE column prior to use. Keep the silica bed wet with solvent and do not store prewashed SPE columns for more than 30 min Mild vacuum may be applied to SPE columns to pull the solvent through the column Pipette 2 ml of hexane (top) layer into solvent washed SPE columns. Drain the sample extract into the column bed at 1-2 drips per second. Wash with one column volume of hexane and discard the hexane wash.

Place clean collection tubes below the silica SPE columns and elute the dyes into the collection tubes with two column volumes of 10 % acetone in hexane.

Evaporate the solvent in the collection tubes to dryness under a stream of dried nitrogen or other inert gas Redissolve the residue in each collection tube with 2 ml of acetonitrile.

Filter through 0.45 μ Nylon filter into auto sampler vials and inject on the HPLC instrument

4.4.8 Processing / Data Analysis Parameters

Set up a processing / data analysis method to process the calibration standards containing Sudan 1- 4, Para Red and Dimethyl Yellow. Use the following wavelengths – Dimethyl Yellow- 450 nm, Para Red – 450 nm, Sudan I -505nm, Sudan II – 505 nm, Sudan III – 530 nm, Sudan IV – 505 nm (550 nm for paprika oleo resin) Process the corresponding four calibration standards and samples for these six dyes Set up a processing / data analysis method to process the calibration standards containing Sudan Orange G , cis – bixin and Sudan Red B. use the following wavelengths Sudan Orange G -377 nm, cis – bixin – 460 nm , Sudan red B – 505 nm (550 nm for paprika oleoresin) Process the corresponding four calibration standards and samples for these three dyes.

4.4.9 Calculation

Using the data processing technique perform a linear regression analysis for each dye to determine the slope m of the dye's calibration curve. Force the line through the origin. Let the peak area be the y – variable and the concentration be the x – variable.

Calculate the concentration of the dye in the samples with the following formula

$$C_x = (A_x \times 20) / (m \times W)$$

Where,

C_x = concentration of the dye (x) found ug / gm (mg / Kg)

A_x = peak area of dye (x) in the sample

m = slope of the calibration curve for dye (x)

20 = sample extraction volume in ml

W = sample weight in gm

(Ref: - ASTA Analytical Method 28, 0 / European commission News notification 03 / 99 / Chinese National Quality Assurance and Inspection Bureau GB / t 19681 – 2005)

5.0 ANTIOXIDANTS

Antioxidants are added to oils and fats to prevent oxidative rancidity. Ethyl, propyl, octyl and dodecyl gallates, butylated hydroxyanisole (BHA), tertiary butyl hydroquinone (TBHQ) and resin guaic, ascorbic acid, tocopherol are permitted under FSS, Rules and Regulation, 2011.

5.1 Qualitative Method:

5.1.1 Detection of propyl gallate, BHA, BHT and Nordihydroquaiaretic acid (NDGA) in oils and fats:

Reagents:

- (i) Barium hydroxide (1%):** Dissolve 1 gm of $Ba(OH)_2 \cdot H_2O$ in 100 ml distilled water. Acetonitrile (methyl cyanide) solvent (CH_3CN) saturated with petroleum ether.
- (ii) Ehrlich reagent:** Diazobenzene sulfonic acid (0.5%): Prepare 0.5% solution of sodium nitrite in water and 0.5% solution of sulfonilic acid in hydrochloric acid (1+2). Prepare sodium nitrite solution fresh every three weeks. Keep solutions refrigerated. Mix sodium nitrite and sulfonilic acid solution (1+100) daily.
- (iii) Dianisidine solution:** Dissolve 250 mg of dianisidine (3,3 dimethoxybenzidine) in 50 ml of anhydrous methanol. Add 100 mg activated charcoal, shake for 5 min and

filter. Mix 40 ml filtrate with 60 ml of 1N hydrochloric acid. Prepare daily and protect from light.

- (iv) **Activated florisil absorbent:** 60-100 mesh activated at 260°C or 650°C.
- (v) **Test florisil for BHT retention as follows:** Add 0.2 mg of BHT in 25 ml petroleum ether to prepared column, elute with 150 ml petroleum ether and apply BHT test after evaporating the solvent just to dryness. If BHT is not eluted, activate remaining florisil by heating for 2 hrs at 650°C, cool and add 6.5% water by weight and homogenize by shaking for 1 hour in a closed container.
- (vi) **Preparation of florisil column for cleanup of BHT extract:**
Insert small glass wool plug into a chromatographic tube 20 (o.d.) × 250 mm with stopcock. Add 12 gm of florisil with gentle tapping. Wash with two 15 ml portions petroleum ether, adding second portion when liquid level is just above top of florisil. Do not let the column dry.

Tests

- (a) **Propyl gallate (PG):** Weigh about 30 gm of fat or oil, dissolve in about 60 ml of petroleum ether and transfer to 250 ml separator. Add 15 ml of water and shake gently for 1 min. Let layers separate and drain aqueous phase into 125 ml separator, leaving any emulsion in organic phase. Repeat extraction of petroleum ether with two additional 15 ml portions of water and reserve organic phase for further extraction with acetonitrile. Add 15 ml petroleum ether to aqueous extract and shake for 1 min. Discard aqueous phase and evaporate the solvent just to dryness in small beaker. Add 4 ml of 50% alcohol to residue, swirl and add 1 ml NH₄OH. If the solution turns to rose colour, PG is present (colour is unstable and fades after few min)
- (b) **Nordihydroquaiaretic acid (NDGA):** Extract petroleum ether solution from (a) by shaking for 2 min with 20 ml acetonitrile. Let layers separate and drain acetonitrile into 1 L separator. Repeat extraction with 2 additional 30 ml portions of acetonitrile and discard petroleum ether phase. Dilute combined acetonitrile extracts with 400 ml water,

add 2- 3 gm sodium chloride and shake for 2 min with 20 ml petroleum ether. Let layers separate, drain the diluted acetonitrile layer into second 1000 ml separatory funnel. Extract dilute acetonitrile layer with two additional 20 ml portions of petroleum ether and reserve dilute acetonitrile solution for further extraction. Combine petroleum ether extracts in 100 ml beaker and set aside for BHA and BHT tests. Add 50 ml ether + petroleum ether (1+1) to diluted acetonitrile from (b) and shake for 2 min Let layers separate, discard acetonitrile and evaporate the solvent just to dryness in a small beaker. Add 4 ml of 50% alcohol, swirl and then add 1 ml of 1% Barium hydroxide solution and mix. If NDGA is present the solution turns to blue and fades rapidly.

(c) Butylated hydroxyanisole (BHA): Take 1/3 of combined petroleum ether solution reserved for BHA and BHT tests and evaporate just to dryness, using gentle heat, under air current. Add 2.5 ml of alcohol to dissolve residue and dilute with 2.5 ml water. Swirl, add 1 ml of Ehrlich reagent followed by 1 ml of a 1N sodium hydroxide and swirl again. If solution turns red purple, BHA is present.

(d) Butylated hydroxytoluene (BHT): Pass remaining 2/3 combined petroleum ether through florisil column and elute with 150 ml petroleum ether. Collect eluate in 200 ml beaker and evaporate just to dryness. Add 2.5 ml alcohol swirl and dilute to 5 ml with water and mix. Add 2 ml of dianisidine solution and mix. Add 0.8 ml of 0.3% sodium nitrite (Sodium nitrite) solution, mix and let it stand for 5 min, then transfer to a small separator. Add 0.5 ml chloroform (CHCl_3) &-shake vigorously for 30 sec. and let layers separate. If chloroform layer turns pink to red. BHT is present. Confirm BHT by comparing spectrophotometric curve of coloured chloroform extract obtained from reference standard BHT by dissolving approximately 15 mg in 5 ml aqueous alcohol (1+1) and 2 ml dianisidine.

(Ref: - A.O.A.C 17th edn, 2000 Official method 965.28 Antioxidants in Food, Qualitative Colour Tests)

5.1.2 Thin Layer Chromatographic Detection of Antioxidants:

Principle:

The sample oil is dissolved in petroleum ether and extracted with acetonitrile. Acetonitrile extract is evaporated in vacuum in a rotary evaporator at a temperature not exceeding 40°C. The residue is dissolved in alcohol, applied to TLC plates and after development, spots are visualized by spraying with Gibb's reagent.

Apparatus:

- a) Separatory funnel (250 ml); flask;
- b) Rotary evaporator;
- c) TLC equipment; Silica Gel G.

