MANUAL OF METHODS

OF

ANALYSIS OF FOODS

OILS AND FATS



FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA
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MANUAL FOR ANALYSIS OF OILS AND FATS

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Note: The test methods given in the manuals are validated/ standardized test methods. However, it would be the responsibility of the respective testing laboratory to confirm that the above methods are validated in its laboratory and gives proper result in their laboratory.

MANUAL FOR ANALYSIS OF OILS AND FATS

Oils and fats are important parts of human diet and more than 90 percent of the world production from vegetable, animal and marine sources is used as food or as an ingredient in food products. Oils and fats are a rich source of dietary energy and contain more than twice the caloric value of equivalent amount of sugar. Their functional and textural characteristics contribute to the flavour and palatability of natural and prepared foods. They contain certain fatty acids which play an important role in nutrition and are also carriers of fat soluble vitamins.

1.0 TYPES OF OILS AND FATS

Standards for 24 vegetable oils are prescribed in Section 2.2 of Food Safety and Standards (Food Product Standards and Food Additives) Regulations, 2011. Standards have also been laid down for Cocoa butter, Refined Sal seed fat, Mango Kernel fat, Phulwara fat, Interesterified fat, Vanaspati, Table Margarine and Bakery / Industrial Margarine, Ghee, butter, butter oil. Animal fats include Mutton /Goat fat and Lard

2.0 PREPARATION OF TEST SAMPLE

2.1 Liquid Oils:-

Use clear sediment free liquid directly after inverting container several times. If liquid sample contains sediment release all sediment from walls of container and distribute uniformly throughout the oil for determination of moisture. For determinations in which results might be affected by possible presence of water (e. g iodine value) dry sample by adding anhydrous Sodium Sulphate in the proportion of 1 - 2 gm per 10 gm sample and hold it in oven at 50°C. Stir vigorously and filter to obtain clear filtrate.

2.2 Solid and semisolid Samples:-

Soften sample if necessary by gently heating taking care not to melt it. When soft enough mix thoroughly for determination of moisture and volatile matter. For other determinations, melt in drying oven at a temperature at least 10°C above the melting point. If clear, proceed directly. If turbid or contains sediment filter test sample inside oven. For determinations in which results might be affected by possible presence of water (e.g. iodine value) dry sample by adding anhydrous Sodium Sulphate in the proportion of 1-2 gm per 10 gm sample and hold (keep) it in oven at 50°C. Stir vigorously and filter to obtain clear filtrate. To retard rancidity keep oils and fats in cool place and protect from light and air.

(Ref: - AOAC 17th edn, 2000. Official method 981.11 Oils and Fats – Preparation of test sample)

3.0 DETERMINATION OF MOISTURE CONTENT

3.1 Air-Oven Method

3.1.1 Definition:

Moisture content of oils and fats is the loss in mass of the sample on heating at 105 ± 1°C under operating conditions specified.

3.1.2 Apparatus:

Metal dishes 7 – 8 cm diameter and 2 - 3 cm deep provided with tight fitting slip on covers.

3.1.3 Procedure:

Weigh in a previously dried and tared dish about 5 – 10 gm of oil or fat, which has been thoroughly mixed by stirring. Loosen the lid of the dish and heat, in an oven at 105±1°C for 1 hour. Remove the dish from the oven and close the lid.

Cool in a desiccator containing phosphorus pentoxide or equivalent desiccant and weigh. Heat in the oven for a further period of 1 hour, cool and weigh. Repeat

this process until change in weight between two successive observations does not exceed 1 mg.

Carry out the determination in duplicate

$$\text{Moisture and volatile matter} = W1 \times \frac{100}{W}$$

Where,

W1 = Loss in weight (gm) of the material on drying

W = Weight in gm of the material taken for test

(Ref: - ISI Hand book of Food Analysis (Part XIII) – 1984, page 62)

4.0 DETERMINATION OF SPECIFIC GRAVITY

4.1 Preparation of Sample:

Melt sample if necessary. Filter through a filter paper to remove any impurities and the last traces of moisture. Make sure that the sample is completely dry. Cool the sample to 30°C or ambient temperature desired for determination.

4.2 Apparatus:

a) Pycnometer fitted with a thermometer of suitable range, with 0.1 or 0.2°C subdivision or a density bottle

b) Balance

c) Water bath maintained at 30 ± 2.0°C.

The thermometer should be checked against a standard thermometer calibrated and certified by National Physical Laboratory, New Delhi or any other NABL approved institution.

4.3 Standardisation of Pycnometer:

Carefully clean the pycnometer by filling with Chromic acid cleaning solution and letting it stand for several hours. Empty pycnometer and rinse thoroughly with water, fill with recently boiled water, previously cooled to about 20°C and place in constant temperature water bath held at 30°C. After 30 minutes adjust water level to proper point on pycnometer and stopper, remove from bath, wipe dry with chemwipes/clean cloth or towel and weigh.

4.4 Procedure:

Fill the dry pycnometer with the prepared sample in such a manner to prevent entrapment of air bubbles after removing the cap of the side arm. Insert the stopper, immerse in water bath at 30 ± 2.0°C and hold for 30 minutes.

Carefully wipe off any oil that has come out of the capillary opening. Remove the bottle from the bath, clean and dry it thoroughly. Remove the cap of the side arm and quickly weigh ensuring that the temperature does not fall below 30°C.

$$\text{Specific Gravity at 30°C} = \frac{A-B}{C-B}$$

Where,

A = weight in gm of specific gravity bottle with oil at 30°C

B = weight in gm of specific gravity bottle at 30°C

C = weight in gm of specific gravity bottle with water at 30°C

(Ref:- AOAC 17th edn., 2000, Official method 920.212 Specific gravity (Apparent) of Oils, Pycnometer method / ISI Hand book of Food Analysis (Part XIII) 1984, page 72)

5.0 DETERMINATION OF THE REFRACTIVE INDEX

5.1 Definition:

The ratio of velocity of light in vacuum to the velocity of light in the oil or fat; more generally, expresses the ratio between the sine of angle of incidence to the sine of angle of refraction when a ray of light of known wave length (usually 589.3 nm, the mean of D lines of Sodium) passes from air into the oil or fat.

Refractive index varies with temperature and wavelength.

5.2 Principle:

Measurement of the refractive index of the sample is done by means of a suitable refractometer.

5.3 Apparatus: Refractometer - Abbe or Butyro Refractometer

By Abbes Refractometer: - Open double prism with the help of the screw head and place a drop of oil on the prism. Close prisms firmly by tightening screw heads.

As refractive index is greatly affected by temperature, the temperature of the refractometer should be controlled to within ± 0.1°C and for this purpose it should be provided with a thermostatically controlled water bath and a motor driven pump to circulate water through the instrument.

When a Butyro refractometer is used its reading can be converted to refractive index with the help of the table.

5.4 Calibration of the Instrument:

The instrument is calibrated with a glass prism of known refractive index (an optical contact with the prism being made by a drop of a bromonaphthalene) or by

using distilled water which has refractive index of 1.3330 at 20.0°C and 1.3306 at 40.0°C, the usual temperature of taking readings.

5.5 Light Source:

If the refractometer is equipped with a compensator, a tungsten lamp or day light may be used. Otherwise a monochoursomatic light such as sodium vapour lamp (589.3 nm) may be used.

5.6 Procedure:

Melt the sample if it is not already liquid and filter through a filter paper to remove impurities and traces of moisture. Make sure sample is completely dry.

Circulate stream of water through the instrument. Adjust the temperature of the refractometer to the desired temperature. Ensure that the prisms are clean and dry.

Place a few drops of the sample on the prism. Close the prisms and allow standing for 1-2 minutes Adjust the instrument and lighting to obtain the most distinct reading possible and determining the refractive index or butyro-refractometer number as the case may be.

5.7 Temperature correction:

Determine refractive index at the specified temperature. If temperature correction is necessary use following formula:

$$R = R^1 + K (T^1 - T)$$

Where,

R = Reading of the refractomter reduced to the specified temperature T°C

R^1 = Reading at T^1C

K = constant 0.000365 for fats and 0.000385 for oils (If Abbe Refractometer is used)

or

= 0.55 for fats and 0.58 for oils (if Butyro-refractometer is used)

T^1 = temperature at which the reading R^1 is taken and

T = specified temperature (generally 40°C.)

5.8 Significance

Refractive index of oils increases with the increase in unsaturation and also chain length of fatty acids.

Table 1: Butyro refractometer reading and indices of refraction (n_D)[a]

Fourth Decimal of n_D										
nD	0	1	2	3	4	5	6	7	8	9
Butyro Scale Readings										
1.422	0.0	0.1	0.2	0.4	0.5	0.6	0.7	0.9	1.0	1.1
1.423	1.2	1.4	1.5	1.6	1.7	1.9	2.0	2.1	2.2	2.4
1.424	2.5	2.6	2.7	2.8	3.0	3.1	3.2	3.3	3.5	3.6
1.425	3.7	3.8	4.0	4.1	4.2	4.3	4.5	4.6	4.7	4.8
1.426	5.0	5.1	5.2	5.4	5.5	5.6	5.7	5.9	6.0	6.1
1.427	6.2	6.4	6.5	6.6	6.8	6.9	7.0	7.1	7.2	7.4
1.428	7.5	7.6	7.7	7.9	8.0	8.1	8.2	8.4	8.5	8.6
1.429	8.7	8.9	9.0	9.1	9.2	9.4	9.5	9.6	9.8	9.9
1.430	10.0	10.1	10.3	10.4	10.5	10.6	10.7	10.9	11.0	11.1
1.431	11.3	11.4	11.5	11.6	11.8	11.9	12.0	12.2	12.3	12.4
1.432	12.5	12.7	12.8	12.9	13.0	13.2	13.3	13.5	13.6	13.7
1.433	13.8	14.0	14.1	14.2	14.4	14.5	14.6	14.7	14.9	15.0
1.434	15.1	15.3	15.4	15.5	15.6	15.8	15.9	16.0	16.2	16.3
1.435	16.4	16.6	16.7	16.8	17.0	17.1	17.2	17.4	17.5	17.6
1.436	17.8	17.9	18.0	18.2	18.3	18.4	18.5	18.7	18.8	18.9

Table 1:	**Butyro refractometer reading and indices of refraction (n_D)[a]**									
	Fourth Decimal of n_D									
nD	0	1	2	3	4	5	6	7	8	9
	Butyro Scale Readings									
1.437	19.1	19.2	19.3	19.5	19.6	19.7	19.8	20.0	20.1	20.2
1.438	20.4	20.5	20.6	20.8	20.9	21.1	21.2	21.3	21.4	21.6
1.439	21.7	21.8	22.0	22.1	22.2	22.4	22.5	22.6	22.7	22.9
1.440	23.0	23.2	23.3	23.4	23.5	23.7	23.8	23.9	24.1	24.2
1.441	24.3	24.5	24.6	24.7	24.8	25.0	25.1	25.2	25.4	25.5
1.442	25.6	25.8	25.9	26.1	26.2	26.3	26.5	26.6	26.7	26.9
1.443	27.0	27.1	27.3	27.4	27.5	27.7	27.8	27.9	28.1	28.2
1.444	28.3	28.5	28.6	28.7	28.9	29.0	29.2	29.3	29.4	29.6
1.445	29.7	29.9	30.0	30.1	30.3	30.4	30.6	30.7	30.8	30.9
1.446	31.1	31.2	31.4	31.5	31.6	31.8	31.9	32.1	32.2	32.3
1.447	32.5	32.6	32.8	32.9	33.0	33.2	33.3	33.5	33.6	33.7
1.448	33.9	34.0	34.2	34.3	34.4	34.6	34.7	34.9	35.0	35.1
1.449	35.3	35.4	35.6	35.7	35.8	36.0	36.1	36.3	36.4	36.5
1.450	36.7	36.8	37.0	37.1	37.2	37.4	37.5	37.7	37.8	37.9
1.451	38.1	38.2	38.3	38.5	38.6	38.7	38.9	39.0	39.2	39.3
1.452	39.5	39.6	39.7	39.9	40.0	40.1	40.3	40.4	40.6	40.7
1.453	40.9	41.0	41.1	41.3	41.4	41.5	41.7	41.8	42.0	42.1
1.454	42.3	42.4	42.5	42.7	42.8	43.0	43.1	43.3	43.4	43.6
1.455	43.7	43.9	44.0	44.2	44.3	44.4	44.6	44.7	44.9	45.0
1.456	45.2	45.3	45.5	45.6	45.7	45.9	46.0	46.2	46.3	46.4
1.457	46.6	46.7	46.9	47.0	47.2	47.3	47.5	47.6	47.7	47.9
1.458	48.0	48.2	48.3	48.5	48.6	48.8	48.9	49.1	49.2	49.4
1.459	49.5	49.7	49.8	50.0	50.1	50.2	50.4	50.5	50.7	50.8
1.460	51.0	51.1	51.3	51.4	51.6	51.7	51.9	52.0	52.2	52.3
1.461	52.5	52.7	52.8	53.0	53.1	53.3	53.4	53.6	53.7	53.9
1.462	54.0	54.2	54.3	54.5	54.6	54.8	55.0	55.1	55.3	55.4
1.463	55.6	55.7	55.9	56.0	56.2	56.3	56.5	56.6	56.8	56.9
1.464	57.1	57.3	57.4	57.6	57.7	57.9	58.0	58.2	58.3	58.5
1.465	58.6	58.8	58.9	59.1	59.2	59.4	59.5	59.7	59.8	60.0

Table 1:	**Butyro refractometer reading and indices of refraction (n_D)[a]**									
Fourth Decimal of n_D										
nD	0	1	2	3	4	5	6	7	8	9
Butyro Scale Readings										
1.466	60.2	60.3	60.5	60.6	60.8	60.9	61.1	61.2	61.4	61.5
1.467	61.7	61.8	62.0	62.2	62.3	62.5	62.6	62.8	62.9	63.1
1.468	63.2	63.4	63.5	63.7	63.8	64.0	64.2	64.3	64.5	64.7
1.469	64.8	65.0	65.1	65.3	65.4	65.6	65.7	65.9	66.1	66.2
1.470	66.4	66.5	66.7	66.8	67.0	67.2	67.3	67.5	67.7	67.8
1.471	68.0	68.1	68.3	68.4	68.6	68.7	68.9	69.1	69.2	69.4
1.472	69.5	69.7	69.9	70.0	70.2	70.3	70.5	70.7	70.8	71.0
1.473	71.1	71.3	71.4	71.6	71.8	71.9	72.1	72.2	72.4	72.5
1.474	72.7	72.9	73.0	73.2	73.3	73.5	73.7	73.8	74.0	74.1
1.475	74.3	74.5	74.6	74.8	75.0	75.1	75.3	75.5	75.6	75.8
1.476	76.0	76.1	76.3	76.5	76.7	76.8	77.0	77.2	77.3	77.5
1.477	77.7	77.9	78.1	78.2	78.4	78.6	78.7	78.9	79.1	79.2
1.478	79.4	79.6	79.8	80.0	80.1	80.3	80.5	80.6	80.8	81.0
1.479	81.2	81.3	81.5	81.7	81.9	82.0	82.2	82.4	82.5	82.7
1.480	82.9	83.1	83.2	83.4	83.6	83.8	83.9	84.1	84.3	84.5
1.481	84.6	84.8	85.0	85.2	85.3	85.5	85.7	85.9	86.0	86.2
1.482	86.4	86.6	86.7	86.9	87.1	87.3	87.5	87.6	87.7	88.0
1.483	88.2	88.3	88.5	88.7	88.9	89.1	89.2	89.4	89.6	89.8
1.484	90.0	90.2	90.3	90.5	90.7	90.9	91.2	91.2	91.4	91.6
1.485	91.8	92.0	92.1	92.3	92.5	92.7	92.9	93.0	93.2	93.4
1.486	93.6	93.8	94.0	94.1	94.3	94.5	94.7	94.8	95.0	95.2
1.487	95.4	95.6	95.8	96.0	96.1	96.3	96.5	96.7	96.9	97.0
1.488	97.2	97.4	97.6	97.8	98.0	98.1	98.3	98.5	98.7	98.9
1.489	99.1	99.2	99.4	99.6	99.8	100.0	…	….	…	….