Reagents:

- (i) Acetonitrile
- (ii) Petroleum ether
- (iii) **Developing solvent:** Petroleum ether : benzene : acetic acid (2 : 2 :1)
- (iv) **Spray reagent:** 2, 6-dichloroquinone chlorimide (Gibb's reagent); 0.1% in alcohol
- (v) **Standard solution (0.1%):** Dissolve propyl gallate (PG), octyl gallate (OG), dodecyl gallate (DG), butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) in methanol.
- (vi) **Developing solvent:**

A - Petroleum ether -benzene - Glacial acetic acid (2: 2: 1)

B - Petroleum ether -benzene -ethyl acetate - gl. acetic acid (40: 40:25: 4)

C - Chloroform - methanol - gl. Acetic acid (90: 10: 2)

Procedure:

Dissolve 10 gm of oil or melted fat in 100 ml of petroleum ether and transfer into a 250 ml separatory funnel. Add 25 ml acetonitrile saturated with petroleum ether to the separator and shake gently. Run off the acetonitrile into a second separator and repeat the extraction three times. Transfer acetonitrile extracts to rotary evaporation flask and evaporate at less than 40°C temperature just to dryness. Dissolve the residue in 2 ml methanol, filter if not entirely soluble Prepare 20 × 20 cm silica gel G plates with a 0.25 mm layer using 30 gm in a slurry with 60 ml 1% citric acid solution. Dry the plates in air, activate at 30°C for 1 hour and store in a desiccator. Saturate the developing chamber with a freshly prepared solvent mixture. Line the tank with filter paper, allow to stabilize for 1-2 hrs in the dark. Apply 10 to 20 µg extract solution along with standards (4 µl) 2 cm apart on a start line 2 cm above the bottom edge.

Develop the plate to a distance of 15 cm and allow it to air dry. Spray with Gibb's reagent and dry at 103 ± 2°C for 15 min Compare the colour and R_f values with standards. Cool the plate and place in a tank containing ammonia and note the characteristic colour change as indicated below:

Antioxidant	R _f value with developing solvents			Gibb's reagent	Gibb's reagent followed by ammonia
	A	B	C		
PG	0.12	0.25	0.55	Brown	Gray-Green
OG	0.22	0.40	0.64	Brown	Gray-Green
DG	0.27	0.45	0.66	Brown	Gray-Green
BHA	0.62	0.87	0.92	Brown-red	Gray

BHT	0.82	0.99	0.95	Brown- Violet	Gray

(Ref: - Pearson's composition and Analysis of Foods 9th edn, 1991 Page 100)

5.2 Quantitative method:

5.2.1 Spectrophotometric Determination of Propyl Gallate:

Principle: Oil or melted fat is dissolved in petroleum ether and extracted with ammonium acetate solution and water. The combined extract is treated with ferrous tartarate and the absorbance of the coloured solution is read at 540 nm. The amount of PG present in the sample is calculated from the calibration graph.

Reagent:

Petroleum ether reagent: Mix one volume of 40-60°C 167C petroleum ether with 3 volumes of 80-100°C petroleum ether. Shake for 5 min with 1/10th of its volume of sulphuric acid. Discard acid layers, wash several times with water, then again with water until washings are subsequently neutral. Discard all washings and distill petroleum ether in all glass apparatus.

Ammonium acetate solutions: 1.25%, 1.67% and 10% aqueous solutions. Solution containing 1.67% NH₄OAC in 5 % alcohol may also be required.

Ferrous tartarate reagent: Dissolve 0.1 gm of FeSO₄.7H₂O and 0.5 gm of Rochelle salt (NaKC₄H₄O₆.4H₂O) in water and dilute to 100 ml. Reagent must be used within three hrs of preparation.

Propyl gallate standard solution: 50 µg/ml. Dissolve 50 mg in water and dilute to 1000 ml with water.

Preparation of standard curve:

Place 7 aliquots of standard solution ranging from 50 to 1000 µg in 50 ml Erlenmeyer flasks. Add exactly 2.5 ml of 10% NH₄OAC to each flask, dilute exactly to 24 ml with water and pipette 1 ml of ferrous tartarate solution into each flask. Let the solution stand for 3 min. Measure the absorbance at 540 nm against solution containing 20 ml of 1.25% ammonium acetate solution, 4 ml water and 1 ml ferrous tartarate solution. Plot the calibration curve.

Procedure:

Dissolve 40 gm of fat or oil in the petroleum ether reagent and dilute to 250 ml with reagent (gentle warming may be necessary to obtain complete solution). Pipette 100 ml of fat solution into 250 ml separator. Extract with 20 ml of aqueous 1.67 % ammonium acetate solution by gentle shaking for 2.5 min. Allow the layers to separate and drain aqueous layer into 100 ml volumetric flask (some shortenings show strong tendency to emulsify during aqueous extraction). To prevent emulsification, add 2 ml n-octanol to fat solution before beginning extraction and use 1.67 % ammonium acetate solution in 5% alcohol for extraction in place of aqueous solution. This procedure is used only when usual method fails. Repeat extraction twice with 20 ml portion of ammonium acetate solution and collect in volumetric flask.

Finally, extract fat solution with 15 ml water for 30 sec. and combine aqueous layer. Add exactly 2.5 ml of 10% NH₄OAC solution to combined extract and dilute to volume with water. Filter through filter paper (No.4) to remove any turbidity and develop colour the same day the extract is prepared.

(If combined extracts stand several hrs, yellow colour may develop and solutions must be discarded).

Pipette aliquot of extract (about 20 ml) into 50 ml Erlenmeyer flask. Dilute to 20 ml with 1.25% ammonium acetate solution. Add exactly 4 ml water and pipette 1 ml ferrous tartarate solution. Mix well and measure absorbance at 540 nm against a solution containing 1.25% NH₄OAC solution, 4 ml water and 1 ml ferrous tartarate solution. Calculate the amount of propyl gallate from the calibration curve.

(Ref: - A.O.A.C 17th edn, 2000 Official Method 952.09 Propyl Gallate in Food - Colorimetric Method) / Manual Methods of Analysis for Adulterants and Contaminants in Foods I.C.M.R 1990 Page65)

5.2.2 Spectrophotometric determination of BHA:

Principle:

BHA is extracted from oil or melted fat sample with 95 % methanol. The extract gives colour with Gibb's reagent which has maximum absorption at 610 nm.

Reagents:

- (i) 95% (v/v) methanol
- (ii) Sodium tetraborate decahydrate: 0.5%
- (iii) 2, 6-dichloroquinone chlorimide (Gibb's reagent): 0.01% in 95% methanol.
- (iv) N-butanol
- (v) BHA-standard: Prepare 25 mg/L in 95% methanol.

Procedure:

Vigorously shake 10 gm of warm liquid sample or melted fat with 25 ml of 95% methanol for one minute in a centrifuge tube. Place in a water bath at 40-50°C and allow to separate for about 15 min Pour the upper layer into a 50 ml calibrated flask, repeat the extraction with 20 ml of 95% methanol, transfer the upper layer to the flask and dilute to mark. Add one gram of calcium carbonate, shake and filter through a paper (Whatman No.1 or equivalent) rejecting the first few ml of filtrate. The amount of calcium carbonate is not critical but must be enough to ensure a clear filtrate. To exactly 2 ml of this extract add 2 ml of 95%

methanol, 8 ml of borax solution and 2 ml of Gibb's reagent. After 15 min dilute exactly to 20 ml with n- butanol. Prepare the blank and standard using the 25 mg/L BHA solution.

Read the absorbance at 610 nm and calculate the amount of BHA present in the sample from the absorbance of sample and the standard.

Note: Excess of gallates reduce the intensity of the colour. For example, 200 mg/L of propyl gallate reduces the colour from 200 mg/L BHA to about one half under the standard conditions of the test.

(Ref: - F.A.O Manuals of Food Quality Control 1980, 14/2 Page 49 / Manual Methods for Adulterants and Contaminants in Foods I.C.M.R 1990, Page 66)

5.2.3 Spectrophotometric determination of BHT:

Principle:

The sample is steam-distilled and BHT in the steam distillate is determined by the colour reaction with Q-anisidine and sodium nitrite.

Apparatus:

- (i) Distillation and volumetric flasks;
- (ii) Separatory funnels 60 ml capacity;
- (iii) steam distillation apparatus;
- (iv) oil bath at 160°C;
- (v) Steam generator.

Reagents:

(i) Chloroform

- (ii) **Magnesium chloride solution:** Dissolve 100 gm of magnesium chloride hexahydrate in 50 ml water.

- (iii) **O-dianisidine solution:** Dissolve 0.25 gm in 50 ml methanol, add 100 gm of activated charcoal, shake for 5 min and filter. Mix 40 ml of this clear solution with 60 ml of 1 N hydrochloric acid. Prepare daily and protect from light.
- (iv) **Sodium nitrite:** Prepare 0.3% solution in water.
- (v) **Standard solution of BHT:** Dissolve 50 gm in methanol and dilute to 100 ml with methanol. Prepare working standards containing 1-5 µg/ml by diluting with 50% (v/v) methanol.