(Ref: - AOAC 17th edn, 2000, Official method 921.08 – Index of refraction of oils and fats / ISI Handbook of Food analysis (Part XIII) – 1984, page 70) Table for conversion of B.R. readings to Refractive Index

6.0 DETERMINATION OF FLASH POINT: PENSKY MARTEN (CLOSED CUP) METHOD

6.1 Principle:

The method determines the temperature at which the sample will flash when a test flame is applied under the conditions specified for the test.

6. 2 Outline of method:

The sample is heated in a test cup at a slow and constant rate with continual stirring. A small test flame is directed into the cup at regular intervals with simultaneous interruption of stirring. The flash point is taken as the lowest temperature at which the application of the test flame causes the vapour above the sample to ignite momentarily.

6.3 Apparatus:

Pensky-Martens closed cup apparatus with thermometer.

6.4 Preparation of sample:

Samples containing dissolved or free water may be dehydrated with Calcium chloride or by filtering through a suitable filter paper or a loose plug of dry absorbent cotton. Warming the sample is permitted but it shall not be heated for prolonged periods or above the temperature of 16°C below its expected flash point.

6.5 Procedure:

Thoroughly clean and dry all parts of the cup and its accessories before starting the test, being sure to remove any solvent which had been used to clean the apparatus.

Support the tester on a level steady table.

Fill the cup with the oil to be tested up to the level indicated by the filling mark.

Place the lid on the cup and properly engage the heating devices. Insert the thermometer, light the test flame and adjust it to 4.0 mm in diameter. Heat the sample so that the temperature increase is about 5 to 6°C per minutes During the heating, turn the stirring device from one to two revolutions per second. Apply the test flame when the temperature of the sample is a whole number not higher than 17°C below the flash point. At every 5°C rise in temperature, discontinue stirring and apply the test flame by opening the device which controls the shutter and lowers the test flame into the shutter opening. Lower the test flame in for 0.5 second and quickly return to the raised position. Do not stir the sample while applying the test flame. As soon as the test flame has been returned to the raised position, resume stirring. The flash point is the temperature indicated by the thermometer at the time of the flame application that causes a distinct flash in the interior of the cup.

(Ref: - IS 1448 – 1970 Methods of test for petroleum and its products (P: 21) Flash Point (Closed) by Pensky Martin apparatus)

7.0 DETERMINATION OF COLOUR

7.1Principle:

The method determines the colour of oils by comparison with Lovibond glasses of known colour characteristics. The colour is expressed as the sum total of

the yellow and red slides used to match the colour of the oil in a cell of the specified size in the Lovibond Tintometer.

7.2 Apparatus:

(a) Lovibond Tintometer

(b) Glass cells (cell size 0.25 inch, 0.5 inch. 1.0 inch, 5.25 inch or 1.0 cm, 2.0 cm, 5.0 cm as required)

7.3 Procedure:

Melt the sample if it is not already liquid and filter the oil through a filter paper to remove any impurities and traces of moisture. Make sure sample is absolutely clear and free from turbidity. Clean the glass cell of desired size with carbon tetrachloride and allow it to dry. Fill it with the oil and place the cell in position in the tintometer. Match the colour with sliding red, yellow and blue colours.

Report the colour of the oil in terms of Lovibond units as follows:-

Colour reading = (a Y + 5 b R) or (a Y + 10 b R) in (* cell)

Where,

a = sum total of the various yellow slides (Y) used

b = sum total of the various red (R) slides used

Y + 5R is the mode of expressing the colour of light coloured oils; and

Y + 10 R is for the dark-coloured oils

Although the yellow and red slides required to match the colour shade of an oil in a tintometer are assessed separately, it is found that to a certain extent these slides are mutually compensatory. Consequently different workers may report different values for the yellow and red units for the same oil and the same workers

may report different values for the yellow and red units for the oil examined at different times. To obviate such personal errors a composite factor is used for checking the colour comprising the sum total of the yellow(Y) units and 5 or 10 times the total of red units as specified for the oil or fat.

(Ref: - ISI Hand book of Food Analysis (Part XIII) – 1984 page 75 / IS 548 (Part 1) – 1964, Methods of sampling and test for Oils and Fats)

8.0 DETERMINATION OF MELTING POINT OF FAT

Oils and fats are chiefly mixtures of triglycerides. They do not exhibit either a definite or sharp melting point. Therefore the melting point does not imply the same characteristics that it does with pure crystalline substances. Fats pass through a stage of gradual softening before they become completely liquid. The melting point is therefore defined by the specific conditions of the method by which it is determined.

8.1 Open-tube Capillary-Slip Method

8.1.1 Principle:

The melting point is the temperature at which the oil or fat softens or becomes sufficiently fluid to slip or run as determined by the open-tube capillary-slip method.

8.1.2 Apparatus:

a) Melting point tubes -thin walled with uniform bore capillary glass tubes open at both ends with following dimensions:

Length 50 to 80 mm

Inside diameter 1.0mm

Outside diameter 2.0 mm

b) Thermometer with 0.2°C sub-divisions with a suitable range. The thermometer should be checked against a standard thermometer that has been calibrated and certified by National Physical Laboratory, New Delhi or any other laboratory approved for calibration of instruments.

c) Beaker with a side tube heating arrangement – Thiele melting point tube may be used. Alternatively a Melting point apparatus may also be used

d) Heat source: Gas burner or Spirit Lamp or electric hot plate with rheostat control.

8.1.3 Procedure:

Melt the sample and filter it through a filter paper to remove any impurities and last traces of moisture. Make sure that the sample is absolutely dry. Mix the sample thoroughly. Introduce a capillary tube into the molten sample, so that a column of the sample, about 10 mm long, is sucked into the tube. Dip atleast 3 clean capillary tubes in the completely liquid sample so that the sample rises about 10 mm high in tubes. Chill the sample at once by holding the ends of the tubes that contain the sample against a piece of ice until the fat solidifies. Place the tube in a small beaker and hold it in a refrigerator at 4°C to 10°C for 16 hours (Ref. AOCS Official Method Cc 3-25 – Slip melting point-AOCS Standard Open Tube Melting Point). Remove the tube from the refrigerator and attach with a rubber band to the thermometer bulb, so that the lower end of the capillary tube and the thermometer bulb are at the same level. Suspend the thermometer in 600 mL beaker of clear distilled water. The bottom of thermometer is immersed in the water to the immersion mark. Take water at 10°C in the 'Thiele' tube and immerse the thermometer with the capillary tube containing the sample of fat. Gradually increase the temperature by heating at the side-tube of the Thiel Tube at the rate of 2°C per min, till the temperature reaches 25°C, and thereafter at the rate of 0.5°C per minutes Note the temperature of the water when the sample column begins to rise in the capillary tube. Report the average of two such separate determinations as the melting point, provided that the readings do not differ by more than 0.5°C.

(Ref :- ISI Handbook of Food Analysis (Part XIII) – 1984 , page 68/IS : 548 (Part 1) – 1964, Methods of Sampling and test for Oils and Fats page 33, AOCS Official Method Cc 3-25 – Slip melting point-AOCS Standard Open Tube Melting Point)

9.0 DETERMINATION OF SAPONIFICATION VALUE

9.1 Definition:

The saponification value is the number of mg of potassium hydroxide required to saponify 1 gram of oil/fat.

9.2 Principle:

The oil sample is saponified by refluxing with a known excess of alcoholic potassium hydroxide solution. The alkali required for saponification is determined by titrating the excess potassium hydroxide with standard hydrochloric acid.

9.3 Analytical importance:

The saponification value is an index of mean molecular weight of the fatty acids of glycerides comprising a fat. Lower the saponification value, larger the molecular weight of fatty acids in the glycerides and vice-versa.

9.4 Apparatus:

a. 250 mL capacity conical flask with ground glass joints.
b. 1 m long air condenser, or reflux condenser (65 cm minimum in length) to fit the flask (a).
c. Hot water bath or electric hot plate fitted with thermostat.

9.5 Reagents:

i) Aldehyde free alcohol**:** conforming to IS: 323-1959 Specification for Rectified Spirit (*Revised*)

ii) Alcoholic Potassium Hydroxide Solution - Dissolve 35 to 40 gm of potassium hydroxide in 20 mL of distilled water, and add sufficient aldehyde-free alcohol to make up to 1000 mL. Allow the solution to stand in a tightly stoppered bottle for 24 hours. Then quickly decant the clear supernatant into a suitable, tight container, and standardize the solution and keep in a bottle closed tight with a cork or rubber stopper.

iii) Phenolphthalein indicator solution - Dissolve 1.0 gm of phenolphthalein in 100 mL rectified spirit.

iv)Standard hydrochloric acid: approximately 0.5N

9.6 Procedure:

Melt the sample if it is not already liquid and filter through a filter paper to remove any impurities and the last traces of moisture. Make sure that the sample is completely dry. Mix the sample thoroughly and weigh about 1.5 to 2.0 gm of dry sample into a 250 mL Erlenmeyer flask. Pipette 25 mL of the alcoholic potassium hydroxide solution into the flask. Conduct a blank determination along with the sample. Connect the sample and blank flasks with air condensers; keep on the water bath, gently and steadily boiling until saponification is complete, indicated by absence of any oily matter and the appearance of a clear solution. Clarity may be achieved within one hour of boiling. After the flask and condenser have cooled, wash down the inside of the condenser with about 10 mL of hot ethyl alcohol neutral to phenolphthalein. The excess potassium hydroxide is determined by titration with 0.5N hydrochloric acid, using about 1.0 mL phenolphthalein indicator.

9.7 Calculation:

$$\text{Saponification Value} = \frac{56.1\,(B-S)\,N}{W}$$

Where,

B = Volume in mL of standard hydrochloric acid required for the blank.

S = Volume in mL of standard hydrochloric acid required for the sample

N = Normality of the standard hydrochloric acid and

W = Weight in gm of the oil/fat taken for the test.

Note: - When titrating oils and fats, which give dark coloured soap solution the observation of the end point of titration may be facilitated either (a) by using thymolphthalein or alkali blue 6B in place of phenolphthalein or (b) by shaking 1mL of 0.1% (w/v) solution of methylene blue in water to each 100mL of phenolphthalein indicator solution before the titration.

(Ref: - AOAC 17th edn, 2000, Official method 920.160 Saponification number of oils and fats / IUPAC 2. 202 / ISI Handbook of Food Analysis (Part XIII) 1984, page 78)

10.0 DETERMINATION OF UNSAPONIFIABLE MATTER

10.1 Principle:

Unsaponifiable matter is defined as the substances soluble in the oil, which after saponification are insoluble in water but soluble in the solvent used for the determination. It includes lipids of natural origin such as sterols, higher aliphatic alcohols, pigments, vitamins, and hydrocarbons as well as any foreign organic matter non-volatile at 100°C e.g. (mineral oil). Light Petroleum or diethyl ether is used as a solvent but in most cases results will differ according to the solvent selected and generally the use of diethyl ether will give a higher result.

(Ref: - FAO Manual of Food quality control 14/8, page 261)

10.2 Apparatus:

a) Flat bottom flask or conical flask with a ground glass joint, 250 mL capacity

b) Air condenser 1 meter long to fit the flask

c) Separating funnel, 500 mL capacity

d) Weighing balance

The weighing balance should be accurately calibrated to measure 10 mg of sample on a tare weigh of 100 gm.

10.3 Reagents:

a. Alcoholic potassium hydroxide solution: Dissolve 7 to 8 gm of potassium hydroxide in an equal quantity of distilled water and add sufficient aldehyde free ethyl alcohol and make up to 100 mL.
b. Ethyl alcohol: Ninety-five percent
c. Phenolphthalein indicator solution: Dissolve one gram of phenolphthalein in 100 mL of ethyl alcohol.
d. Petroleum ether (40 - 60°C): Analytical reagent grade
e. Aqueous alcohol: 10% of ethyl alcohol in water
f. Standard sodium hydroxide solution: Approximately 0.02N
g. Acetone: Analytical reagent grade
h. Anhydrous sodium sulphate

10.4 Procedure:

Weigh accurately 5 gm of well mixed oil/fat sample into a 250 mL conical flask. Add 50 mL of alcoholic potassium hydroxide solution. Boil the content under reflux air condenser for one hour or until the saponification is complete (complete saponification gives a homogeneous and transparent medium). Take care to avoid loss of ethyl alcohol during the saponification. Wash the condenser with about 10 mL of ethyl alcohol. Transfer the saponified mixture while still warm to a separating funnel, wash the saponification flask first with some ethyl alcohol and then with cold water, using a total of 50 mL of water to rinse the flask. Cool to 20 to 25°C. Add to the flask 50 mL of petroleum ether, shake vigorously, and allow the layers to separate.

Transfers the lower soap layer into another separating funnel and repeat the ether extraction 3 times, using 50 mL portions of petroleum ether. Some oils high in unsaponifiable matter, e.g., marine oils, may require more than three extractions to

completely remove Unsaponifiable matter. Wash the combined ether extract three times with 25 mL portions of aqueous alcohol followed by washing with 25 mL portions of distilled water to ensure ether extract is free of alkali (washing are no longer alkaline to phenolphthalein). Transfer washed ether extract to 250 mL beaker, rinse separator with ether, and add rinsing to main solution. Evaporate to about 5mL and transfer quantitatively using several portions of ether to a previously dried and weighed 50 mL Erlenmeyer flask. Evaporate ether. When all ether has been removed add 2-3 mL acetone and while heating on steam or water bath completely remove solvent under a gentle air. To remove last traces of ether, dry at 100°C for 30 minutes till constant weight. Dissolve residue in 50 mL of warm ethanol, which has been neutralised to a phenolphthalein end point. Titrate with 0.02N Sodium hydroxide.

10.5 Calculation:

Weight in g of the free fatty acids in the extract as oleic acid = 0.282 V×N

Where,

V = Volume in mL of standard sodium hydroxide solution

N = Normality of standard sodium hydroxide solution

$$\text{Unsaponifiable matter} = \frac{100\,(A-B)}{W}$$

Where,

A = Weight of the residue in gm

B = Weight of free fatty acids in the extract in gm

W = Weight of the sample in gm

(Ref: - ISI Handbook of Food Analysis (Part XIII)-1984, page 67 /AOAC 17th edn, 2000, Official method 933.08, Residue (unsaponifiable) of oils and fats)

11.0 DETERMINATION OF ACID VALUE

11.1 Definition:

The acid value is defined as the number of milligrams of Potassium hydroxide required to neutralize the free fatty acids present in one gram of fat. It is a relative measure of rancidity as free fatty acids are normally formed during decomposition of triglycerides. The value is also expressed as per cent of free fatty acids calculated as oleic acid, lauric, ricinoleic and palmitic acids.

11.2 Principle:

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

11.3 Analytical Importance:

The value is a measure of the amount of fatty acids, which have been liberated by hydrolysis from the glycerides due to the action of moisture, temperature and/or lipolytic enzyme lipase.

11.4 Apparatus:

250 mL conical flasks

11.5 Reagents:

a) Ethyl alcohol: Ninety-five percent alcohol or rectified spirit neutral to phenolphthalein indicator.

b) Phenolphthalein indicator solution: - Dissolve one gram of phenolphthalein in 100 mL of ethyl alcohol.

When testing rice bran oil based blended oils or oils or fats, which give dark colored soap solution, the observation of the end point of the titration may be facilitated, by using Alkali Blue 6B in place of Phenolphthalein.

c) Standard aqueous potassium hydroxide or sodium hydroxide solution 0.1 or 0.5 N. The solution should be colourless and stored in a brown glass bottle. For refined oils, the strength of the alkali should be fixed to 0.1 N.

11.6 Procedure:

Mix the oil or melted fat thoroughly before weighing. The mass of the test sample shall be taken based on the colour and expected acid value.

Expected Acid Value	Mass of Test portion (gm)	Accuracy of weighing of test portion (gm)
<1	20	0.05
1 to 4	10	0.02
4 to 15	2.5	0.01
15 to 75	0.5	0.001
>75	0.1	0.0002

Weigh accurately appropriate amount of the cooled oil sample in a 250 mL conical flask and add 50 mL to 100 mL of freshly neutralised hot ethyl alcohol and about one ml of phenolphthalein indicator solution. Boil the mixture for about five minutes and titrate while hot against standard alkali solution shaking vigorously during the titration. The weight of the oil/fat taken for the estimation and the strength of the alkali used for titration shall be such that the volume of alkali required for the titration does not exceed 10 mL.

11.7 Calculation:

$$\text{Acid value} = \frac{56.1\ V \times N}{W}$$

Where,

V = Volume in mL of standard potassium hydroxide or sodium hydroxide used

N = Normality of the potassium hydroxide solution or Sodium hydroxide solution; and

W = Weight in gm of the sample

11.8 Free Fatty Acids (FFA)

The acidity is frequently expressed as the percentage of FFA in the sample. The percentage of FFA in most oils and fats is calculated on the basis of oleic acid; although in coconut oil and palm kernel oil it is often calculated as lauric acid, in castor oil in terms of ricinoleic acid and in palm oil in terms of palmitic acid. The calculation in terms of different oils are given below

Free fatty acids as oleic acid % by weight =	$\frac{28.2\ V \times N}{W}$
Free fatty acids as lauric acid % by weight =	$\frac{20\ V \times N}{W}$
Free fatty acids as ricinoleic acid % by weight =	$\frac{29.8\ V \times N}{W}$
Free fatty acids as palmitic acid % by weight =	$\frac{25.6\ V \times N}{W}$

Acid value = % fatty acid (as oleic) ×1.99

Oryzanol has its own acidity and contributes to the measured FFA content when present in oil. FFA content determined by using phenolphthalein as the indicator needs to be corrected. The formula for calculating real FFA content is shown below.

Real FFA = observed FFA (for phenolphthalein) – (% oryzanol in the oil) ×0.425

For determination of acid value in case of rice bran oil and blended oils containing rice bran oil, the correction factor provided above must be used to account for oryzanol's acidity or alkali blue may be used as an indicator for the titration which is most suitable.

(Ref: - ISI Handbook of Food Analysis (Part XIII)-1984 Page 67/ IUPAC 2.201(1979) /IS: 548 (Part 1) – 1964, Methods of Sampling and Test for Oils and Fats/ ISO 660:1996 Determination of acid value and acidity)

12.0 DETERMINATION OF IODINE VALUE

12.1 Definition:

The iodine value of an oil/fat is the number of grams of iodine absorbed by 100gm of the oil/fat, when determined by using Wijs solution.

12.2 Principle:

The oil/fat sample taken in carbon tetrachloride is treated with a known excess of iodine monochloride solution in glacial acetic (Wijs solution). The excess of iodine monochloride is treated with potassium iodide and the liberated iodine estimated by titration with sodium thiosulfate solution.

12.3 Analytical importance:

The iodine value is a measure of the amount of unsaturation (number of double bonds) in a fat.

12.4 Apparatus:

500 mL Erlenmeyer flask

12.5 Reagents:

i) Potassium dichromate AR

ii) Concentrated hydrochloric acid AR

iii) Glacial acetic acid, free from ethanol

iv) Carbon tetrachloride, analytical reagent grade

v) Iodine mono-chloride (ICl)

vi) Potassium iodide (free from potassium iodate) - 10% solution prepared fresh

vii) Starch solution - Mix 5 gm of starch and 0.01 gm of mercuric iodide with 30 mL of cold water and slowly pour it with stirring into one litre of boiling water. Boil for three minutes. Allow to cool and decant the clear supernatant.

viii) Wijs Iodine monochloride solution - Dissolve 10 mL of iodine monochloride in about 1800 mL of glacial acetic acid and shake vigorously. Pipette 5 mL of Wijs solution, add 10 mL of potassium iodide solution and titrate with 0.1N standard sodium thiosulphate solution using starch as indicator. Adjust the volume of the solution till it is approximately 0.2 N or prepare Wijs iodine solution by dissolving 13 gm resublimed Iodine in 1000 mL acetic acid and pass in dried chlorine (dried through H_2SO_4.) until original Sodium thiosulphate titre value of the solution is not quite doubled (characteristic colour change at the end point indicates proper amount of Chlorine. Convenient method is to reserve some amount of original I solution, add slight excess of Cl to bulk of solution and bring to desired titre by re additions of reserved portion). Store in an amber colored bottle sealed with paraffin until ready for use. Wijs solutions are sensitive to temperature, moisture and light. Store in the dark below 30°C. Determine I/Cl ratio as follows Iodine Content – Pipette 5 mL Wijs solution into 500 mL Erlenmeyer flask containing 150 mL saturated Cl – water and some glass beads. Shake heat to boiling point and boil briskly for 10 minutes. Cool, add 30 ml H_2SO_4 (1+ 49) and 15 mL 15 % Potassium iodide solution and titrate immediately with 0.1 N Sodium thiosulphate.

Total Halogen content – Pipette 20 mL Wijs solution into 500 Erlenmeyer flask containing 150 mL recently boiled and cooled water and 15 mL 15 % Potassium iodide solution. Titrate immediately with 0.1 N Sodium thiosulphate.

I/Cl = 2 X / (3B – 2 X) where X = mL of 0.1 Sodium thiosulphate required for I content and B = mL required for total halogen content.

I / Cl ratio must be 1.10 ± 0.1

ix) Standard sodium thiosulphate solution (0.1N) - Dissolve approximately 24.8 gm of sodium thiosulphate crystals ($Na_2S_2O_3.5H_2O$) in distilled water and make up to 1000 mL. Standardise this solution by the following procedure:

Weigh accurately about 5.0 gm of finely powdered potassium dichromate, which has been previously dried at 105°C ± 2°C for one hour, dissolve it in distilled water and make up to 1000 mL. For standardisation of sodium thiosulphate, pipette 25 mL of this solution into a 250 mL conical flask. Add 5 mL of concentrated hydrochloric acid and 15 mL of a 10 % potassium iodide solution. Allow to stand in dark for 5 min and titrate with sodium thiosulphate solution using starch as indicator. End point is change of blue colour to green.

$$N = \frac{25W}{49.03\ V}$$

Where,

N = Normality of the sodium thiosulphate

W = Weight in gm of the potassium dichromate, and

V = Volume in mL of sodium thiosulphate solution required for titration

12.6 Procedure:

Oil/fat may be weighed accurately following the Table given below:

Expected Iodine Weight to be take for Value estimation (gm)

Expected Iodine Value	Weight to be taken for estimation (g)	
	Maximum	Minimum
5	6.3460	5.0770
10	3.1730	2.5384
50	0.6612	0.5288
100	0.3173	0.2538
150	0.2125	0.1700
200	0.1586	0.1269

Weigh accurately an appropriate quantity of the dry oil/fat as indicated in the Table above, into a 500 mL glass stoppered conical flask, to which 25 mL of carbon tetrachloride has been added. Mix the contents well. The weight of the sample shall be such that there is an excess of 50 to 60 % of Wijs solution over that actually needed. Pipette 25 mL of Wijs solution and replace the glass stopper after wetting with potassium iodide solution. Swirl for proper mixing and keep the flasks in dark for 30 minutes for non-drying and semi-drying oils and one hour for drying oils. Carry out a blank simultaneously. After standing, add 15 mL of potassium iodide solution, followed by 100 mL of recently boiled and cooled water, rinsing in the stopper also. Titrate the liberated iodine with standardized sodium thiosulphate solution, using starch as indicator until the blue colour formed disappears after thorough shaking with the stopper on.

Conduct blank determinations in the same manner as test sample but without oil/fat. Slight variations in temperature appreciably affect titre of iodine solution as chloroform has a high coefficient of expansion. It is thus necessary that blanks and determinations are made at the same time.

12.7 Calculation:

$$\text{Iodine value} = \frac{12.69\ (B - S)\ N}{W}$$

Where,

B = volume in mL of standard sodium thiosulphate solution required for the blank.

S = volume in mL of standard sodium thiosulphate solution required for the sample.

N = normality of the standard sodium thiosulphate solution.

W = weight in gm of the sample.

(Ref: - AOAC 17th edn, 2000, Official method 920. 159 – Iodine absorption number of oils and fats / ISI Handbook of Food Analysis (Part XIII) – 1984 page 76)

*The following methods can also be referred:

AOCS Official Method Cd 1b-87: Iodine value of fats and oils: Cyclohexane

AOCS Official Method Cd 1D-92: Iodine value of fats and oils: Cyclohexane Acetic acid method

13.0 DETERMINATION OF REICHERT-MEISSL AND POLENSKE VALUE

Butter is distinguished from other fats by the presence of glyceryl esters of relatively low molecular weight fatty acids, especially butyric but also caproic, capric, caprylic, lauric and myristic acids. These acids are wholly or partially steam volatile and water soluble. The Reichert-Meissl value reflects the amount of butyric and caproic acids present and Polenske value chiefly capryilic, capric and lauric acids, with some contribution from myristic and even palmitic acid.

13.1 Definition:

The Reichert-Meissl value is the number of mLs of 0.1N aqueous sodium hydroxide solution required to neutralize steam volatile water soluble fatty acids distilled from 5gm of an oil/fat under the prescribed conditions. It is a measure of

water soluble steam volatile fatty acids chiefly butyric and caproic acids present in either an oil or fat.

The Polenske value is the number of mililiters of 0.1N aqueous alkali solution required to neutralise steam volatile water insoluble fatty acids distilled from 5 gm of the oil/fat under the prescribed conditions. It is a measure of the steam volatile and water insoluble fatty acids, chiefly caprylic, capric and lauric acids present either in an oil or fat.

13.2 Principle:

The material is saponified by heating with glycerol sodium hydroxide solution and then split by treatment with dilute sulfuric acid. The volatile acids are immediately steam distilled. The soluble volatile acid in the distillate are filtered out and estimated by titration with standard sodium hydroxide solution.

13.3 Analytical Importance:

These determinations have been used principally for analysis of butter and margarines. Butter fat contains mainly butyric acid glycerides. Butyric acid is volatile and soluble in water.

No other fat contains butyric acid glycerides, and therefore, the Reichert-Meissl value of the butter fat is higher than that for any other fat. Coconut oil and palm kernel oil contain appreciable quantities of caprylic capric and lauric acid glycerides. These fatty acids are steam volatile but not soluble in water, and hence give high Polenske value.

13.4 Apparatus:

a. An all-glass distillation assembly conforming to specifications given in AOCS Official Methods Cd 5 - 40 or Methods of Analysis, AOAC- 17th Edn.,2000 (925.41, Chapter 41 page 14) or distillation apparatus as shown in the diagram below

b. 25 mL beaker

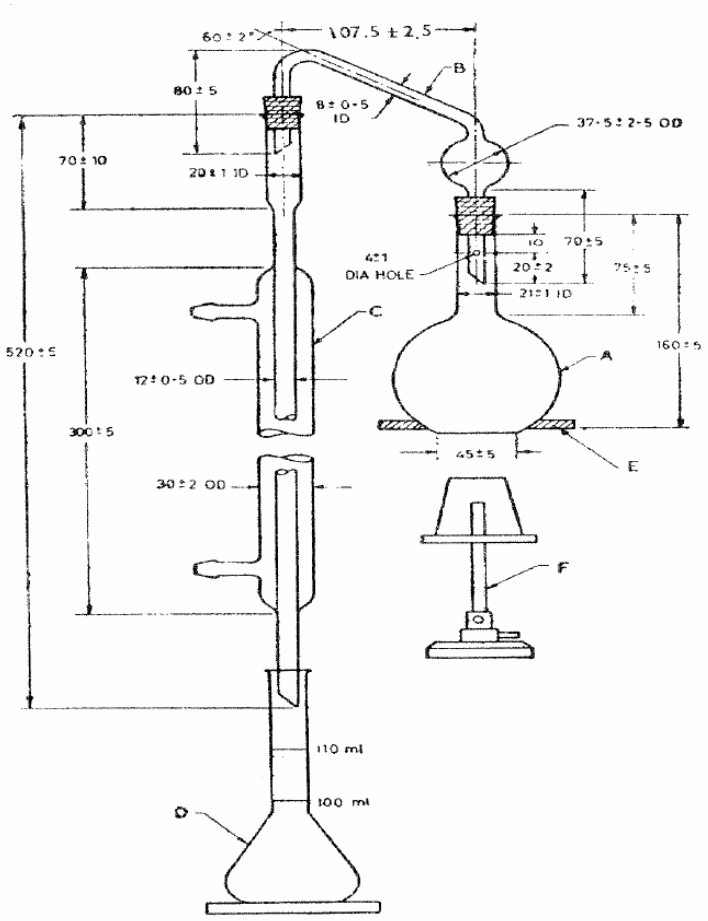
c. 100 mL graduated cylinder

d. 100 mL pipette

e. Graduated burette

f. Asbestos board with a hole about 65 mm dia for supporting the flask over the burner. During distillation the flask shall fit snugly into the hole of the board to prevent the flame from impinging on the surface of the flask above the hole.

g. Bunsen burner sufficiently large to allow completion of distillation in the prescribed time.



Reichert-Meissl Distillation Apparatus

13.5 Reagents:

a.) Glycerine:

b.) Concentrated sodium hydroxide solution: 50 % (w/w) Dissolve Sodium Hydroxide in equal weight of water and store solution in a polypropylene bottle. Use clear solution free from deposit.

c.) Pumice stone grains

d.) Dilute sulfuric acid solution: Approximately 1.0 N

e.) Sodium hydroxide solution: 0.1N solution in water, accurately standardised

f.) Phenolphthalein indicator: Dissolve 0.1 gm of phenolphthalein in 100 mL of ethyl alcohol

g.) Ethyl alcohol: 90% by volume and neutral to phenolphthalein

13.6 Procedure:

Weigh accurately 5 ± 0.1gm of filtered oil or fat sample into a clean, dry, 300ml distilling flask. Add 20 mL of glycerine and 2 mL of concentrated sodium hydroxide solution, and heat with swirling over a flame until completely saponified, as shown by the mixture becoming perfectly clear. Cool the contents slightly and add 90 mL of boiling distilled water, which has been vigorously boiled for about 15 minutes After thorough mixing, the solution should remain clear. If the solution is not clear (indicating incomplete saponification) or is darker than light yellow (indicating over-heating), repeat the saponification with a fresh sample of the oil or fat. If the sample is old, the solution may sometimes be dark and not clear.