Procedure:

Add 15 ml of magnesium chloride solution and 5 gm of fat to the distillation flask. Preheat the bath for the distillation flask to $160 \pm 10^\circ\text{C}$. Adjust the steam generator to distill about 4 ml water per minute. Connect condenser and steam-generator to the distillation flask and immediately immerse the later in the oil bath. Steam distillation must be vigorous. Collect the first 100 ml of the distillate in a 200 ml volumetric flask containing 50 ml methanol. Disconnect the distillation flask from the steam generator and remove it from the oil bath. Wash the condenser with 5 ml portions of methanol adding washing to volumetric flask. Cool to room temperature and adjust the volume to 200 ml with methanol and mix.

Clean and dry three 60 ml separating funnels of low actinic glass or painted black. To the first one add 25 ml of 50% methanol, to the second one add 25 ml of 1-3 µg/ml standard and to the third one add 25 ml of distillate. To each funnel add 5 ml of O-dianisidine solution, stopper the funnel and carefully mix the contents. Then to each funnel add 2 ml of 0.3% sodium nitrite solution, stopper the funnels and thoroughly mix the contents. Let them stand for 10 min then add 10 ml of chloroform to each funnel. Extract the coloured complex by vigorously shaking funnels for 30 sec. Let the layers separate.

Mark 10 ml volumetric flasks of low actinic glass for blank, standard and sample. Draw off the chloroform layer to the corresponding flasks to reach the mark on the flask. Read the absorbance at 520 nm using 2 ml methanol and 8 ml chloroform as blank.

Calculate the BHT content of the sample from the formula.

BHT content of sample (mg/kg)

$$= \frac{\text{A sample - A blank} \times \text{Standard (} \mu\text{g/ml)} \times \text{wt. of sample}}{\text{A standard- A blank} \times 200}$$

(Ref: - F.A.O Manuals of Food Quality Control 1980 14 / 2 Page 43 / Manual Methods of Analysis for Adulterants and Contaminants in Foods I.C.M.R 1990 page 66)

5.2.4 Antioxidants by High Performance Liquid Chromatography

(Applicable to propyl gallate (PG), trihydroxybutyrophenone (THBP) Ter- butyl hydroquinone (TBHQ), nor dihydroguaritic acid (NDGA), butylated hydroxyl anisole (BHA), butylated hydroxyl toluene(BHT) at 20- 200 ug / gm in oils and fats 10-100 ug /gm in butteroil and to octyl and dodecyl gallate at 10 – 100 ug / gm in butter oil)

Principle:

Antioxidants are extracted into acetonitrile Extract is concentrated and diluted with 2 propanol. Antioxidants are separated by liquid chromatograph and measured by uv detection at 280 nm.

Apparatus

(a) Liquid Chromatograph (LC) – equipped with 10 mV recorder or integrator to electronically measure peak heights, 10 µl sample loop injection valve and detector to measure absorbance at 280 nm. Typical operating conditions - detector sensitivity 0.05AUFS, temp ambient , flow rate 2ml / min

(b) LC column - Packed with C 18 bonded spherical(preferred) silica or equivalent

- (c) Glass ware – Rinse all glass ware with chloroform, acetone and methanol successively and blow dry with nitrogen.

Reagents

- a) Solvents – Acetonitrile, 2 propanol, hexane (HPLC grade)

- b) Mobile phase – (1) 5 % acetic acid in water (LC grade),

- (2) Acetonitrile – methanol (1+ 1, v /v) LC grade

Run linear gradient from 30 % (1) to 100 % (2) over 10 min with hold until last antioxidant (DG) is eluted. For test solutions only increase flow rate to 4 ml/ min at 100 % (2) over 6 min or until non polar liquids are eluted. For test solutions and standards return to 30 % (2) in (10 over 1 min at 2 ml / min and let baseline and pressure stabilize. Reduce flow rate and proportionally increase rinsing and equilibrium times if excessive back pressure results. Run blank solvent gradient (no injection) to ensure that no peaks interfering with any antioxidant are present. To remove or reduce peaks arising from elution solvent (1) replace in let filter with pre rinsed solid – phase C 18 extraction cartridge and use in line filter. If small interfering peaks are not eliminated, subtract peak height of gradient interference from that of relevant standard or test solution

(c) Standard solutions

Prepare in 2 propanol – acetonitrile (1 + 1, v/v). (Caution – TBHQ is readily oxidized especially in light. Refrigerate all antioxidant solutions and store out of direct light). Monitor TBHQ response relative to PG or THBP and prepare fresh standards if response decreases more than 5 %.

- (1) Stock standard solution - 1 mg / ml accurately weigh 50 mg to nearest 0.1 mg each antioxidant and transfer to single 50 ml volumetric flask. Dissolve dilute to volume and mix.

(2) Working standard solution - 0.01 mg / ml. Pipette 1 ml of stock standard solution into 100 ml volumetric flask, dilute to volume and mix.

(d) Extraction Solvents – (1) Saturated hexane – Saturate about 300 ml in separatory funnel by adding acetonitrile until two layers remain after shaking 2 mins. Discard acetonitrile lower layer.

(3) Saturated acetonitrile = saturate about 300 ml acetonitrile in separatory funnel by adding hexane until two layers remain after shaking 2 mins.

Discard hexane upper layer.

Determination

(a) Extraction

Accurately weigh to the nearest 0.01 gm, 50 ml beaker containing about 5-6 gm liquid or butter oil or 3 gm lard or shortening (liquefied in water bath at 60°C and swirled or shaken to ensure homogeneity). Decant as much test portion as possible into 125 ml separatory funnel containing 20 ml saturated hexane. Reweigh beaker to determine test portion weight, Mix test portion with hexane and extract with three 50 ml portions of saturated acetonitrile. If emulsion forms hold separatory funnel under hot tap water for 5-10 seconds. Collect extracts in 250 ml separatory funnel and let combined extracts slowly drain into 250 ml round bottom flask to aid removal of hexane – oil droplets.

(Note: - At this point 150 ml acetonitrile extract may be stored overnight in refrigerator). Evaporate to 3- 4 ml using flash evaporator with 40 °C water bath within 10 min (Note :- (a) Prolonged evaporation time may cause TBHQ losses. To decrease evaporation time use efficient vacuum source and water – ice condenser cooling. (b) Use 500 ml flask to reduce bumping losses. Take care to ensure quantitative transfer of extract after evaporation

(a) Chromatography

Using sample loop injection valve, inject 10 µl sample extract and eluate with solvent gradient programme with mobile phase 1 and 2. Before and after every 3-4 test injections or more frequently if differences between standard peak heights are found to be more than 5 %, inject 10 µl of antioxidant working standard solution (10 µl / ml) and elute with solvent gradient programme for standards. For analyte peaks off scale or more than 3 x standard, quantitatively dilute test extract with 2 propanol – acetonitrile (1+1) and re inject. Identify peaks by comparison with retention times of standard.

For reagent blank determination, take 25 ml of saturated hexane and follow extraction from “extract with three 50 ml portions saturated acetonitrile, Inject 10 µl of reagent blank extract and elute with solvent gradient programme. The reagent blank should have no peaks interfering with antioxidant determination.

Calculate concentration of antioxidant as follows:-

$$\text{Antioxidant } \mu\text{g / gm} = (R_x / R_s) \times (C_x / W_s) \times D$$

Where,

R_x and R_s are peak heights from test portion and standard respectively

C_x is concentration standard µg / ml

W_s is test portion wt gm / ml in undiluted 10 ml extract and

D is dilution factor, if solution injected is diluted.

(Ref: - A.O.A.C 17th edn, 2000 Official method 983.15 Phenolic Antioxidants in oils fats and butter oil – Liquid Chromatography Method)

Note :- Another Liquid chromatographic method for determination of Propyl Gallate (PG), 2,4,5, trihydroxybutyrophenone (THBP), tert, butyl hydroquinone (TBHQ), nordihydroguaiaritic acid (NDGA), 2 and 3 ter- 4- hydroxyanisole(BHA), 2,6- di – tert- butylmethylphenl (Inox 100), 2, 6 di- ter- hydroxy toluene (BHT) has been described in A.O.C.S Method Ce 6 -86

6.0 EMULSIFIERS, STABILIZERS AND THICKENERS

A variety of organic compounds form the group of emulsifiers, stabilizers and thickening agents. Compounds such as stearyl tartarate, glycerol esters like glycerylmonostearate,

propylene glycol esters, and sorbitan esters of fatty acids, cellulose ethers and sodium carboxymethyl cellulose are in use for making food emulsions and to stabilize them. Pectin, alginates, agar, Irish moss, cellulose, carboxy methyl cellulose, starch and certain gums like guar gum, gum Arabic, karaya gum, gum ghati, tragacanth gum, locust bean gum, gelatin etc. are being used as thickening agents.