Add about 0.6 - 0.7 gm of pumice stone grains, and 50 mL of dilute sulfuric acid solution. Immediately connect the flask to the distillation apparatus. Place the flask on asbestos board so that it fits snugly into the aperture. This will prevent the flame from impinging on the surface of the flask above the level of the liquid and

avoid super heating. Heat very gently until the liberated fatty acids melt and separate. Then set the flame so that 110 mL of distillate shall be collected within 19 to 21 minutes. The beginning of the distillation is to be taken as the moment when the first drop of the distillate falls from the condenser in the receiving flask. Keep the water in the condenser flowing at a sufficient speed to maintain the temperature of the outgoing water from the condenser between 15 and 20°C. Collect the distillate in a graduated flask.

When the distillate exactly reaches the 110 mL mark on the flask, remove the flame and quickly replace the flask by a 25 mL measuring cylinder. Stopper the graduated flask and without mixing place d it in a water bath maintained at 15°C for 10 min so that the 110 mL graduation mark is 1 cm below the water level in the bath. Swirl round the contents of the flask from time to time. Remove the graduated flask from the cold water bath, dry the outside and mix the content gently by inverting the flask 4 to 5 times without shaking. Avoid wetting the stopper with the insoluble acids. Filter the liquid through a dry, 9 cm Whatman No. 4 filter paper or equivalent. Reject the first 2-3 mL of the filterate and collect the rest in a dry flask. The filtrate should be clear. Pipette 100 mL of the filtrate and add 5 drops of the phenolphthalein solution, and titrate against standard 0.1N sodium hydroxide solution.

Run a Blank Test without the fat, but using the same quantities of the reagents.

13.7 Calculation:

Reichert-Meissl Value = (A – B) x N x 11

where,

A = Volume in mL of standard sodium hydroxide solution required for the the test;

B = Volume in mL in standard sodium hydroxide solution required for the blank; and

N = Normality of standard sodium hydroxide solution.

13.8 Determination of Polenske Value:

After titrating, the soluble volatile acids detach the still head and rinse the condenser with three successive 15 mL portions of cold distilled water passing each washing separately through the measuring cylinder, 110 mL graduated flask and the filter paper and allow all of it to pass through. Discard all the washings. Place the funnel on a clean conical flask. Dissolve the insoluble fatty acids by three similar washings of the condenser, the measuring cylinder, the 110 mL flask with stopper, and the filter paper with 15 mL portions of ethyl alcohol. Combine the alcoholic washings in a clean flask, add 5 drops of phenolphthalein indicator solution, and titrate with standard (0.1N) sodium hydroxide solution.

Polenske value = 10 x V x N

where,

V = Volume in mL of standard sodium hydroxide solution required for the test; and

N = Normality of the standard sodium hydroxide solution.

Note: - Unless the directions are followed in every detail reproducible results cannot be obtained.

(Ref: - ISI Handbook of Food Analysis (Part XIII) – 1984 page 81) / AOAC 17th edn, 2000. Official method 925.41 Acids (volatile) in oils and fats)

14.0 BELLIER TEST (TURBIDITY TEMPERATURE) ACETIC ACID METHOD

14.1 Principle:

Oils containing long chain saturated fatty acids give a precipitate at a particular temperature, which is specific for the oil when their alcoholic soap solution is treated with dilute acetic acid solution and 70% ethyl alcohol.

14.2Apparatus:

a) Conical flask - 100 mL capacity with cork

b) Thermometer (0 - 60°C calibrated to read 0.5°C)

c) Water bath

14.3 Reagents:

i) Purified / Rectified spirit:

Reflux 1.2 liters of rectified spirit for 30 minutes in a distillation flask with 10 gm of caustic potash and 6 gm of granulated aluminium (or aluminium foil pieces). Distil and collect one liter after discarding the first 50 mL. Use this purified rectified spirit for preparation of all the reagents.

ii) Alcohol 70 % (by volume):

Dilute 700 mL of alcohol to 950 mL with distilled water and check the strength by specific gravity determination and adjust if necessary. The specific gravity of 70% alcohol at 15.5°C is 0.8898 and 30 °C is 0.8807. The final strength should be checked accurately.

iii) Alcoholic potash (1.5 N): Dissolve 8.5 gm potassium hydroxide in 100 mL purified rectified spirit. It is preferable to keep this solution in a dark colour bottle.

iv)Dilute acetic acid: Mix one volume of glacial acetic acid with two volumes of distilled water.

v) Phenolphthalein indicator: Dissolve 0.5 gm of phenolphthalein in 50 mL of purified rectified spirit and mix the solution with 50 mL of distilled water.

14.4 Procedure:

Measure with the aid of a pipette one ml of the filtered sample of oil in a flat-bottom 100 mL conical flask (preferably with a long neck), add 5 mL of 1.5N alcoholic potash and saponify completely by heating over a boiling water-bath using an air condenser (about 1.3 meters long) to avoid loss of alcohol as far as possible. Complete saponification usually takes about 10 minutes.

During saponification swirl the flask several times. Cool, add 0.1 mL of phenolphthalein indicator, neutralise exactly by adding carefully dilute acetic acid and then add an extra amount of 0.4 mL (accurately measured). Add 50 mL of 70 % alcohol and mix. Fit a thermometer (0° to 60°C reading to 0.5°C, accurately calibrated) into the flask, with the aid of a velvet cork in such a way that the bulb of the thermometer is immersed in the liquid but does not touch the bottom of the flask. Heat the flask gently over the water-bath until the temperature reaches 50°C and the solution is clear. Allow the flask to cool in air with frequent shaking until the temperature falls gradually to 40°C (in case of pure groundnut oil turbidity appears at 39 to 41°C). Then, cool the flask with constant shaking by occasional immersion in a cooling bath maintained at 15 °C (±1°C) so that the temperature drops roughly at the rate of 2°C per minute. Note the temperature at which the first distinct turbidity appears is the turbidity temperature. This turbidity temperature is confirmed by a little further cooling, which would result in deposition of the precipitate.

Dissolve the precipitate by gently heating the contents to 50°C in a water-bath, again cool as described above and make a duplicate determination of the turbidity temperature. The mean of the two values is taken as the true turbidity temperature. Duplicate shall agree within ±0.5°C.

Note: It is essential that stirring is continuous and moderate while the contents are being cooled in the cooling bath. Violent shaking or agitation would be avoided as it will affect the result adversely.

(Ref: - ISI Handbook of Food Analysis (Part XIII) 1984 - page 90)

15.0 TEST FOR PRESENCE OF SESAME OIL (BAUDOUIN TEST)

15.1 Principle:

The development of pink colour with furfural solution in the presence of hydrochloric acid indicates the presence of sesame oil. The color is produced on account of reaction with sesamolin present in sesame oil.

15.2 Apparatus:

(a) Glass stopper test tubes / measuring cylinders

15.3 Reagents:

i) Hydrochloric acid (concentrated) Sp. Gr. 1.19

ii) Furfural solution (2 percent furfural–freshly distilled in ethyl alcohol)

15.4 Procedure:

Take 5 mL of the oil or melted fat in a 25 mL measuring cylinder (or test tube) provided with a glass stopper, and add 5 ml of concentrated hydrochloric acid and 0.4 mL of furfural solution. Insert the glass stopper and shake vigorously for two minutes.

Let it stand and allow the mixture to separate. The development of a pink or red colour in the lower acid layer indicates presence of sesame oil. Confirm by adding 5 mL of water and shaking again. If the colour in acid layer persists, sesame oil is present and if the colour disappears it is absent. (As furfural gives violet tint with hydrochloric acid, it is necessary to use the dilute solution specified)

Note: Test the sample for the presence of colouring matter that are chromogenic in presence of Hydrochloric acid. For this purpose, take 5 mL of the sample in a 25mL

measuring cylinder provided with a glass stopper and shake with 5mL of concentrated hydrochloric acid. If there is no development of pink or red color in the aqueous layer apply the test as above. If pink or red color develops in the aqueous layer, remove the red acid layer which collects at the bottom and repeat the procedure until no further coloration takes place. After complete removal of Hydrochloric acid layer perform the test as prescribed above.

(Ref: - ISI Handbook of Food Analysis (Part XIII)-1984 Page 86 / AOAC 17th edn, 2000, Official method 893.01-Oil (sesame) in Oils and Fats Modified Villavecchia Test))

16.0 TEST FOR PRESENCE OF COTTONSEED OIL (HALPHEN'S TEST)

16.1 Principle:

The development of red colour on heating the oil with a solution of sulphur in carbon disulphide indicates the presence of cottonseed oil. The test is also given by Hempseed oil, Kapokseed oil / oils and fats containing cyclopropenoid fatty acids (such as sterculic and malvalic acid). Hydrogenation and deodorization wholly or partially destroy the chromogens and react with diminished intensity. A positive reaction is not given by oil heated to 250°C or above. The fat of animals fed on cottonseed meal (butter, lard) or other cottonseed products may give faint positive reaction by this test.

16.2 Apparatus:

(a) Test tubes

(b) Water bath

(c) Oil bath or Brine bath maintained at 110 – 115°C

16.3 Reagent:

(a) Sulphur solution: Prepare a one % (w/v) solution of sulphur in carbon disulphide and then add an equal volume of amyl alcohol.

16.4 Procedure:

Take about 5 mL of the oil or melted fat in a test tube and add to it an equal volume of the sulphur solution. Mix thoroughly by shaking and heat gently on a water bath (70 - 80°C) for a few minutes with occasional shaking until the carbon disulphide has boiled off and the sample stops foaming. Place the tube in an oil bath or a saturated brine-bath maintained at 110 -115°C and hold for 2.5 hours. A red colour at the end of this period indicates the presence of cottonseed oil. The test is sensitive to the extent of 0.5 % cottonseed oil in other oils.

(Ref: - ISI Handbook of Food Analysis of (Part XIII)-1984 Page 86 / AOAC 17th edn, 2000, Official method 197.02-oil (cottonseed) in oils and fats /FAO Manuals of Food Quality Control 14 / 8 Page 271)

17.0 DETERMINATION OF CLOUD POINT IN PALMOLEIN (AND TEST FOR PRESENCE OF PALMOLEIN IN OTHER OILS)

17.1 Principle:

The cloud point is that temperature at which (under the conditions of this test) a cloud is induced in the sample caused by the first stage of crystallization.

17.2 Apparatus:

a) Oil sample bottle, 115 mL (4 oz)

b) Thermometer, range 2 – 68°C

c) Water bath made up of water, chipped ice and water or chipped ice, salt and water, depending upon the temperature required. The temperature of the water bath shall not be less than 2°C and not more than 5°C of the cloud point.

17.3 Procedure:

The sample must be completely dry before conducting the test. Heat 60 - 75 gm of sample to 130°C just before the test. Pour ca 45 mL of the heated fat into an oil sample bottle. Place the bottle in a water bath. Begin to cool the bottle in the water bath, stirring enough using the thermometer to keep the temperature uniform.

When the sample has reached a temperature ca 10°C above the cloud point, begin stirring steadily and rapidly in a circular motion so as to prevent super-cooling and solidification of fat crystals on the sides or bottom of the bottle. From this point on, do not remove the thermometer from the sample, since doing so may introduce air bubbles which will interfere with the test. Maintain the test bottle in such a position that the upper levels of the sample in the bottle and the water in the bath are about the same. Remove the bottle from the bath and read the temperature. The bottle should be inspected regularly. The cloud point is that temperature at which that portion of the thermometer immersed in the oil is no longer visible when viewed horizontally through the bottle.

Note: This test is useful for the detection of palmolein in groundnut oil. Presence of palmolein over 10 percent in groundnut oil readily gives cloud at a higher temperature than that of groundnut oil due to the presence of palmitic glycerides in higher amounts in palmolein / palm oil.

(Ref:- AOCS (1989) Official Method Ce 6 – 25 / Manual methods of Analysis for Adulterants and Contaminants in Foods , ICMR (1990) Page 4)

18.0 TEST FOR PRESENCE OF RICE-BRAN OIL

18.1 Principle:

Oryzanol in rice-bran oil is isolated using 30% aqueous potassium hydroxide solution and detected on thin-layer Chromatographic plate.

18.2 Apparatus:

a) Conical flasks, 250 mL capacity - 4 Nos., 100 mL capacity - 2 Nos,

b) Thin layer Chromatographic plates (0.25 mm) prepared by coating slurry of silica gel G. on glass plate of 20 x 10 cm dimension.

c) Iodine chamber for visualization of spots

d) Spotting capillaries

e) Separating funnel (100 mL capacity)

f) Hot water bath

18.3 Reagents:

i) Aqueous potassium hydroxide solution 30%

ii) Hydrochloric acid dilute

iii) Blue litmus paper

iv) Diethyl ether AR grade

v) Sodium sulphate anhydrous

vi) Benzene - acetic acid (100: 1 v/v)

vii) Chloroform AR grade

18.4 Procedure:

Take 20 mL of the oil in a 100 mL capacity separating funnel and add to it equal volume of aqueous potassium hydroxide solution. Shake the contents gently but constantly for 10 minutes. Keep the separating funnel on a stand for about 45 minutes to allow the separation of alkali layer. Draw the alkali layer and neutralise with dilute hydrochloric acid solution. Confirm the neutralization with blue litmus paper.

Extract this salt solution with diethyl ether (20 ml x 3 times). Wash the diethyl ether extract with distilled water and dry on anhydrous sodium sulphate. Evaporate the solvent on hot water bath and spot the residue in chloroform on TLC/HPTLC plate.

Develop the TLC/HPTLC plate in pure benzene: acetic acid mixture. Allow the solvent front to move a distance of 15 cm. Visualise the spots in iodine chamber.

Appearance of a spot between Rf 0.7 to 0.75 indicates the presence of rice bran oil.

Run a control by taking a sample of rice bran oil and compare the spot given by test sample under identical conditions.

18.5 Sensitivity:

The above method can detect rice-bran oil in other edible vegetable oils up to the minimum of 5% level.

(Ref: - Manual methods of Analysis for Adulterants and Contaminates in Foods ICMR (1990) Page 5)

19.0 TEST FOR PRESENCE OF LINSEED OIL (HEXABROMIDE TEST)

19.1 Principle:

The formation of a precipitate of hexabromide when the oil in chloroform is treated with bromine, followed by alcohol and ether in cold condition indicates the presence of linseed oil.

19.2 Apparatus:

a) Boiling tubes

b) Ice water bath

19.3 Reagents:

i) Chloroform – A.R

ii) Liquid bromine – A.R

iii) Ethyl alcohol

iv) Diethyl ether

19.4 Procedure:

Pipette one mL of the oil into a boiling tube (wide-mouthed 100 mL capacity). Add 5 mL of chloroform and about one mL of bromine drop-wise till the mixture becomes deep red in colour and cool the test-tube in an ice water-bath. Add about 1.5 mL of rectified spirit drop-wise while shaking the mixture until the precipitate which was first formed just dissolves and then add 10 mL of diethyl ether. Mix the contents and place the tube with in the ice water-bath for 20 minutes. Appearance of precipitate indicates the presence of linseed oil.