6.1 Thin Layer Chromatographic Detection of Emulsifiers

Principle

Emulsifier together with fat is extracted by blending with chloroform and methanol. On adding a calculated amount of water, chloroform containing fat and emulsifier separates. The chloroform layer is evaporated to dryness and emulsifier present extracted with methanol. The methanol extract is subjected to TLC

Apparatus

- (1) TLC equipment including Polygram Sil G. plates
- (2) Short wave UV lamp, 256 nm
- (3) Rotary film evaporator

Reagents

- (1) Chloroform
- (2) Methanol
- (3) Magnesium Chloride solution, 2 N- in water
- (4) 1, 2 - dichloromethane
- (5) Cyclohexane
- (6) Butan-2-one
- (7) Glacial acetic acid
- (8) Dibromofluorescein, 0.2 % in 95 % ethanol

(9) TLC solvent 1:- Pipette 12 ml methyl alcohol into a dry 200 ml volumetric flask and dilute to mark with 1, 2 dichloromethane

(10) TLC Solvent 2:- Pipette 4 ml water and 16 ml acetic acid into a dry 200 ml volumetric flask. Add from a measuring cylinder 80ml dry butan -2 -one and dilute to mark with cyclohexane (This solvent separates below 15°C.)

Procedure

Into a dry macerator, put about 20 gm sample, 40 ml chloroform, 80 ml methanol and 4 ml magnesium chloride solution. Macerate about 2 min Add further 40 ml chloroform and macerate about 2 min more. Filter through Whatman filter paper No1. A reasonably clear filtrate should be obtained. Wash the macerator with chloroform and pass through the filter. Transfer the filtrate to a 250 ml separator. Add sufficient water to make a total of 72 ml. Mix thoroughly and allow to stand until the chloroform has completely separated and is clear (takes at least 1 hr). Run off the chloroform into a tared round bottom flask. Evaporate on a water bath and dry at 100°C to obtain an estimate of the fat and emulsifier content. Add sufficient methanol to give a 10 % solution. Heat to boiling on a water bath. Stopper and shake the flask. Unstopper and heat again to boiling, then allow to stand and cool for 10 min until the undissolved fat has settled. Decant off the methanol into a tared flask, evaporate on a water bath, and then dry at 100°C to give the weight of emulsifier concentrate. Dissolve concentrate in chloroform / methanol 1:1 to give a 10 % solution TLC plates supplied as 20 × 20 cm need washing to remove fluorescent impurities. This is done by developing them with chloroform / methanol 1:1 in an inclined tank for 24 hrs dry at 100°C for about half hour. The plate is then cut into quarters. The cut edges are tidied by removing a 3 mm wide strip of silica gel using a ruler and razor blade. The origin is placed at 8 cm from both cut edges. In this way the distortion due to edge effect is minimized. The repaired plates can be stored at 100°C until required.

0.5 µl of emulsifier solution is spotted at the origin in as small a spot as possible. The spot is dried at 100°C for 10 mins. The plates can be bent so that they can be run in a larger glass jar. About 25 ml solvent is needed and development takes about 20 min each way. The plates should be removed as soon as the solvent front reaches the top. The first solvent is removed at 105°C in vacuum for 10 min before running in the second solvent. The second

solvent must also be removed under the same conditions otherwise it interferes with spot location. The spots are located by spraying with a 0.02 % dibromofluorescien solution in 95% ethanol until pale orange. Dry briefly at 100°C and view under short wave light. It often helps to re spray the plate lightly and allow to dry under UV.

Note: - It is essential to run standard commercial emulsifier preparations as comparisons.

(Ref: - F.A.O Manuals of Food Quality Control 1986 14 / 7 Page118)

6.2 Detection of Alginates in foods (chocolate, ice-cream and frozen products):

Reagents:

Ferric hydroxide - sulphuric acid (Fe -sulphuric acid reagent):

Dissolve 10 gm of $\text{FeCl}_3 \cdot \text{H}_2\text{O}$ in approximately 100 ml of water in each of 2 centrifuge bottles and precipitate ferric hydroxide by adding excess ammonium hydroxide (by odour). Wash precipitate with approximately 5 portions of water, centrifuging and decanting until little odour of ammonia remains. Break up centrifuged precipitate each time before washing. Dry precipitate on steam bath or in oven overnight, break up and dry again. Grind and place 0.5 gm dry powder in 50 ml graduated flask, add 50 ml sulphuric acid. Shake vigorously and let it settle until it is clear (4-7 days). Some ferric sulphate appears to stick to sides, but reagent is ready for use after seven days. Prepare fresh after 3 weeks. Check the reagent as follows.

Dissolve small amount 1-5 mg) of alginate in water containing 5 drops of 0.1N sodium hydroxide, add 4 volumes of alcohol to precipitate alginate. Centrifuge, decant and dry on a steam bath until no odour of alcohol remains. Add 3 drops of 0.1N sodium hydroxide, dissolve with the aid of glass rod, and add 2 ml Fe- sulphuric acid reagent. Solution turns purple slowly, usually within one hour, depending on amount of alginate present, but it may take longer. If solution appears to be turning brown, add additional 2 ml reagent, mix with glass rod and let it stand.

Procedure:

Weigh sample containing 10-20 mg alginate into 250 ml centrifuge bottle, add water to volume of 40-50 ml and dissolve by swirling. Adjust pH to 8-9 with saturated Na₃PO₄ solution, usually 5 drops is enough. Add about 0.5 gm of Pancreatin and 3 drops of formalin and shake vigorously for 1 min let it stand for 2-16 hrs.

Centrifuge at 1200 rpm for 2-3 min, decant into 250 ml centrifuge bottle, and discard residue. Add 3 volumes alcohol, shake and let it stand for 1 hr. Shake several times, centrifuge as before and discard the liquid. Add 3 gm of decolourising charcoal and shake vigorously for 1 hour on a mechanical shaker. Do not centrifuge but pour directly into folded filter paper, collecting filtrate in 250 ml centrifuge bottle. If filtrate is not clear pour back through filter paper several times. If filtration is slow let it filter over night. To the filtrate add 4 volumes of alcohol, shake and let it stand for an hour, or overnight if convenient. Centrifuge and decant saving the residue. Residue contains alginates, gums and gelatin. Dry residue on steam bath, using air current if desired, until no odour of alcohol can be detected. Cool, add 3 drops of 0.1 N sodium hydroxide and dissolve residue, using glass rod, as completely as possible. Add 2 ml Fe-sulphuric acid reagent, solution turns to purple if alginate is present. If brown appears, add additional 2 ml reagent. Let stand overnight as colour develops slowly. Deep purple is positive test for alginate. If test is negative repeat determination using twice the size sample.

(Ref: - A.O.A.C 17th edn, 2000 Official Method 959.06 Alginates in Chocolate Products)

6.3 Stabilizing agents and Gums:

6.3.1 Extraction of gums from fruit and vegetable products:

Grind solid samples in a blender and weigh 50 gm of the sample. To solid or semisolid samples add equal quantity of water. Filter or centrifuge and decant the supernatant liquid. To about 10 ml, add 20% tannic acid solution and to another 10 ml, add 40% trichloroacetic acid and compare the volume of the precipitates. Tannic acid precipitates only proteins and alginic acid. Depending on the results of the tests, treat the rest of the supernatant liquid either with tannic acid or with trichloroacetic acid.

Allow it to stand for 15 min and centrifuge for 5 to 10 min Test a portion of the

supernatant liquid for complete precipitation either with tannic acid or trichloroacetic acid. If precipitate is formed, add more, re-centrifuge and filter. To the filtrate add 5 volumes of alcohol with constant stirring and allow the solution to stand for 5 to 10 min. Add ammonia drop by drop, until the mixture is alkaline, allow it to stand for 5 min and then add 10 drops of conc. hydrochloric acid with vigorous stirring. A precipitate at this time indicates the presence of gums, starches, dextrans etc.

Allow the mixture to stand overnight. Filter, wash the precipitate thoroughly with alcohol and dissolve the residue in 30 ml of boiling water and boil, if necessary to make into solution. Use this solution for identification.