Note:

1. This test is not applicable for detecting linseed oil in Mahua oil.

2. The use of safe and suitable pipette i.e. Lunge-Ray pipette is suggested for the handling and addition of bromine.

3. The test is also given by fish oils and fats containing highly unsaturated fatty acids. It has been observed that in low erucic rapeseed oil, and Mahua oil having linolenic acid content greater than 12.0% may also give positive test. The results obtained in such cases have to be viewed with caution. Experiments conducted with these oils with or without added linseed oil have shown that, if linseed oil is present even at 1% level, hexabromide insoluble in cold ether are formed within 20 minutes. Any hexabromides insoluble in cold ether formed after 20 min need not be taken for the presence of linseed oil.

An explanation for this behavior of these oils can be given on the basis of glyceride structure. Oils and fats are mixed triacylglycerides i.e. different fatty acid are present in each of the positions of the glycerol molecule. Exception to this rule is the oil /fat containing a particular fatty acid in amounts greater than 50 % where such a fatty acid may take all the three positions of the glycerol molecule giving rise to simple triacylglycerides. Linseed oil is such an example containing greater than 50 % Linolenic acid.

(Ref: - Manual of Methods of Analysis for Adulterants and Contaminants in Foods, ICMR (1990) Page 5 / ISI Handbook of Food Anaysis Part (XIII) – 1984 page 86)

20.0 POLYBROMIDE TEST FOR MUSTARD OIL

This test for the presence of fatty acids with more than two non conjugated double bonds is more reliable on fatty acids than on glycerides in which one of the three fatty acids in combination may be polyunsaturated.

20.1 Principle:

An ethereal solution of the fat or fatty acid is treated with bromine. The formation of a precipitate gives a qualitative indication of the presence of fatty acids with three or more non conjugated double bonds

20.2 Reagents:

(1) Diethyl ether

(2) Bromine

20.3 Apparatus:

(1) Conical Flask 100 mL capacity

(2) Burette with a finely drawn out jet

20.4 Procedure:

Dissolve approximately 3 gm of clear fat in 25 mL diethyl ether in the conical flask. Place the flask in a melting ice bath for 15 minutes and then slowly add 1 mL bromine drop wise from burette with continuous swirling and cooling (the first half ml in 20 minutes and the remainder in 10 minutes). Cool the flask and keep it in the ice bath for a further 3 hours. If a precipitate forms, the reaction is considered positive.

(Ref: - Laboratory Handbook for Oil and Fat Analysis, Cocks and Reid, page 147-148)

21.0 DETERMINATION OF FATTY ACID COMPOSITION OF OILS AND FATS BY GAS LIQUID CHROMATOGRAPHY

21.1 Principle:

The methyl esters of fatty acids are formed using boron trifluoride or methanol and alkali and separated by gas – liquid chromatography using a flame ionization detector. The elution pattern of methyl esters can be compared with authentic oils for identification

21.2 Apparatus:

(1) Gas liquid chromatograph with the following characteristics:

a) Injection system heated to a temperature of 20 – 50°C higher than the column.

b) Oven – capable of heating the column to at least 220°C and maintaining the temperature to within ±1°C. If temperature programming is to be employed, twin columns are recommended.

c) Packed column - may be glass or stainless steel. However glass is preferred as steel may decompose polyunsaturated fatty acids having more than 3 double bonds. Some successful column packing with column length, internal diameter and operating temperature are as follows

i) 12- 15 % ethylene glycol succinate on 100 / 120 mesh gas chrom P (2m x 4 mm, at 180 degree C)

ii) 2- 10 % Apizon –L on 80/ 100 mesh Chromosorb W or Celite (2 m x 4 mm at 220 degree C)

iii) 10 % Butan-1-4 diol succinate on 80 / 100 mesh Chromosorb W or celite (2 m x 4 mm at 175 degree C)

iv) 3 % SE – 30 on 100 / 120 mesh Chromosorb –G silanised (2m x 3mm at 190 ° C)

Condition the newly prepared column by disconnecting the detector and heating the column in the oven to the normal operating temperature for 16 hours while running the carrier gas at a rate of 20 – 60 mL/minutes

v) Detector – Flame ionization detector – capable of being heated to a temperature above that of the column

2) Syringe – 10 μL graduated in 1/10th of a microlitre

3) Recorder – electronic with high precision with rate of response below 1.5 second, width of paper 25cm, paper speed 25-150 cm/hours

4) Integrator or calculator for rapid and accurate calculations.

5) 50 and 100 mL boiling flasks

6) Reflux condenser

7) Graduated pipette – 10 mL

8) Test tubes with ground stoppers

9) 250 mL Separating funnels

21.3 Reagents:

(1)Carrier Gas – Inert gas (nitrogen, helium, argon) thoroughly dried and containing less than 10 mg / kg of oxygen

(2)Auxillary gas Hydrogen 99.9 % minimum purity. Free from organic impurities, air or oxygen

(3)Reference standards – a mixture of methyl esters or methyl esters of oils of known purity preferably similar to the fatty matter being analysed

(4)Methanolic Sodium hydroxide solution - approx 0.5 N. Dissolve 2 gm of Sodium Hydroxide in 100 mL methanol containing not more than 0.5 % m/m water. When the solution has to be stored for considerable time, a small amount of white ppt of Sodium Carbonate may be formed. This has no effect on the preparation of the methyl esters

(5)Methanolic solution of Boron trifloride – 12 - 15 % m/m, 14 and 50 % solutions are commercially available. The metahnolic solution of boron trifloride should be stored in a refrigerator

(6)Heptane- Chromatographic quality

(7)Redistilled petroleum Ether 40 – 60°C

(8)Anhydrous Sodium sulphate.

(9)Saturated solution of Sodium chloride.

(10)Methyl red – 1 gm / litre in 60 % alcohol

21.4 Procedure:

Prepare the methyl esters of the fatty acids. The method using boron trifluoride gives good results and is preferable to alternative methods which may be used when boron trifluoride is not available. Because of the toxic character of boron trifluoride various operations must be performed under a ventilated hood. All glass ware must be washed with water immediately after use. If the oil or fatty acids include fatty acids containing more than 2 double bonds it is advisable to purge the air from the methanol and the flask by passing a stream of nitrogen into the methanol for a few minutes.

Transfer about 350 mg of clear oil to a 50 mL conical flask, and add 6 mL of 0.5 N methanolic sodium hydroxide solution, 7 mL of boron trifluoride solution and a boiling chip. Fit the condenser to the flask. Boil under reflux until the droplets of oil disappear (5- 10 minutes). Add the appropriate amount of boron trifluoride solution with a bulb or automatic pipette through the top of the condenser. Continue boiling for 2 minutes. Add 2- 5 mL of heptane to the boiling mixture through the top of the condenser. Continue boiling for 1 minute. Withdraw the source of heat and then remove the condenser. Add a small amount of saturated Sodium Chloride solution to the flask in order to bring the level of liquid into the neck of the flask.

Transfer about 1 mL of the upper layer (Heptane solution) into a test tube with a ground glass neck and add a little anhydrous Sodium Sulphate to remove any trace of water. This solution will contain about 5 – 10 % of methyl esters and may be injected directly into the column of gas liquid chromatograph.

21.4a –Alternate method for preparation of methyl esters:

Methyl esters can also be prepared without the use of boron trifluoride. This involves methyl esterification of the fatty acids in an alkaline medium and is suitable for neutral oils and fats with an acid value less than 2.

Reagents:

(1) Methanol containing not more than 0.5 % water

(2) Methanolic potassium hydroxide solution – approx 1 N. Dissolve 5.6 gm Potassium hydroxide in 100 mL of methanol containing not more than 0.5% m/m water (anhydrous methanol)

(3) Heptane chromatographic quality

(4) Anhydrous Sodium Sulphate

(5) Nitrogen, containing not more than 0.5 mg/Kg of oxygen

Procedure:

If the oil includes fatty acids containing more than 2 double bonds, it is advisable to purge the air from the methanol and the flask by passing a stream of nitrogen into the methanol for a few minutes.

Transfer about 4 gm of clear sample oil into a 100 mL round bottomed or conical flask. Add about 40 mL of methanol, 0.5 mL of methanolic Potassium hydroxide solution and a boiling chip. Fit under a reflux condenser, stir and bring to boil. The solution should become clear (5-10 minutes). Cool under running water

and transfer the contents to a 125 mL separating funnel, rinsing the flask with 20 mL of heptane.

Add about 40 mL water, shake and allow to separate. The esters pass into the upper heptane layer. Separate. Extract the aqueous layer again with 20 mL heptane.

Combine the two extracts and wash them with several 20 mL portions of water. Separate and dry the ester solution over anhydrous Sodium sulphate. Filter through cotton wool into a 50 mL conical flask and evaporate solution to approx 20 mL on a water bath while passing a stream of nitrogen.

21.5 Determination of fatty acids:

Programme GC to maintain column temperature of 185°C and detector temperature at 200°C. Inject 0.1 – 2 µL of 5- 10% of heptane solution of methyl esters by piercing the septum of the inlet port. Withdraw needle and note formation of a small peak on the chart paper due to solvent making start reference point. It is possible to work with lower column temperature where the determination of acids below C_{12} is required or higher temperature when determining fatty acids above C_{20}.

It is also possible to employ temperature programming to take care of both situations. Analyse reference standard mixture of known composition in the same operating conditions as those employed for the sample and measure the retention distances or retention times for the common fatty esters. Identify the peaks for the sample from the graph. If an integrator is used obtain the figures from it. Fatty acids appear on the chart in increasing number of carbon atoms and increasing unsaturation. Thus C_{16} appears before C_{18}, $C_{18:1}$ before $C_{18:2}$ and so on.

(Ref : - IUPAC 2.301 , 2.302 (1979)/FAO Manuals of Food quality Control 14/8 , pages 274 – 281/AOAC 17th edn , 2000 Official method 969.33 and 969.22 Fatty acids in oils and fats Preparation of methyl esters/Gas chromatographic method)

22.0 TEST FOR THE PRESENCE OF ANIMAL BODY FAT IN VEGETABLE FAT

22.1 Microscopic examination of fat crystals

22.1.1 Principle:

Animal body fats such as beef tallow and lard have been shown to contain trisaturated glycerides. On crystallization these glycerides exhibit a characteristic crystalline appearance when viewed under microscope. The procedure recommended by Williams Sutton for the microscopy of fat crystals have been suitably modified and given.

22.1.2 Procedure:

Take about 2 gm of melted fat samples in test tubes and mix with 10 mL diethyl ether. Plug the tubes with cotton and allow to stand for 30 min in ice water for 24 hours at 20°C (slow crystallization gives bigger crystals). In certain cases it is preferable to first crystallize with a stronger solution of fat from a mixture of ether and ethyl alcohol (1:1). In such cases separate the crystals by filtration and recrystallise in ether. Place the crystals on a drop of glycerine previously taken on a microscopic slide. Cover the crystals immediately with cover glass. Examine the crystals under x 160 and finally x 400 magnifications. The typical appearance of beef tallow crystallized into characteristic fan like tufts, the ends of which are more or less pointed can be seen. Lard crystals are of chisel shaped. Hydrogenated fats deposit smaller size crystals. The size and shape of the crystals depend upon the strength of solution, amount of fat taken and the time allowed for crystallization.

(Ref: -Manual Methods of Analysis for Adulterants and Contaminants in Foods ICMR (1990) page 6)

22.2 Separation of Cholesterol by Reversed Phase Thin Layer Chromatography

22.2.1 Principle:

A preliminary separation of total sterols from the unsaponifiable matter is achieved on silica gel-G thin layer chromatography. Subsequently the sterols are separated by reversed phase chromatography on Kieselghur-G using liquid paraffin as stationary phase and aqueous acetone saturated with liquid paraffin as the mobile phase.

22.2.2 Procedure:

Separation of total sterols from unsaponifiable matter:

Extract the unsaponifiable matter from the fat sample as per the method described elsewhere. Evaporate the ether and dissolve the residue in 5 mL of chloroform.

Prepare 20 × 20 cm glass plates coated with 0.5 mm thick silica Gel-G (BDH). Air-dry the plates and activate at 110°C for 2 hours. Cool the plates to room temperature spot the unsaponifiable matter along with the standard cholesterol on the plate. Develop the plates in diethyl ether: petroleum ether (1: 1) solvent system. Remove the plates when the solvent front reaches 14 cm height (it takes about 30 minutes).

Air-dry the plates and expose to iodine vapors for a while. Total sterols spot corresponding to standard spot of cholesterol appear as brown colour spots. Mark spots and scrape off with stainless steel blade into a test tube. Extract the sterols using chloroform from silica gel. Separate the sterols by reversed phase thin-layer chromatography.

22.2.3 Preparation of equilibrated aqueous acetone with liquid paraffin:

Take 300 mL of 4: 1 acetone: water in a separating funnel and add 30 mL of liquid paraffin (heavy grade). Shake well and keep for 18 hours at room temperature for equilibration. Separate the lower layer of liquid paraffin and dilute to 5 per cent proportion with petroleum ether. Use this for treating Kieselghur-G coated thin-layer chromatographic plates. The upper acetone-water mixture serves solvent system to develop the paraffin treated plates.

22.2.4 Preparation of the plates for reversed phase TLC/HPTLC:

Coat 20 × 20 cm glass plates of 0.5 mm thick layers with Kieseghur-G and water (1:2) slurry. Air-dry the plates and then activate at 110°C for an hour. Cool the plates to room temperature in a desiccator. For treatment of TLC/HPTLC plates with liquid paraffin, carefully dip the plate holding horizontally for a few seconds in a tray containing 5 percent liquid paraffin solution in petroleum ether as described above. Air-dry the plates.

Spot the sterols in chloroform isolated from unsaponifiable matter by a preliminary separation on silica gel-G thin layer chromatography on paraffin treated plates along with standard cholesterol. Develop the plate using the solvent system of acetone: water (4:1) which was earlier equilibrated with paraffin. After the solvent front has ascended to a height of 15 cm remove the plate and air-dry. Spray with p-Anisaldehyde reagent (1.5 g. p-anisaldehyde and 1.5 mL concentrated sulphuric acid in 27 mL ethyl alcohol) followed by heating at 110°C for 5 minutes The sterol spots appear as blue spots on pale pink background. Cholesterol appears at R_f 0.48 distinctly separated from other closely related sterols.

(Ref: - Manual Methods of Analysis of Adulterants and Contaminants in Foods, ICMR (1990) Page 7)

22.3 Test for presence of animal body fat in vegetable fat based on the presence of unusual fatty acids in animal fats by gas liquid chromatography

22.3. 1 Preparation of fatty acid methyl esters:

Take 30 to 50 mg of melted fat (1 drop) in a glass-stoppered test tube and add 1 mL of dichloromethane/benzene followed by 2 mL of 1% sodium methoxide solution (1gm sodium dissolved in 100 mL of anhydrous methanol). Hold the test tube at 60°C for 10 minutes Cool and add 0.1 mL of glacial acetic acid followed by 5 ml of distilled water and 5 mL petroleum ether (40- 60°C). Mix the contents. Allow the layers to separate. Take out about 2 mL of the upper layer containing the methyl esters in a small tube and concentrate it by passing nitrogen gas before injecting to gas chromatograph.

22.3.2 Gas chromatography:

The instrument fitted with flame ionisation detector and stainless steel column of 10 ft packed with 15% diethylene glycol succinate on C W (80-100 mesh), or any other intermediate polar stationary phase column. Maintain the column temperature at 185°C, flow rate of carrier gas nitrogen at 2.8 kg/cm^2 (25 mL/min) and chart speed at 1 cm/minutes.