Chemical nature of the commonly used thickening agents

Sl No	Material	Source	Principal compound
1	Pectic substances	Fruit	Galacturonic acid
2	Algin (Sodium alginate)	Seaweeds	Mannuronic acid (Na Salt)
3	Irish moss	Seaweeds	Galactose, galactose-4 sulphate (K and Ca Salts)
4	Agar	Seaweeds	Galactose, D and – galactose 6-sulphate (Ca and Mg salts)
5	Tragacanth	Plant gum	alpha fructose, D-xylose, alpha arabinose, D-galactose
6	Methyl cellulose	Modified cellulose	Methyl D-glucose
7	Starch	Plants	D-glucose
8	Carboxymethyl	Modified cellulose	Carboxy methyl D-glucose cellulose
9	Locust-bean gum (carob gum)	Seed endosperm	Mannose and galactose
10	Guar gum	Seed endosperm	Mannose and galactose
11	Karaya Gum	Plant gum	Galactose, acetic acid,

			galactonuric acid tagatose rhamnose,
12	Arabica (acacia)	Plant gum	D-glucuronic acid, D-galactose, D-arabinose, (mixed Ca, Mg and K salts)
13	Ghatti	Plant gum	alpha arabinose, galactose, galacturnoc acid (Ca salt)
14	Gelatin	Modified protein	Amino acids

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990 Page 68)

6.3.2 Identification of Gums:

Wet 0.25 to 0.50 gm of the material to be identified with 1 to 2 ml of 95% alcohol and add 50 ml distilled water. Suspend the solid material in the water by shaking or stirring and heat when the sample dissolves. Discontinue heating, otherwise hold at 85 to 95°C for 15 min

Group A: Treat 3 to 5 ml aliquot of the solution with 0.2 volumes of 0.25M calcium chloride. A gelatinous precipitation or gel indicates alginates or deesterified pectin. If no reaction is apparent with calcium chloride alone, add 1 volume of 3N ammonium hydroxide. Slow formation of a gel or gelatinous precipitate indicates pectin.

If the above test is positive, mix a fresh 3 to 5 ml aliquot of sample with 1 volume of 3 N sodium hydroxide. Observe the reaction and then heat the mixture in a boiling water bath for 10 min immediate formation, in the cold, of a gelatinous or flocculent precipitate indicates either pectin or deesterified pectin. No precipitate indicates alginates. All three mixtures become yellow on heating but the precipitates with pectic substances do not dissolve.

Group B: If the material is not an alginate or a pectic substance, mix 3 to 5 ml aliquot of the sample with one volume of saturated barium hydroxide. Observe in the cold and heat in boiling water bath for 10 min Formation in the cold of a non-setting, almost opaque gelatinous

precipitate indicates Irish moss. Carry out confirmatory test for Irish moss.

A small amount of flocculent precipitate or cloudiness in the cold and definite lemon yellow on heating indicates gum tragacanth. If the colour changes during heating to yellow, then to green and finally to gray, it indicates agar. Carry out confirmatory test for agar.

If the mixture is cloudy or forms a gel on heating but becomes clear on cooling, methyl cellulose is indicated. Carry out confirmatory test for methyl cellulose. An opaque flocculent precipitate which may tend to redisperse on heating and reprecipitate on cooling indicates starch. Carry out confirmatory test for starch. Precipitates which disappear when the barium hydroxide is thoroughly mixed with the sample may be disregarded at this point.

If the material has not been identified, mix a fresh 3 to 5 aliquot of sample with 1 volume of saturated barium hydroxide. Observe whether there is an immediate precipitation and examine again after letting it stand for 5 min at room temperature.

A voluminous opaque, stringy precipitate which tends to form a clot indicates locust bean gum. This precipitate may appear flocculent if the mixture is shaken vigorously. Carry out confirmatory tests for carboxy methyl cellulose.

An opaque flocculent precipitate which becomes sloppy and is not voluminous indicates gum karaya. Carry out confirmatory test for karaya.

Group C: If the sample has yet not been identified, it may be gum Arabic, gum ghatti or gelatin, Mix a fresh 3 to 5 ml aliquot of sample with 1 ml of basic lead acetate solution. Immediate formation of a voluminous opaque precipitate indicates gum Arabic.

If there is only a small amount of flocculent precipitate or no precipitate with the basic lead acetate, add 1 ml of 3N ammonium hydroxide to the lead containing sample. A voluminous opaque flocculent precipitate indicates gum ghatti. If there is no precipitate, the sample, probably, is gelatin. Carry out confirmatory test for gelatin.

Reactions useful for characterising the gums are summarized:-

The reactions with stock's acid, mercuric nitrate illustrate the effects of low pH on precipitation of heavy metal salts of the polysaccharide acids. An excess of the reagent makes the solutions strongly acidic and thus the weakly dissociated acid redisperse. Alginic acid and pectic acid are insoluble and thus are not dissolved by an excess of stock's reagent.

Gelatin gives pronounced precipitation reaction only with those gums having anionic components. The precipitates are found only if the pH of the mixture is below the isoelectric point of the protein and may be more correctly called co extractives. They are usually dispersed by a few drops of mineral acid or dilute ammonium hydroxide. Ammonium sulphate give pronounced precipitation tests with tragacanth, karaya, arabic or ghatti, each of which probably contains uronic acid components. The characteristic nature in which the gums are precipitated by alcohol may also be of use in their identification.

Confirmatory Tests:

Alginates and de-esterified pectin's:

Add 0.2 ml of 3N hydrochloric acid (or other mineral acid) to 3 to 4 ml of the sample. A gelatinous precipitate confirms alginates or de-esterified pectin.

Irish Moss: Add 2 to 3 drops of 0.5% methylene blue solution in water to 1 ml of the sample solution. Precipitation of purple stained fibres confirms Irish Moss.

Methyl cellulose: Mix 5 ml of sample with 25 ml of 95% alcohol and 2 to 3 drops saturated sodium chloride. No precipitate confirms methyl cellulose.

Agar: Precipitate gum from 5 ml of sample with alcohol and stain with tincture of iodine. A blue colour is formed. Starch is also stained blue by the reagents.

Starch: Add 1 to 2 drops of iodine solution to 1 ml of sample. A blue or purple colour confirms starch. Some samples of gum tragacanth may give a faint blue test.

Carboxy methyl cellulose:

- (a) Add 2 ml of 1M copper sulphate solution to 5 ml of the solution. An opaque, slightly bluish clotted precipitate confirms carboxy methyl cellulose.
- (b) To 5 ml of sample and uranyl zinc acetate, yellow precipitate forms in the presence of carboxy methyl cellulose.

Locust bean gum: Add 1 ml of 4% borax to 3 to 5 ml of gum solution, if the mixture gelatinizes locust bean gum is confirmed. Guar gum also forms a gel in similar conditions.

Karaya: Precipitate gum from 5 ml of solution with alcohol and stain with ruthenium red. If sample swells considerably and is stained pink, karaya is confirmed.

Gelatin: Add 2 to 3 drops of gum solution to 2 ml of saturated picric acid. A fine yellow precipitate confirms gelatin.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990 Page 70)

7.0 FLAVOURS AND FLAVOUR ENHANCERS

Flavours and flavour enhancers form a divergent group of organic compounds both natural and synthetic in nature. They are used in trace amounts to impart a characteristic flavour. Menthol, vanillin and monosodium glutamate are of interest as they are extensively used in various foods. Menthol is used mainly to flavour confectionery and panmasala. Vanillin is extensively used in ice creams and monosodium glutamate to enhance flavour of meat, soups etc., and Gas chromatography is extensively used in determination of various flavouring compounds.

7.1 Gas Chromatographic determination of menthol in mentholated sweets and Pan Masala

Principle:

Menthol along with other flavouring matter is steam distilled into chloroform. The dry chloroform extract is directly subjected to gas chromatography on 10% carbowax 20M using FID. The amount of menthol present in the sample is determined using peak height/area of sample and standard.

Glassware and Apparatus:

- a) Distillation and conical flask;
- b) separatory funnel;
- c) Gas chromatograph: CIC Model gas chromatograph fitted with FID or equivalent;
- d) Column: Stainless steel column (6'× 1/8" O.D) packed with 10% carbowax 20M chromosorb- W (80-100 mesh). OR a Capillary GC column DB-225 30 m × 0.53 mm id × 3.0 μ thickness may be used provided standardisation is done with nitrogen flow 10ml/min

Conditions:

Column temperature: 185°C, detector and injector temperature – 240°C; Nitrogen as carrier gas at 25 ml/min Under these conditions retention time for menthol is 3.0 min

Reagents:

- (i) **Standard solution:** Prepare (0.5 mg/ml) menthol solution by accurately weighing 25 mg of menthol, dissolve in chloroform and make upto 50 ml with chloroform.
- (ii) **Chloroform:** Chromatography grade.