The fatty acid composition of animal fat (beef tallow) and vanaspati containing animal fat show the presence of odd chain fatty acids and branched chain fatty acids namely $C_{15:\ 0}$, $C_{15:\ 1}$, $C_{17:\ 0}$ and $C_{17:\ 1}$. These fatty acids are absent in vegetable fats. On the basis of this fact it is possible to detect the presence of animal fat (beef tallow) in vegetable fats.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods, ICMR (1990) Page 8)

23.0 TEST FOR REFINED WINTERIZED SALAD OILS – COLD TEST

23.1 Procedure:

Fill 4 oz (100 mL) sample bottle with oil, cork tightly and seal with paraffin. Completely submerge bottle in bucket containing finely cracked ice and add water until it rises to top of the bottle. Keep bucket filled solidly with ice by removing any excess water and adding ice when necessary. After 5.5 hours remove bottle and examine oil. If it is properly winterised, sample will be brilliant, clear and limpid.

(Ref: - AOAC 17th edn, Official method 929.08 Salad oils (refined, winterized))

24.0 TEST FOR PRESENCE OF TEASEED OIL IN OLIVE OIL

24.1 Principle:

The test is based on the development of red colour by acetic anhydride in the presence of sulphuric acid when a solution of oil in Chloroform is taken (Fitelson Test)

24.2 Apparatus:

1) Test tubes - 150mm x 15 mm

2) Pipette – 2 mL, graduated to 0.1 mL

3) Dropper so calibrated that 7 drops of oil weigh 0.22 gm

4) Water bath maintained at 50°C

24.3 Reagents:

1) Chloroform

2) Concentrated Sulphuric acid

3) Acetic anhydride

4) Diethyl ether, anhydrous peroxide free, stored over Sodium

24.4 Procedure:

Pipette into a test tube 0.8 mL of acetic anhydride, 1.5 mL of Chloroform and 0.2 mL of Sulphuric acid. Cool to 5°C and add approximately 0.22 gm (7 drops) of oil. If any turbidity appears, add acetic anhydride drop by drop with shaking until the solution becomes clear. Keep at 5°C for 5 minutes. Add 10 mL of Diethyl ether previously cooled to 5°C. Stopper the test tube and mix thoroughly by inverting it twice. Return the test tube to the bath at 5°C. An intense red colour which develops about a minute after the addition of ether, reaches a maximum and disappears indicates pure teaseed oil. A less intense colour indicates presence of teaseed oil but caution must be exercised in interpreting results in the presence of olive oil. The test is generally applicable, but some olive oils yield a pink colour and the test is therefore not reliable for the detection of less than 15% of teaseed oil in olive oil.

(Ref: - FAO Manuals of Food Quality Control 14 / 8, page 273 / AOAC 17th edn, 2000, Official Method 936.12 Oil (Teaseed) in olive oil)

25.0 TEST FOR PRESENCE OF OLIVE RESIDUE (POMACE) OIL IN OLIVE OIL

25.1 Principle:

The test is based on the temperature of precipitation of salts of fatty acids after saponification.

25.2 Preparation of sample:

The sample is filtered through paper at a temperature slightly above the melting point of certain solid constituents which could separate from the fluid fatty matter.

25.3 Procedure:

Saponify 1 gm of oil by boiling for 10 minutes with 5 mL alcoholic KOH (42.5 gm KOH in 72 mL water made up to 500 mL with 95% ethyl alcohol). After cooling add 1.5 mL aqueous acetic acid (1+ 2 by volume such that 1.5 mL exactly neutralizes 5 mL of aqueous alcoholic KOH) and 50 mL of 70% ethanol warmed to 50°C. Mix, insert a thermometer and allow to cool. If a precipitate forms above 40°C the test for the presence of olive residue oil is positive. Allow to cool to ambient temperature for 12 hours. Observe solution again. The formation of a flocculent precipitate floating in the middle of the liquid also indicates that the test is positive. A cloudiness not forming into flakes does not indicate the presence of olive residue oil.

(Ref:- Pearsons Composition and Analysis of Foods 9th edn, page 619 / Codex Alimetarious Commission – recommended method 22 -1970)

26.0 TEST FOR SEMI- SICCATIVE OIL IN OLIVE OIL

26.1 Principle:

The test is based on the reaction between semi- siccative (unsaturated) oils and bromine yielding substances, which form an insoluble precipitate at 0°C.

26.2 Apparatus:

(1) Stoppered 50 ml Erlenmeyer flask

(2) Bath of melting ice

26.3 Reagents:

(1) Hexane or Petroleum ether (40-60 degree C free from any residue)

(2) Bromine solution prepared by adding drop by drop while shaking 4 mL of pure Bromine (the presence of Chlorine prevents the reaction) into100 mL of Hexane or Pet. Ether chilled at 0°C and kept in the melting ice bath until required.

26.4 Procedure:

The oil to be tested is filtered and dried. Place 1 mL of oil in a previously dried Erlenmeyer flask and dissolve in 10 mL Hexane. Place the stoppered Erlenmeyer flask in the melting ice bath. After 5 minutes add 10 mL of bromine solution in small quantities at a time while shaking and maintaining the temperature at 0°C.

The colour of the solution must clearly indicate excess of bromine. Leave the Erlenmeyer flask in melting ice for 1 hour, after which note appearance of the solution. If semi- siccative oil is present a flocculent precipitate will form varying in quantity according to the % of adulteration and the nature of adulterant oil. The solution remains clear and transparent in the case of genuine olive oil.

(Ref: - Codex Alimentarious Commission – Recommended method 21 – 1970)

27.0 DETERMINATION OF 9, 10 EPOXY AND 9, 10 DIHYDROXY STEARIC ACID IN SALSEED FAT

Two methods have been prescribed namely

(1)Gas Liquid Chromatography Method and

(2) Preparatory TLC/HPTLC method

The GLC method is to be used as a reference method

27.1 Gas Liquid chromatography method

The method consists of enrichment of triglycerides containing 1 mole of 9, 10 epoxy Stearic acid and 2 moles of predominantly Stearic acid (designated as P) and other triglyceride containing 9, 10 dihydroxy stearic acid in place of epoxy stearic acid (designated as Q) from a known mass of salseed fat by treatment with silicic acid in Hexane and desorbing these by a more polar solvent. The desorbate to which

a known quantity of internal standard is added, is subjected to transmethylation and the methyl esters after silylation are analysed by GLC.

27.1.1 Procedure:

(1) Enrichment of 'P' and 'Q' and transmethylation:

Dissolve 200 mg of refined salseed fat in 2 mL of n – hexane and stir with 600 mg of silicic acid (activated at 110°C for 1 hour before use) for 1.5 hours using a magnetic stirrer. Pipette out the supernatant Hexane and wash the residue with additional 2 mL of hexane and discard the hexane. Add 2 mL of chloroform methanol (3:1 v/v) to the residual silicic acid and stir for 45 minutes. Filter the contents and wash the residue with additional 2x2 mL chloroform- methanol (3: 1) mixture to ensure complete desorption of the adsorbed material. Add a known amount of about 2 mg of n – heptadeconoic acid methyl ester to the combined chloroform- methanol filtrate contained in a 10 mL round bottom flask and evaporate solvent to dryness under a stream of nitrogen. Dissolve the residue in 1 mL of dichloromethane and retreat it with 2 mL of 2M solution of sodium methoxide in methanol and keep at 50°C for 15 minutes with occasional shaking.

During this treatment connect the flask to a condenser and calcium chloride guard tube. Dilute the contents with 2 mL of saturated sodium chloride solution and extract with n – hexane (3 x 2 mL) in a separating funnel. Wash the combined hexane extract with water to ensure complete removal of alkali (by pH paper), dry over anhydrous sodium sulphate and evaporate to dryness under nitrogen.

(2) Silylation:

Transfer the final residue to a 5 mL screw capped vial with the aid of ether and subsequently remove ether by evaporation under nitrogen. Dissolve this in 0.2 mL of dry pyridine and treat with 0.1 mL of hexamethyldisilazane and 0.05 mL of chlorotrimethylsilane and keep at room temperature for 1 hour. Evaporate the reagents to dryness on a waterbath at about 50°C under nitrogen.

27.1.2 GLC analysis:

Dissolve the residue obtained above in hexane and inject into GLC column. The instrument should have a flame ionization detector and a 2.5 metre x 4 mm glass column packed with 1% OV 17 on 80-100 mesh Gas Chrosom Q. The temperature of oven and detector shall be 195°C. The carrier gas shall be Nitrogen with a flow rate of 60 mL/min and the chart speed shall be 25 cm/hours.

27.1.3 Calculation:

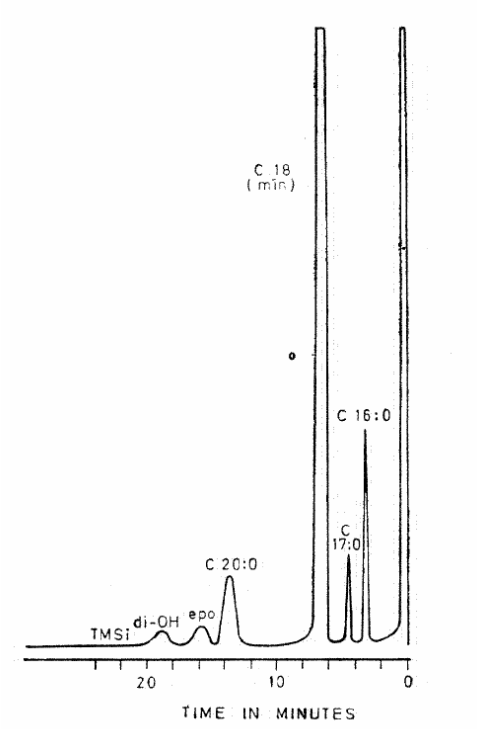
$$P\% = \frac{\text{Peak Area of epoxy Acid Methyl ester}}{\text{Peak Area of the internal standard}} \times \text{Amt of internal standard (in mg)} \times \frac{904}{312} \times \frac{100}{\text{Mass of sample (in mg)}}$$

$$Q\% = \frac{\text{Peak Area of dihydroxy Methyl ester}}{\text{Peak Area of the internal standard}} \times \text{Amt of internal standard (in mg)} \times \frac{922}{330} \times \frac{100}{\text{Mass of sample (in mg)}}$$

Note: - The GLC method of 'P' and 'Q' estimation is applicable to refined fats or fats with FFA less than 2%. In case of high FFA, fat neutralization should precede transesterification. For this purpose spray a small quantity of fat with 3 N sodium hydroxide (10 % excess) containing 10 % sodium chloride at 50 – 60°C under gentle stirring. After allowing the soap to settle for a while, transfer the material to a tube and centrifuge. Wash the supernatant oil free of soap and take for trans-esterification.

Epoxy and dihydroxy fatty acids % by mass = $\underline{P + Q}$

3



Typical GLC Scan

27.2 Preparative Thin Layer chromatography method

'P' and 'Q' are separated on preparatory TLC/HPTLC plates and the bands scraped, extracted with solvent, evaporated and weighed. The method is applicable to fats containing approximately 3% each or more of 'P' and 'Q'. Fats containing lesser proportion of 'P' and 'Q' need enrichment prior to preparative TLC/HPTLC in order to get reasonable amounts of 'P' and 'Q' for weighing. The method works well with refined fats or raw fats with F.F.A. upto 3 % or less but fats with high FFA should be refined as per Note under GLC method.

27.2.1 Procedure:

Weigh accurately about 10 gm of salseed fat and dissolve in 100 mL of Hexane and stir with 20 gm of silicic acid (B.D.H Lab reagent suitable for lipid chromatography activated at 110°C for 1 hour before use) for 4 hours on a magnetic stirrer. Filter the slurry using a Buchner funnel and distill the filtrate to obtain the

normal triglycerides. This fraction does not show the presence of 'P' and 'Q' when 2 mg of material is chromatographed on a TLC/HPTLC plate indicating that the 'P' and 'Q' are completely adsorbed.

Note: - in case of silicic acid from other sources it is necessary to do the TLC/HPTLC test to ensure absence of 'P' and 'Q' in the hexane extract and to arrive at the appropriate fat: silicic acid ratio. A ratio of 1: 1 is suitable.

Stir the residual silicic acid with 100 mL of chloroform for 1 hour and filter. Repeat the operation with fresh lot of 100 mL of chloroform. Distill the combined filtrate to obtain the enriched fraction.

Dissolve a known mass (80- 90 mg) of the enriched fat in 0.5 mL of Chloroform and apply as a streak on a preparatory plate of 1 mm thick silica gel and develop 4 such plates using hexane, ether and acetic acid (60 : 40 : 1) as a solvent system.

Visualise the bands in an Iodine chamber and scrape the bands at R_f at 0.84 and 0.28 corresponding to 'P' and 'Q'. Transfer quantitatively into two separate thimbles and extract with chloroform in a soxhlet. It takes about 1 hour for extraction.

After extraction, distill the chloroform and transfer the residue carefully through a Whatman filter paper No 42 or equivalent to a tared 5 mL round –bottom flask using ether. Wash the filter paper thoroughly with ether and collect the washings in the same flask. Evaporate ether under a stream of Nitrogen and weigh the flask to a constant mass by keeping it in the oven at 105°C.

27.2.2 Calculation:

Calculate the amount of 'P' and 'Q' in the original fat from the mass of the residue. Express the results as %age of epoxy and dihydroxy fatty acids by dividing the sum of 'P' and 'Q' by 3.

Note 1:- The specified solvent system (Hexane: Ether: Acetic acid (60:40:1) is satisfactory for resolving 'Q' but the resolution of 'P' from normal triglycerides is occasionally poor and appears to depend upon the activity of silicagel. The close Rf values of 'P' and normal triglycerides is then likely to lead to errors in estimation of 'P'. In such cases a slightly less polar solvent (hexane: ether: acetic acid 80:20:1) will lead to a good resolution. 'P' and 'Q' should then be determined separately using appropriate solvent system.

Note 2:- Generally the pattern of separation of various constituents in descending order of Rf on the plate is as follows:-

i. Normal triglycedides

ii. 'P'

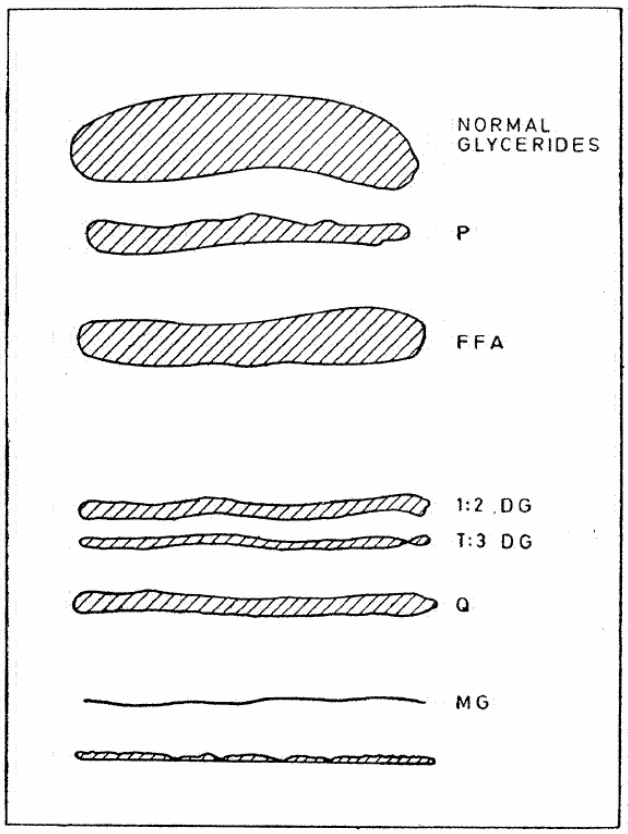
iii. FFA

iv. 1,2 di-glycedides

v. 1,3 di-glycerides

vi. 'Q'

vii. Monoglycerides



A typical chromatogram of Salseed Fat in Hexane; Ether; Acetic Acid (60:40:1)

(Ref: - IS 7375 – 1979 Specification for Salseed fat)

28.0 TEST FOR PRESENCE OF MINERAL OIL

Two methods are used to detect mineral oils in edible oils.