Procedure:

To a known quantity of sample (10 gm) in a distillation flask add 20 ml distilled water

and connect it to a steam generator and condenser. Dip the other end of the condenser in a conical flask containing 15-20 ml of chloroform. Steam distills the contents of the flask slowly until about 50 ml distillate is collected into conical flask. Transfer the distillate to a 100 ml separatory funnel and separate the chloroform layer. Extract the aqueous phase with 2 × 10 ml portions of chloroform. Dry the combined chloroform layer over anhydrous sodium sulphate and make up to a known volume (50 ml). If the menthol concentration is less in the extract, concentrate it under nitrogen to 10 ml.

Inject 1 µl of standard menthol solution in chloroform into gas chromatograph and record the retention time and peak height/area. Inject 1 µl of sample solution in chloroform and record the peak height/area. Carry out the determination in duplicate. Determine the menthol content of sample from average peak height/area of standard and sample.

7.2 Detection of Vanillin, Ethyl Vanillin and Coumarin by Thin Layer Chromatography

Principle:

Vanillin, ethyl vanillin and coumarin in vanillin extracts are separated on TLC and detected by spraying hydrazine sulphate hydrochloric acid, potassium hydroxide alone, and potassium hydroxide followed by diazotized sulfanilic acid.

Apparatus:

- a) Pipette, conical flask;
- b) TLC equipment.

Reagents:

(i) Solvent system:

- a) Pet. ether: ethyl acetate (5: 2.5)
- b) Hexane: Ethyl acetate (5: 2)

(ii) Spray reagents:

- a) 1% hydrazine sulphate in 1N hydrochloric acid
- b) Concentrated methanolic solution of potassium hydroxide
- c) 1% diazotised sulfanilic acid

(iii) Silica Gel G

Procedure:

Spot the sample extract in ethyl acetate on TLC plate coated with silica gel. Saturate the TLC developing chamber with either of the developing solvent system, (refer table below) and develop the plate to about 12 cm height. Air dry the plate and spray with hydrazine sulphate solution. Develop another plate and spray with methanolic potassium hydroxide and view under day light and UV.

Spray the plate previously sprayed with methanolic potassium hydroxide again with diazotised sulfanilic acid and view under day light. Confirm the presence of the above compounds from the Rf of standards.

Sl. No		Rf value		Detection				POTASSIUM HYDROXIDE and there-after diazotised sulfanilic acid
		Solvent A	Solvent B	Hydrazine hydrochloric acid day light	Sulfate UV	Potassium Hydroxide day light	UV	
1	Vanillin	0.30	0.27	Yellow	Orange	--	--	Rose
2	Ethyl Vanillin	0.46	0.42	Yellow	Yellow	--	--	--
3	Coumarin	0.50	0.38	--	--	--	Green	Orange

4	Dihydrocou marin	0.60	0.50	--	--	--		Orange
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(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990
Page (73))

GC Method for vanillin:

The following GC capillary chromatographic conditions can be adopted provided standardization is done:

Use gas chromatograph with thermal conductivity or flame ionization detector. Temperatures: Program column from 70° to 250°C at 4°/min, injector 240°C, detector 240°C. Columns: 6 ft (1.8 m) x1/ 8 in. stainless steel or Cu, packed with 3% silicone oil (SE-30, SF-96, OV-1, or OV-101) on silanized 100–120 mesh Chromosorb W, acid-washed and dichlorodimethylsilane-treated, or Gas-Chrom Q; He carrier gas flow 30–35 mL/min Alternatively, use 1.8 m x 1 4 in. column with same packing material at flow rate of 60–80 mL/min

(Ref: AOAC official method 971.12 nonvanillin vanilla volatiles in vanilla extract direct gas chromatographic method first action 1971 final action 1972.)

7.3 Determination of Monosodium Glutamate in Food

Principle:

Glutamic acid is extracted from foods using water, separated from other amino acids by using an ion-exchange resin chromatography and titrated potentiometrically using 0.1N sodium hydroxide.

Apparatus:

(1) Volumetric flask; (2) beaker; (3) chromatographic column; 500 x 22 mm o.d. tube 30 ml bed volume with Dowex 50W \times 8 (H form) 100-200 mesh; (4) Potentiometer. (5) pH meter

Reagents:

- (i) Activated carbon: Darco-G 600 or equivalent
- (ii) 0.8N, 1N and 4N hydrochloric acid
- (iii) 50 % sodium hydroxide (iv) 0.1 N sodium hydroxide
- (iv) Formaldehyde

Preparation of Samples:

For products in dry form reduce about 40 gm to powder in mortar and weigh 10 gm sample into 250 ml beaker. For undiluted concentrated soups or canned green beans, homogenize entire undiluted content of can in blender and weigh 20 gm sample into 250 ml beaker. For consomme type (clear condensed) soup, weigh 20 gm into 250 ml beaker.

Dilute sample to about 70ml with water at room temperature and mix until all water soluble substances are in solution (15 min). Add 6 gm activated carbon and mix thoroughly (for products containing starch, also add 60 ml acetone to precipitate starch and to aid in making a solution of the sample).

Let it stand for 30 min Filter under vacuum through 60 ml coarse fritted glass funnel containing asbestos pad. Wash flask and reduce with six 25 ml portions acetone water (1+1).

Collect filtrate and washings in 400 ml beaker. Add 2 drops of hydrochloric acid (1+25) and evaporate on steam bath to about 40 ml (Hydrochloric acid prevents conversion of glutamic acid to pyrrolidone carboxylic acid). Quantitatively transfer to 50 ml volumetric flask, dilute to volume with water and mix.

Determination:

Transfer 25 ml aliquot to prepared column and adjust flow to about 0.5 ml/min After all the solution enters the resin, wash the column with 10 ml water. Let the washings pass into resin. Add 120 ml 0.8N hydrochloric acid and maintain flow rate. (0.8N hydrochloric acid will eluate any serine, threonine and aspartic acid) After, all the 0.8N hydrochloric acid passes into resin add 170 ml 1N hydrochloric acid and adjust flow rate to between 25/30 drops/min to elute glutamic acid. Collect the eluate in a 400 ml beaker. (any glycine present will eluate after 200 ml of 1 N hydrochloric acid) Nearly neutralize the eluate with 50% sodium hydroxide and adjust potentiometrically to pH 7 with 0.1N sodium hydroxide.

Neutralize 25 ml of 37% HCHO to pH 7 with 0.1N sodium hydroxide and add this to the beaker. Mix for 10 min on magnetic stirrer and titrate potentiometrically to pH 8.9 with 0.1N sodium hydroxide.

Determine blank by titrating to pH 8.9 a mixture of 25 ml neutralized HCHO and 170 ml 1N hydrochloric acid neutralised to pH 7.0%

$$\text{Glutamic acid} = [(S - B) \times N \times 0.147 \times 100] / W$$

Where,

S = ml sodium hydroxide used to titrate sample B = ml sodium hydroxide used to titrated blank.

N = Normality of sodium hydroxide and

W = gm sample

MSG = % glutamic acid \times 1.15

Note: Before using resin column once again, wash the column with 50 ml 4N hydrochloric acid followed by H₂O. Test the washings with silver nitrate solution for chlorides if any.

(Ref: - F.A.O Manual of Food Quality Control 14 /7, 1986 Page124 /Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990 Page 73)

HPLC Method: An alternative method**Method I.**

Apparatus:

High-performance liquid chromatography column r ODS-C18 bonded to silica, 12.5 cm ×4.6 mm. i.d. Fluorescence detector. Vials and heating module. Reacti-Vials (1 ml) and a Reacti-Therm heating module for derivatization.

Reagents (AR grade)

- a) Glacial acetic acid.
- b) Dansyl chloride solution
- c) Methanol HPLC grade.
- d) Use water which is distilled and filtered through a 0.45-µm millipore filter
- e) Buffer - A pH 10.5 solution of 4 g l⁻¹ sodium hydrogen carbonate.
- f) L-Glutamic acid - A 10 mg mL⁻¹, standard solution in water
- g) Eluting solvents - Pump A, 1% V/V glacial acetic acid in methanol; and pump B, 1% water.
- h) V/V glacial acetic acid in 45% methanol + 55% water.

Dansylation Procedure:

Transfer Aliquots of 10 µl of the standard, sample or blank (water) into a Pierce 1-ml Reacti-Vial and 50µl of the buffer solution and add 100µL of the dansyl chloride solution and mix the solution vigorously using a rotomixer. Place the vials in the heating module at 100°C for 10 min in the dark. The contents in the vial will change from pale yellow before reaction to colourless after reaction. Cool the vials with water and add 300µL of methanol to each to minimise any errors that might occur owing to loss of the reaction mixture through evaporation.

Separation and Detection Procedures:

Perform the elution with a flow-rate of 3 ml min⁻¹. Held the solvent at 45% V/V methanol for 2.5min after injection and then run up to 99.9% V/V methanol over 1 min and hold for 2.5 min Reverse the gradient reversed and allow the column to equilibrate at 45% V/V methanol over 3 min before the next injection. Detect the dansylated glutamic acid with an excitation wavelength of 328 nm and an emission wavelength of 530 nm. Obtain the corrected excitation spectrum and uncorrected emission spectrum of the derivative by collecting the fraction containing the compound from the chromatograph and scanning the spectrum, luminescence spectrometer. Although the peak excitation is at 245 nm, advised to use an excitation wavelength of 328 nm to minimise any interference from compounds that might absorb radiation in the ultraviolet region.

(Ref. A. T. Rhys Williams* and S. A. Winfield Analyst, Vol. 107 1982, 1092-1094)

Method II.

Apparatus:

Laboratory mixer (diameter 3.18 mm). Chromatograph high performance liquid (HPLC): Analytical Column: reversed phase ODS-Hypersil (5 µm), 200 µm × 4.6 mm. visible detector:

Reagents: Use all chemicals analytical reagent grade.

- a) Trichloroethylene. Sodium
- b) bicarbonate (5% w/v). 2,4 dinitrofluorobenzene (DNFB).
- c) Diethylether.
- d) Hydrochloric acid (6M).
- e) Methilic alcoholic.
- f) L-glutamic aminoacide
- g) L- glutamic acid
- h) Standard: 500 mg/ 100 mL.

Sample Preparation:

Process 250 g of food/meat with a laboratory mixer to obtain a homogenous sample. Assay procedure: The proposed method has two steps:

a) MSG isolation and chromatographic analysis.

Isolation of monosodium glutamate from food:

Extract Aliquots of sample (20.0 g) were by homogenizing with 20.0-30.0 mL of distilled water. Filter the resulting slurry filtered through a Buchner filter and a metallic vacuum trap. Repeat this procedure several times until 100 mL was collected. Add A 10 mL aliquot of this extract and 2.0 mL of trichloroethylene to a centrifuge tube, stirred and centrifuge at 3000 rpm for 15 min Discard the organic phase and use the aqueous phase to chromatography analysis.

b) High Performance liquid chromatography analysis (HPLC).

c) Pre-column derivatization:

To detect the analite at 254 nm it was necessary to derivatize the glutamic acid. There are several derivatizing reagents as dinitrophenyl (DNP), phenylthiohydantion (PTH), ortophtaldehyde (OPA), and dinitroflorobenzene (DNFB) and dansyl chloride (DNS).

Adjust the pH of the supernatant to 7.50 -8.00 by adding appropriate amount of sodium bicarbonate 5%. Transfer A small aliquot sample (0.50 mL) to a test tube and 10 μ L of 2,4-DNFB, then shake the mixture in the dark at 40°C for 3 hrs. Remove the excess of DNFB by extracting it with diethyl ether. Acidify the remaining aqueous fraction 50 μ L of 6 M hydrochloric acid and extract the DNP-amino acid with diethyl ether until the ether no longer becomes colored. Evaporate the ether and take up the residue in 0.50 mL of methanol and inject into the HPLC apparatus.

d) HPLC Conditions:

All chromatographic runs should be made at ambient temperature (25°C) using a solvent flow rate of 1.2 mL/min Set the ultraviolet detector at 254 nm. Prepare the mobile phase with 25% acetonitrile and 75% glacial acetic acid (1% w/v).

(Ref: - Rodriguez et al. The Journal of the Argentine Chemical Society - Vol. 91 -NO 4/6, 41-45 (2003)

8.0 QUININE

Quinine is an important alkaloid of *Cinchona officinalis* L. and is permitted in soft drinks and tonic waters. Quinine is determined usually by fluorimetry but can also be determined spectrophotometrically.

8.1 Fluorimetric determination of Quinine in soft drinks and tonic waters.

Principle:

Quinine is determined by direct fluorescence spectrophotometry after dilution of the sample with sulphuric acid.

Apparatus:

1. Volumetric flasks;
2. Beakers;
3. Fluorimeter with 365 nm excitation filter and 415 nm emission filter or Spectrofluorimeter.

Reagents:

- (i) Sulphuric acid (0.05M): Dilute 2.78 ml to 1000 ml with water.
- (ii) Sulphuric acid (1M): Dilute 55.6 ml to 1000 ml with water.
- (iii) Standard solution of quinine sulphate.

Stock solution:

Weigh accurately 50 mg of quinine or 60.4 mg of quinine sulphate dihydrate. Transfer into a 500 ml volumetric flask, add 25 ml of 1M sulphuric acid and dilute to volume with water. Prepare working standard solution by dilution with 0.05M sulphuric acid.

Preparation of Sample:

Remove carbonation by stirring and weigh accurately 5 gm of sample and dilute to 250 ml in a volumetric flask with 0.05M sulphuric acid. Pipette 5 ml of this solution and dilute to 25 ml using 0.05M sulphuric acid.

Procedure:

Using 365 nm excitation filter and 415 nm emission filter, adjust the fluorimeter to zero with 0.05 sulphuric acid. Measure the emission intensity of concentration (μg) VS emission intensity. Read the fluorescence of the sample solution and calculate the amount of quinine present in the sample from the calibration curve.

8.2 Spectrophotometric determination of quinine in soft drinks**Principle:**

Quinine is extracted from soft drinks using chloroform after making it alkaline with sodium hydroxide. It is re - extracted from organic layer with 1N sulphuric acid. An aliquot of acid extract is adjusted to pH 1.0, alizarin brilliant violet R solution is added and the complex is extracted into chloroform. The absorbance of chloroform layer is measured at 578 nm. Amount of quinine present in the sample is computed from the calibration graph.

Reagents:

- (i) **Buffer (pH 1):** Add 97.0 ml of 1.0N hydrochloric acid to 50 ml of 1.0M potassium chloride and make upto 1000 ml.
- (ii) Alizarin brilliant violet R (ABVR): 1×10^{-3} M: Prepare in distilled water.
- (iii) Quinine Standard solution: Prepare 1 mg/ml by dissolving quinine sulphate in 0.1N sulphuric acid. Dilute to a working standard containing 40 $\mu\text{g}/\text{ml}$.

Procedure:**(a) Preparation of Standard Curve:**

To a 30 ml buffer solution in a 100 ml separatory funnel, add aliquot of quinine working

standard solution, ranging from 10-200 µg and 5 ml of dye solution. Adjust the volume to 50 ml with distilled water. Extract with 10 ml of chloroform. Read the absorbance of chloroform layer at 578 nm and plot the calibration graph.

(b) Determination in soft drinks:

Accurately weigh 50 gm of soft drink and transfer to 250 ml separatory funnel. Add 10 ml of 10% sodium hydroxide and extract with 3 × 20 ml portions of chloroform. Shake the combined extract with 3 × 30 ml of 1N sulphuric acid in another separator and make up the acid solution to 100 ml in a volumetric flask. Taking an aliquot of this solution proceed as given under preparation of standard curve.

Directly take 1 to 5 ml of soft drink and proceed as given under preparation of standard curve. Calculate the amount of quinine present in the sample from the absorbance of sample using standard curve.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods, I.C.M.R 1990 Page 75)

OR

A HPLC-UV method may also be used instead of U.V. spectrophotometric for determination of Quinine.

9.0 4 -METHYL IMIDAZOLE IN CARMEL

Caramel samples produced by ammonia and ammonium sulphite processes are found to contain a process contaminant namely 4-methyl imidazole. The compound has been subjected to toxicological evaluation by Bureau of Indian Standards.

Determination of 4-methyl imidazole in caramel samples:

Principle:

Caramel colour is added to a basic celite column and eluted with a mixture of chloroform

and alcohol. The eluant is extracted with dilute sulphuric acid and the aqueous extract is made to a known volume. Taking aliquot of the extract, the colour is developed with diazotised sulphphanilic acid in alkaline medium. The amount of 4-methyl imidazole present is calculated from the standard curve.

Apparatus:

Volumetric flasks, 100 ml capacity and 25 ml capacity; separatory funnel, 250 ml capacity; beaker, 250, 100 ml capacity; conical flask, 250 ml capacity; pipettes, 2 ml and 5 ml capacity; spectrophotometer chromatographic column with stop cock, 25.0×2.5 cm.