Method A - Holde's test

Method B - TLC/HPTLC test

Method A is for rapid detection of mineral oil in vegetable oils and fats. It is sensitive when mineral oil is present to the extent of 1 % or more. The test is not

sensitive in the case of oils with high content of unsaponifiable matter. Method B shall be used where confirmation is required.

28.1 Method A (Holde's Test)

28.1.1 Principle:

The presence of mineral oil is indicated by the development of turbidity when hot distilled water is added to a freshly made alcoholic solution of the soap formed by the oil.

28.1.2 Apparatus:

(a) Conical flask (100 mL) with standard joint

(b) Air condenser/Water Condenser to fit above

28.1.3 Reagent:

Alcoholic potassium hydroxide solution, 0.5 N

28.1.4 Procedure:

Take 25 mL of the alcoholic KOH solution in a conical flask and add 1 mL of the sample of oil to be tested. Boil on a water bath using an air or water cooled condenser till the solution becomes clear and no oily drops are found on the sides of the flask. Take out the flask from the water-bath, transfer the contents to a wide mouthed warm test tube and carefully add 25 mL of boiling distilled water along the sides of the test tube. Keep on shaking the tube lightly from side to side during the addition. The turbidity indicates presence of mineral oil, the depth of turbidity depends on the percentage of mineral oil present.

(Ref: - ISI Handbook of Food Analysis (Part XIII)-1984 Page 89/ AOAC 17th edn, 2000, Official Method 945.102 – Oil (mineral) in fats – Qualitative Test)

28.2 Method B (Thin Layer Chromatographic test)

28.2.1 Principle:

Being non-polar, mineral oils give faster moving spots on thin layer chromatographic plates, than the triglycerides.

28.2.2 Apparatus:

a. Glass slides (7.6 x 2.5 cm) or glass plates of 20 x 5 cm or 20 x 10 cm may be used

b. Developing tank

c. Ultra-violet lamp (365 nm). This should be placed in a darkened enclosure

28.2.3 Reagents:

a. Silica-gel 'G' with calcium sulphate as binder (commercially available)

b. Petroleum ether

c. Spray reagent: 0.2 % solution of 2', 7'-dichloro-fluoresein in 95 % ethanol

28.2.4 Procedure:

Hold two slides together face to face and dip them in a slurry of silica gel G (45gm) in a mixture of chloroform and methanol (80 + 20 mL). Withdraw the slides, separate them and allow drying in air and activating at 110°C for 15 minutes and cooling in a desiccator. Apply 10 mL of a 10 % solution of oil in chloroform on the glass slide/glass plate using a capillary tube. Allow to dry and place the slide in a developing tank containing petroleum ether. Cover the tank and allow the solvent to travel for 6 cm from the origin (about 4 minutes). Remove the plate from the tank, dry in air, spray with the fluorescein solution and view under UV light.

Appearance of a yellow fluorescent spot on the solvent front indicates the presence of mineral oil. The vegetable oil forms a yellow streak about 2-3 cm long from the point of spotting.

Note: If desired, a standard sample containing 1 % by mass of liquid paraffin in a sample of pure oil under test may be prepared and tested simultaneously as reference sample.

(Ref: - ISI Handbook of Food Analysis (Part XIII)-1984 Page 89)

29.0 TEST FOR PRESENCE OF CASTOR OIL

29.1 Principle:

'Triricinolein' a characteristic and predominate triglyceride component of castor oil is separated on silica gel TLC/HPTLC and visualized by iodine vapours.

29.2 Apparatus:

a) Separating funnel

b) Slides: microscopic slides (7.6 X 1.5 cm) or glass plates of 20 X 5 cm or 20 X10 cm may be used

c) Developing tank: a tall beaker of at least 10 cm height/TLC/HPTLC developing chamber

d) Visualisation tank (Iodine chamber): A dry beaker or developing tank saturated with iodine vapour by placing a few crystal at the bottom and leaving for an hour

e) Beaker: 25 mL

29.3 Reagents:

(i) Absolute Alcohol

(ii) Silica gel containing 15 % Calcium sulphate as binder (silica gel G) passing 75 micron IS sieve

(iii) Developing solvent: Hexane: Solvent ether (1:1)

(iv)Standard castor oil solution – 1 % castor oil dissolved in 100 ml absolute alcohol

29.4 Procedure:

Coat microscopic slides or TLC/HPTLC plates with a slurry of silica gel G and water (1:2) with the help of an applicator. Activate at 110°C for one hour. Cool and keep in a desiccator. Take 10 mL of suspected oil in a separating funnel and add 10 mL of absolute alcohol. Shake vigorously for one minute and allow to separate the two layers. Discard the lower oil layer and draw of the upper alcohol layer into a 25 ml beaker. Concentrate alcohol extract to about 2 mL. Spot 10 µL of alcoholic extract and 10 µL of standard Castor oil solution on TLC/HPTLC plate and develop in developing tank containing Hexane: Solvent ether (1:1) upto 15 cm. Air dry the plate and put in iodine chamber. Occurrence of a spot at R_f of about 0.25 shows presence of castor oil. All other spots will be above this. The spot shall be noticed in the visualization tank since it fades on removing. This method has a sensitivity of one per cent.

This method is specific for castor oil, but rancid or oxidized oils give spots with the R_f values similar to those given by Castor oil. Hence, care should be taken when applying the TLC/HPTLC test to rancid oil and interpretation of result. In such cases the rancid oil has to be purified by "refining" as described below.

29.5 Detection of Castor Oil in rancid oils

The suspected rancid oil (5 mL) may be taken in a round bottom flask and treated with activated charcoal (2 gm). The contents are mixed thoroughly and heated on boiling water bath for about 30 min with constant shaking. The bleached oil is filtered to separate the charcoal. The filtered oil may now be passed through a mini column packed with neutral alumina (10 gm) using hexane (50 mL) as eluent.

This bleached and neutralized oil may be spotted on the TLC/HPTLC plate for detecting presence of castor oil as above.

(Ref: - ISI Handbook of Food Analysis (Part XIII) –1984 Modified test for presence of Castor oil, page 91)

30.0 TEST FOR PRESENCE OF ARGEMONE OIL

Argemone (*Argemone mexicana L.*), yellow poppy, is a wild herb, which grows in mustard field and bears capsules full of brown black seeds.

Because of its resemblance with black mustard, it is often used as an adulterant.

The oil is reported to cause glaucoma, dropsy and sometimes total blindness due to the presence of alkaloids namely, sanguinarine and dihydrosanguinarine.

30.1 Principle:

The hydrochloric acid extract of the oil sample containing argemone oil when subjected to TLC/HPTLC for separation of alkaloid gives fluorescent spot under UV light.

30.2 Apparatus:

a) TLC//HPTLC plates coated with silica gel G or precoated ready-made plates cut to suitable size

b) Ultraviolet lamp (long wave – 366 nm) in a visualization chamber

c) Pear-shaped flask

d) Hot water bath

e) Separating funnel – 50 mL capacity

f) Glass beaker – 10 mL capacity

30.3Reagents:

i) Solvent mixture (mobile phase)

a) Butanol : Acetic acid : water 70:20:10 (v/v)

b) Hexane or Heptane: Acetone 60:40 (v/v)

ii) Diethyl ether

iii) Hydrochloric acid, cons. Sp. Gr. 1.19

iv) Chloroform: Acetic acid (90 : 10 v/v) mixture

v) Aqueous sodium hydroxide solution 1 N

vi) Standard Argemone oil extract

30.4 Procedure:

Take 10 mL sample in a separating funnel and dissolve in 15 mL Diethyl ether. Add 5 mL concentrated Hydrochloric acid and shake vigorously for 2 – 3 minutes. Allow to separate.

Contents of the separator funnel may be heated cautiously over the vent of heating water bath for some time for quick separation. Transfer the acid layer to a 25 mL beaker. Place the beaker into a boiling water bath and evaporate till dryness.

Dissolve the residue obtained after evaporation of hydrochloric acid in 1 mL of a mixture of chloroform and acetic acid 9:1) and spot on TLC/HPTLC plate with the help of spotting capillary. Spot side by side standard Argemone oil extract (0.1 % in ether). Develop the plate in (a) Butanol: Acetic acid: water; or (b) Hexane: Acetone mixture. Allow the solvent front to move up a distance of 10 cm and allow the plate to dry. Place the plate under UV light in the visualization chamber.

Bright yellow or orange yellow fluorescent spots having R_f similar to the standard argemone oil will confirm presence of argemone oil. The spot gives blue fluorescence under UV-light if plate is sprayed with 1% aqueous sodium hydroxide solution.

The method is very sensitive and can detect argemone oil upto 50 ppm level.

(Ref: - Manual methods of Analysis for Adulterants and Contaminants in Foods ICMR (1990) page 12)

31.0 TEST FOR PRESENCE OF KARANJA (*PONGAMIA GLABRA*) OIL

31.1 Principle:

Extraction of glabrin, karanjin, karanjone, pongaglabrone and pongamol using concentrated hydrochloric and their detection on TLC /HPTLC UNDER ultra-violet light.

31.2 Apparatus:

a) All-glass separating funnel (100 mL capacity)

b) Glass beaker (50 mL capacity)

c) Measuring cylinder for separating funnel

d) Wooden stand for separating funnel

e) Hot water-bath

f) Capillary tubes

g) TLC/HPTLC plates (0.25 mm). Prepared by coating a slurry of silica gel G on glass plate of 10 x 20 cm diameter, activated at 110°C for 1 hour and stored in a desiccator.

h) Ultra-violet lamp long wave (366 nm) in a visualization chamber.

31.3 Reagents:

i) Hydrochloric acid AR Sp. Gr. 1.18

ii) Solvent Mixture as mobile phase, petroleum ether: diethyl ether:acetic acid 60 : 40 : 1 (v/v)

iii) Standard Karanja oil extracts (1.0 % oil in any other oil extracted simultaneously with the sample)

31.4 Procedure:

Take 20 mL of the suspected oil in a 100 mL capacity separating funnel and add to it 10 mL concentrated hydrochloric acid. Shake the content gently, but consistently for 15 minutes. Keep the separating funnel on a wooden stand for about 30 minutes to allow the separation of acid layer. Draw out the acid layer in a glass beaker. Keep the beaker on a boiling water bath and evaporate the hydrochloric acid till dryness.

Dissolve the residue in 0.5 mL of chloroform. Spot the chloroform solution on a pre-activated TLC/HPTLC plate with the aid of capillary tube. Spot standard Karanja oil extract side by side. Develop the plate in solvent system petroleum ether: diethyl ether: acetic acid 60:40:1 v/v for 20 minutes

Remove the plate, dry at room temperature and view under ultra-violet lamp. Appearance of three bluish green spots at R_f 0.34, 0.22 and 0.17 confirms the presence of Karanja oil.

Note: - The test is sensitive to the extent of 0.01 % Karanga oil.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants ICMR (1990) page 12)

32.0 TEST FOR PRESENCE OF HYDROCYANIC ACID

32.1 Principle:

Hydrocyanic acid is sometimes present as an impurity in synthetic allyl-isothiocyanate which is commonly used as an adulterant to enhance the flavour of poor quality mustard oil. Two methods have been prescribed for the purpose of this test. Method A shall be used as referee method and method B as routine method.

32.2 Method: A

The hydrocyanic acid in the oil when heated over water bath is displaced by bubbling air and is absorbed in potassium hydroxide solution. The cyanide is then tested with ferric chloride solution.

32.2.1 Reagents:

(a) Potassium hydroxide solution - approximately 2N

(b) Lead acetate solution - approximately 2 N

(c) Ferrous sulphate solution - approximately 2%

(d) Hydrochloric acid

(e) Ferric chloride solution - 20 % (w/v) in water to which sufficient hydrochloric acid has been added to prevent hydrolysis.

32.2.2 Procedure:

Heat about 50 mL of the oil in a distillation flask by placing it on a water bath.

During heating pass through the oil for about 30 min, air which has been purified by scrubbing through solution of potassium hydroxide and lead acetate. Connect the distillation flask to an absorption tube containing 5 mL of potassium hydroxide solution. The air bubbling through the oil carry with it the hydrocyanic

acid and this is absorbed by the potassium hydroxide solution. Shake the solution with few drops of ferrous sulphate solution, acidify with few drops of hydrochloric acid and warm gently for 5 minutes. Filter and add a few drops of ferric chloride solution. A blue or bluish-green colour or precipitate in the solution indicates the presence of cyanide.

32.3 Method: B

This method is based on the reaction of hydrocyanic acid on picric acid paper which acquires a red colour.

32.3.1 Reagents:

(a) Picric acid paper: Soak a filter paper (Whatman No. 1 or equivalent) in a saturated aqueous solution of picric acid, draining the excess liquid and drying the dyed paper in air.

(b) Tartaric acid solution - 10 % (w/v)

(c) Sodium carbonate solution - 5 % (w/v)

32.3.2 Procedure:

Pour 30 mL of the oil into a 250 mL conical flask and mix well with about 50 mL of water. Add 15 mL of 10 % tartaric acid solution and mix. Stopper the flask, with a velvet cork from which hangs a picric acid paper (about 7.5 cm long) previously wetted with a drop of 5% sodium carbonate solution. The flask is placed on a hot water bath by the side of the steam vent and not directly on the steam for 30 to 45 minutes in presence of hydrocyanic acid, the picric acid paper acquires red colour. Ignore pink or light reddish hue which may, at times, appear at the periphery of the picric acid paper.

(Ref: - ISI Handbook of Food Analysis (Part XIII) – 1984, page 88)

33.0 TEST FOR PRESENCE OF TRICRESYL PHOSPHATES AND DETERMINATION OF TRI-O-CRESYL PHOSPHATE IN EDIBLE OILS

33.1 Principle:

Tricresyl phosphate in contaminated edible oils is extracted using acetonitrile and detected by thin-layer chromatography as well as gas liquid chromatography.

33.2 Apparatus:

(a) Separatory funnels - 250 mL capacity

(b) TLC/HPTLC Plates - Prepare slurry of silica gel G with water (1:2 w/v) and spread over glass plates (0.3 25mm layer on 20 x 20 cm plates) with applicator. Let the plates set at room temperature. Activate at 110°C for 1 hour, cool and store in a desiccator.

(c) Gas Chromatograph - Fitted with flame ionization detector; stainless steel column (10' x 1/8') packed with 10% OV - 101 on 60 to 80 mesh Chromosorb-AW-DMCS; nitrogen carrier gas 30 mL/min, column temperature 250°C, detector and injector temperature 300°C; chart speed 1 cm/minutes

33.3 Reagents:

i) Developing solvent - Iso-octane-ethyl acetate (90:10). Developing chamber linedwith filter paper

(ii) Spray reagent: 0.5% solution of 2, 6-dichloro-quinone chlorimide. A.R in absolute ethyl alcohol (Gibbs reagent). Store reagent at 10°C and use within 5 days

(iii) Standard Tricresyl phosphate (TCP) and tri-O-cresyl phosphate (TOCP)

33.4 Procedure:

Take 10 mL oil sample containing Ca. 50 µg TCP or TOCP into separatory funnels; add 50 mL petroleum ether (40 - 60°C) to dissolve the oil followed by 10 mL acetonitrile previously saturated with petroleum ether. Shake contents vigorously and let stand 10 minutes. Collect lower acetonitrile layer in beaker and evaporate solvent on hot water bath. Dissolve residue in Ca. 1 mL ethyl or methyl alcohol.