Reagents:

- (i) Sodium hydroxide , 2N
- (ii) Sodium carbonate, 20% m/v and 5% m/v
- (iii) Elution solvent: chloroform: Alcohol (80: 20)
- (iv) Sulphuric Acid : 0.05/0.1N

(v) Chromogenic reagent:

- (a) Dissolve 1 gm of sulphhanilic acid in 9 ml of conc. hydrochloric acid and make upto a known volume and cool to 4 °C.
- (b) Prepare by dissolving 1 mg of sodium nitrite in 100 ml water and cool to 4°C. Prepare freshly by mixing 25 ml of (a) and 25 ml of (b).
- (vi) **(a) Stock solution of 4-methyl imidazole:** Dissolve 100 mg of 4- methyl imidazole in 100 ml of 0.1N sulphuric acid and store in a refrigerator.
- (b) Standard solution:** Pipette out 5 ml stock solution into a 100 ml volumetric flask and make upto volume (50 µg/ml).
- (c) Working standard solution:** Pipette out 10 ml of standard solution (50 µg/ml)

into a 100 ml volumetric flask and make upto volume (5 µg/ml)

(vii) Celite 547.

Procedure:

Prepare a basic column packing material by mixing well, celite 545 + 2N sodium hydroxide in the proportion of 2 ml of 2N sodium hydroxide to 3 gm of celite. Place fine glass wool plug at the base of the chromatographic column followed by 5 gm of basic column packing. Tag the packing firmly to a uniform mass.

Take 10 gm of the caramel sample in a 100 ml beaker and add 6 gm of 20% Sodium carbonate solution and pack it on the prepared column. Place a plug of glass wool at the top of the column. Elute with elution mix, until 150 ml of eluate is collected into a 250 ml beaker. Transfer the eluate to a 250 ml separatory funnel and extract with three 20 ml portions of 0.05N sulphuric acid {this extract should be strongly acidic (pH 3), test this by test paper}. Concentrate the combined aqueous extract using flash evaporator at a temperature below 50°C. Make up the residue to known volume.

Preparation of standard curve:

Into a series of 25 ml volumetric flasks containing 0.0, 1.0, 2.0, 3.0 and 5.0 of working standard solution, add 1.0 ml each of diazotised sulphanilic acid (Chromogenic reagent) and 2.0 ml of sodium carbonate (5%) solution. Make up to volume and read the absorbance at 505 nm and plot the standard graph.

Take 2.0 to 5.0 ml solution of the aqueous extract and develop the colour. Read the absorbance of the sample solution and calculate the quantity of 4- methyl imidazole present in the sample from the standard curve.

10.0 Detection of brominated vegetable oils in soft drinks:

Principle:

Brominated vegetable oil (BVO) in the soft drinks is extracted using diethyl ether. The concentrated ethereal solution is treated with a small quantity of zinc dust to convert the organic bromide to inorganic form and subsequently treated with lead dioxide to liberate bromine. The bromine evolved is detected by means of fluorescein treated filter paper strip which turns pink due to formation of eosin. The test can detect as low as 1 ppm BVO under the experimental conditions described.

Apparatus and Reagents:

- (i) Separatory funnels: 500 ml capacity and glass stoppered conical flask – 250 ml/100 ml capacity.
- (ii) Diethyl ether: Analytical reagent grade, distill before use.
- (iii) Dilute acetic acid: Dilute the glacial acetic acid (Analytical reagent grade) 1: 10 with distilled water.
- (iv) Zinc dust: Analytical reagent grade
- (v) Lead dioxide (PbO₂): Analytical reagent grade
- (vi) Fluorescein treated filter paper strips: Prepare 0.1 per cent solution of fluorescein in 1: 1 ethyl alcohol (freshly distilled) and water. Make the solution alkaline by adding 1 ml 0.1N sodium hydroxide solution per 100 ml. Filter the solution. Soak Whatman No. 1 filter paper strips (2 × 10 cm) for 10 min. Air dry the strips before use. If required for routine use, store the dried strips in air tight brown bottle. Discard pale or discoloured strips.
- (vii) Brominated vegetable oils: Brominated sesame oil and cottonseed oil (refined)

prepared in the laboratory. Standard solution of BVO 1 mg= 1 ml of ether.

Procedure:

Extraction of BVO from Soft Drinks:

Transfer 200 ml or a suitable aliquot of the soft drink (containing about 2 mg BVO) into a separatory funnel. Extract with two 75 ml portions of diethyl ether. Combine extracts in a separatory funnel, wash with 25 ml water and dry it by filtering through anhydrous sodium sulphate.

Detection of BVO:

Concentrate ether extract to approximate 5 ml in a conical flask, add 25-30 mg Zinc dust (use a small spatula) and 5 ml dilute acetic acid. Heat for 5 min over a steam bath. Cool and filter the contents of the flask using Whatman No. 1 filter paper into another glass stoppered conical flask (100 ml capacity). Wash the filter paper with 2-3 ml dilute acetic acid rinsings of the original flask. Add to this flask about 25-30 mg Lead dioxide and immediately hang a fluorosein treated filter paper strip inside the flask along with the stopper, Taking care that the strip does not touch the liquid in the flask. Heat the contents of the flask over a steam bath. Observe the colour change in the strip. The yellow strip turns to pink within 2-3 min if BVO is present in the sample.

(Ref: - Method developed by CFTRI Mysore)

An alternative GC method is given below:

Gas chromatograph.—

Varian Model 1740-10, or equivalent, with flame ionization detector, strip chart recorder fitted with disk integrator, 0.9 m (3 ft) 1/8 in. od stainless steel column packed with 3% JXR or SE-30 (Supelco, Inc.) on 80-90 mesh Anakrom ABS.

Operating conditions: temperatures—injector 260°C, detector 270°C, column programmed from 150°C to 270°C at 10°C/min; He carrier gas 40 mL/min

(Ref: AOAC Official Method 973.27. Brominated Vegetable Oils in Non-alcoholic Beverages Gas Chromatographic Method First Action 1973, Final Action 1974.)

11.0 Detection and Estimation of Magnesium Carbonate in Pan Masala and Gutka

11.1 Determination of Magnesium

Reagents

1. 0.1 M EDTA - Dissolve 37.23 gm EDTA (dihydrate) in distilled water and make up to 1 L
2. Eriochrom Black T – Dissolve 0.2 gm of the dye in 15 ml of triethanolamine and 5 ml absolute ethanol
3. Buffer solutions - pH 10 – add 142 ml concentrated ammonia solution (sp gr 0.88) to 17.5 gm of Ammonium chloride and dilute to 250 ml with water.
4. Standard Magnesium ion solution (0.1M) – Dissolve 0.61 gm of pure Magnesium turnings in hydrochloric acid and nearly neutralise with sodium hydroxide (M) and dilute to 250 ml with distilled water.

Procedure

Weigh accurately about 5 gm of pan masala / gutka in a platinum basin, carbonize on a burner or heater and transfer to a muffle furnace at 550°C for 2 hrs for complete ashing. Add 25 ml of dilute hydrochloric acid to the ash, boil for 5 min, cool and filter. Wash the residue with distilled water and add the washings to the filtrate. Add 2 gm of ammonium chloride to the filtrate and dissolve by swirling. Make the solution alkaline with ammonium hydroxide to allow formation of a precipitate. Filter and collect filtrate. Wash ppt with water and add washings to filtrate.

The filtrate is treated with ammonium carbonate solution for precipitation of group (iv) metals and kept on a water bath for 15 min, cooled and filtered. The ppt is washed with water and washings added to the filtrate. The filtrate is made up to 100 ml with water in a volumetric flask 25 ml of filtrate is taken in a beaker, 75 ml water and 2 ml buffer solution is added followed by 2-3 drops of Eriochrom Black –T indicator. Titrate with 0.1 M EDTA solution

until colour changes from red to blue. Carry out titration slowly under slightly warm condition (40° C).

Calculation

1 ml (0.1M) EDTA = 2.432 mg of Mg

Test for presence of Carbonate

Add hydrochloric acid (1:1) to a small portion of the sample in a closed test tube fitted with a glass tube. Put the other end of the glass tube into clear lime water.

Warm the test tube. The presence of carbonate is detected by presence of effervescence or change in the colour of lime water.

(Ref: - Method developed by Central food Laboratory, Kolkata)

12.0 Determination of Aluminium content in Lake colour

Determine Aluminium content by Atomic Absorption Spectrophotometer. For details refer to the Laboratory manual on metals.

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*The methods mentioned in the manual needs to be verified/ validated before they are put in use by the laboratory.



*Food Safety and Standards Authority of India
(Ministry of Health and Family Welfare)
FDA Bhawan, Kotla Road,
New Delhi-110002
www.fssai.gov.in*