Thin layer chromatography: Spot ca 0.1 mL (Ca. 5 mg TOCP) of solution on TLC/HPTLC plate. Develop plate in glass chamber containing iso-octane ethyl acetate (90:10) ca. 45 minutes to a height of 10 cm. Remove plate and dry in air. Spray plate with Gibbs reagent and heat in 100°C oven Ca. 15 minutes Observe for characteristic blueviolet spot at R_f 0.27 corresponding to standard TCP or TOCP.

33.5 Gas chromatography:

Inject about 1 mg (2.5 mg TOCP) of acetonitrile extract of the oil sample into GC apparatus; compare retention time and peak area of sample with that of standard T.C.P or T.O.C.P for quantitation.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants in Foods ICMR (1990) page 14)

33.6 TLC/HPTLC Method based on alkaline hydrolysis of oil

33.6.1 Apparatus:

a) Conical flask 250 mL capacity fitted with air condenser

b) TLC plates 10x 20 cm or 20x 20 cm/HPTLC plates and a developing tank

c) Sprayer

d) Air oven

e) Pipette 5 and 50 mL capacity

f) Capillary tubes

33.6.2 Reagents:

(1)Dissolve 70 – 80 gm of KOH in an equal quantity of distilled water and add 2 litres of aldehyde free alcohol. Allow to stand overnight, decant the clear liquid and keep in a bottle closed tightly with cork or rubber stopper

(2)Alcoholic Potassium Hydroxide solution 1.5 N. Add 8.5 g KOH in 100 mL of aldehyde free alcohol

(3)Diazonium reagent - Dissolve 0.8 gm p-nitroaniline A.R) in 250 mL lukewarm water. Add 20 mL of 20 % HYDROCHLORIC ACID and mix properly to dissolve p-nitroaniline. Decant to remove any residual slick which remains. Cool and then add 50% Sodium Nitrite solution until reagent is completely colourless. Store in a refrigerator.

(4) Iso-octane

(5) Ethyl acetate-AR

(6) Tricresyl Phosphate Standard- Prepare a 0.5% solution of tricresyl phosphate in pure rapeseed oil

33.6.3 Procedure:

Weigh accurately 5 gm of sample in a conical flask and add 50 mL of alcoholic KOH. Take 15 mL of standard TCP solution in another flask and add 50 mL of alcoholic KOH. Fit both the flasks with air condenser and boil gently on water bath or hot plate for 1 hour or till saponification is complete.

Prepare a mixture of iso-octane ethyl acetate in the proportion of 90:10 as developing solvent. Spot 10-20 µL of saponified sample as well as standard with a

capillary tube and develop the plates in the solvent mixture for about 15 minutes so that the solvent front reaches 10 cm. Dry the plates and spray with 1.5N alcoholic KOH. Keep in the air oven at 60°C. Spray the plates with Diazonium reagent. Appearance of red spot at the same R_f as the standard sample confirms the presence of tricresyl phosphate.

(Ref: - IS Specification No. IS 548(Part II/ (Sec 22) 1993-Test for Detection of Tricresyl Phosphate in edible oils)

34.0 DETERMINATION OF PHOSPHOROUS IN SOYA BEAN OIL

34.1 Principle:

The method determines Phosphorous or the equivalent phosphatide by ashing in the presence of zinc oxide followed by spectrophotometer measurement of phosphorous as blue phosphomolybdic acid.

34.2 Apparatus:

(1) Platinum basins or crucibles suitable to withstand temperature of 600°C

(2) Electric hot plate and muffle furnace

(3) Watch glass 75 mm diameter and Funnel short stem 50 mm diameter

(4) Filter paper – ashless, Whatman No 42 or equivalent, 90 mm diameter

(5) Wash bottle – 1 litre for use with hot water

(6) Volumetric flasks – 50mL, 100 mL, 250 mL and 500 mL with glass stoppers

(7) Pipette – Mohrs type 10 mL with 0.1 mL subdivision

(8) Spectrophotometer with 1.0 cm cuvettes. For use in the visible region

34.3 Reagents:

(1)Concentrated Hydrochloric acid, sp. gr 1.18

(2) Zinc Oxide, reagent grade

(3) Potassium Hydroxide, reagent grade

(4) Concentrated Sulphuric acid, sp .gr 1.84

(5) Sodium molybdate, reagent grade

(6) Hydrazine sulphate, reagent grade

(7) Potassium dihydrogen phosphate, reagent grade dried for 2 hours at 101°C

34.4 Solutions:

(1)Sodium molybdate - Carefully add 140 mL of concentrated sulphuric acid to 300 mL distilled water. Cool to room temperature and add 12.5 gm of Sodium molybdate. Dilute to 500 mL with distilled water. Mix thoroughly and allow to stand for 24 hours before use.

(2) Hydrazine sulphate – 0.015% Dissolve 0.150 gm hydrazine sulphate in 1 litre water.

(3) Potassium Hydroxide – 50% solution Dissolve 50 gm KOH in 50 mL distilled water

(4) Standard Phosphate solution

(a)Stock solution – Dissolve 1.0967 gm of dry Potassium dihydrogen phosphate in distilled water and make up to 250 mL in a volumetric flask

The solution contains 1 mg phosphorous per mL

(b) Working Solution – Dilute 5 mL of standard stock solution with distilled water to 500 mL in a volumetric flask. This solution contains 0.01 mg phosphorous per mL.

34.5 Procedure:

Weigh accurately 3 – 4 gm of sample in a crucible or Pt basin, add 0.5 gm Zinc oxide and heat slowly on the hot plate until the sample thickens, then gradually increase the heat until the mass is completely charred. Place in a muffle furnace at 550 – 600°C and hold for 2 hours. Remove and cool to room temperature. Add 5 mL distilled water and 5 mL Hydrochloric acid to the ash. Cover the crucible with a watch glass and heat gently to boiling for 5 minutes. Filter the solution in a 100 mL volumetric flask. Wash the inside of the watch glass and the crucible with about 5 mL of hot water using a wash bottle with a fine stream of water. Wash the crucible and filter paper with 4 additional portions of hot distilled water.

Cool the solution to room temperature and neutralise to a faint turbidity by drop-wise addition of 50 % KOH solution. Add concentrated Hydrochloric acid drop-wise until the zinc acetate precipitate is just dissolved, then add 2 additional drops. Dilute to volume with water and mix thoroughly.

Pipette 10 mL of this solution into a clean, dry 50 mL volumetric flask. Add 8 mL of hydrazine sulphate solution and 2 mL of sodium molybdate solution in this order.

Stopper and invert 3 – 4 times. Loosen the stopper and heat for 10 ± 0.5 minutes in a vigorously boiling water bath. Remove from bath , cool to 25 ± 5°C in a water bath, dilute to volume and mix thoroughly Transfer the solution to a clean dry cuvette and measure the absorbance at 650 nm in a spectrophotometer adjusted to read 0 % absorbance (100 % transmittance) for distilled water.

Prepare a reagent blank without the oil test sample. Measure the phosphorus content of the sample and the blank by comparison with the standard curve.

34.6 Preparation of standard curve:

Pipette 0.0, 1.0, 2.0, 4.0, 6.0, 8.0 and 10.0 mL of standard working solution into 50 mL volumetric flasks. Dilute each to 10 mL with distilled water using a measuring pipette. Add hydrazine sulphate and sodium molybdate as above. Plot the absorbance of each standard against its phosphorous content in mg on a linear graph paper.

34.7 Calculation:

$$\text{Phosphorous \%} = \frac{10 \times (a - B)}{W \times V}$$

Where

A = Phosphorous content of sample aliquot in mg

B = Phosphorous content of the blank aliquot in mg

W = Weight of sample in gm

V = Volume of solution taken for colour development

Note: - Phosphorous content can also be determined by Atomic Absoption Spectrophotometer at a wave length of 213 nm following other instrumental parameters.

(Ref: - AOCS (1989) Official Method Ca 12 -55, Phosphorous)

35.0 DETERMINATION OF NICKEL IN VANASPATI

35.1 Reagents:

- Concentrated Hydrochloric acid

- Saturated Bromine Water
- 0.1% Dimethly glyoxime solution in 95% alcohol
- Nickel Sulphate(A.R, 99.9% Pure)

35.2 Procedure:

Nickel can be determined both by spectrophotometric method as well as by Atomic Absorption Spectrophotometer using graphite furnace.

The spectrophotometric method involves burning of 20 – 25 gm of vanaspati in a platinum dish on a low flame, followed by ashing in muffle furnace at 500°C, dissolving the ash in about 5 mL of concentrated Hydrochloric acid followed by removal of excess acid by evaporation to dryness and dissolving the residue in water and making upto a known volume. An aliquot of the solution (5 - 10 mL) is taken in a 25 mL volumetric flask. 0.5 mL saturated Bromine water is added, allowed to stand for 1 minute, followed by addition of 1 ml of ammonia and 2 mL of 0.1% dimethyl glyoxime solution in 95% alcohol and mixed. The final volume is made up to 25 mL with alcohol and the 'absorbance maxima' is recorded at 445 nm within 10 minutes of addition of the dimethyl glyoxime solution.

A standard stock solution of Nickel is prepared separately by dissolving 2.2617 gm of Nickel Sulphate (A.R, 99.9% Pure) in 30 mL of concentrated Hydrochloric acid and making up the volume to 500 mL with distilled water. This solution contains 1000 µg Ni/mL Working standards are prepared by diluting the stock solution to give 0.1 – 1.0 µg/mL Nickel. A calibration graph is prepared with different working standards and the amount of nickel in the sample is extrapolated from the standard graph.

(Ref: - J. Fd Sci Technology 1991, Vol 28, No 1 page 42-43)

Note: - For AAS method refer to AOAC 17th edn 2000, Official Method 990.05 Copper, Iron and Nickel in Edible Oils and Fats, Direct Graphite Furnace AAS Method.

36.0 QUALITATIVE TEST FOR VITAMIN A IN VANASPATI: ANTIMONY TRICHLORIDE METHOD

36.1 Principle:

The melted sample is treated with antimony trichloride and observed for appearance of blue colouration, which indicates presence of Vitamin A.

36.2 Reagents:

Antimony Trichloride Solution: Prepare by dissolving 113.4 gm antimony trichloride in 300 to 400 mL of chloroform. Add 5 gm of anhydrous calcium chloride and filter while hot. Dilute the filtrate to 500 mL with chloroform.

36.3 Procedure:

Take 10 mL of antimony trichloride solution in a test tube and add 15 mL of melted Vanaspati. The material shall be considered to have passed the test if a blue colouration appears immediately at the interface, indicating the presence of vitamin A.

Notes

1 *Antimony trichloride solution is made in chloroform and this phase is heavier than Vanaspati. Therefore, Vanaspati should be added to antimony trichloride solution.*

2 Special care should be taken in carrying out this test since the reaction is spontaneous and the blue colour developed is very unstable

37.0 DETERMINATION OF CAROTENOID CONTENT OF RAW PALM OIL

37.1 Principle:

The absorption of a solution of the fatty material in cyclohexane is measured at 445 nm. The %age content of total carotenoids (m/m) is calculated as beta carotene

37.2 Apparatus:

(1) Spectrophotometer capable of measurement at 445 nm and using matched or paired parallel sided glass or silica cells of 1 cm path

(2) Volumetric flask 100 mL

37.3 Reagents:

(1) Cyclohexane - spectroscopic grade

37.4 Procedure:

Weigh to the nearest 1 mg between 0.5 and 1.0 gm of the oil into a 100 mL volumetric flask. Dissolve the oil in cyclohexane and make upto mark. Fill a 1 cm glass or silica cell with the solution of the oil and fill a second matched cell with cyclohexane. Take absorption readings in the spectrophotometer at 445 nm. If necessary dilute the original solution to a measured volume and take further readings so that the observed absorptions are between 0.2 and 0.8 optical density.

37.5 Calculation:

Carotene content (mg/kg (ppm) as beta carotene) = $\frac{383\ E}{t\ c}$

Where,

E = Observed difference in absoption between sample solution and cyclohexane

t = path length of the cell

c = concentration used for absorption measurement

(Ref: - British standard Methods of Analysis – BS 684, section 2.2:1977 Determination of carotene in vegetable oils)

38.0 TEST FOR PRESENCE OF RANCIDITY

In routine work apart from the free fatty acids determination, the analysis should include the determination of peroxide value, Kries test and ultra-violet absorption at 234 nm and 268 nm to establish rancidity

38.1 Peroxide value

This is an indication of the extent of oxidation suffered by oil.

38.1.1 Reagents:

i) Acetic acid - chloroform solvent mixture (3: 2). Mix 3 volumes of glacial acetic acid with 2 volumes of chloroform.

ii) Freshly prepared saturated potassium iodide solution.

iii) 0.1 N and 0.01 N sodium thiosulphate solutions. Weigh 25 gm of sodium thiosulphate and dissolve in 1000 mL of distilled water. Boil and cool, filter if necessary. Standardise against standard potassium dichromate solution.

iv) Starch solution - 1% water-soluble starch solution

38.1.2 Procedure:

Weigh 5 gm (±50 mg) sample into a 250 mL stoppered conical flask. Add 30 mL acetic acid chloroform solvent mixture and swirl to dissolve. Add 0.5 mL saturated potassium iodide solution with a Mohrs pipette. Let stand for one minute in dark with occasional shaking, and then add about 30 mL of water. Slowly titrate

the liberated iodine with 0.1 N sodium thiosulphate solution with vigorous shaking until yellow colour is almost gone. Add about 0.5 mL starch solution as indicator and continue titration shaking vigorously to release all I_2 from chloroform layer until blue colour disappears. If less than 0.5 mL of 0.1 N sodium thiosulphate is used repeat using 0.01 N sodium thiosulphate. Conduct blank determination (must be less than 0.1 ml 0.1 N sodium thiosulphate).

38.1.3 Calculation:

Peroxide value expressed as milliequivalent of peroxide oxygen per kg sample (meq/kg):

$$\text{Peroxide value} = \frac{\text{Titre} \times N \times 1000}{\text{Weight of the sample}}$$

Where,

Titre = mL of Sodium Thiosulphate used (blank corrected)

N = Normality of sodium thiosulphate solution.

Fresh oils usually have peroxide values well below 10 meq/kg. A rancid taste often begins to be noticeable when the peroxide value is above 20 meq/kg (between 20 – 40 meq/Kg). In interpreting such figures, however, it is necessary to take into account the particular oil or fat.

(Ref: - AOAC 17th edn, 2000, Official Method 965.33 Peroxide Value in Oils and Fats / Pearsons Composition and Analysis of Foods 9th edn page 641)

38.2 Kries Test

38.2.1 Qualitative:

Shake 5 mL of the oil vigorously with 5 mL of 0.1% phloroglucinol solution in diethyl ether and add 5 mL of concentrated hydrochloric acid. A pink colour indicates incipient rancidity.

(Ref: - Pearsons Composition and Analysis of Foods 9th edn, page 642 / Manual Methods of Analysis for Adulterants and Contaminants ICMR (1990) page 16)

38.2.2 Quantitative:

Weigh 0.8 – 1.02 gm of oil or fat into a 100 mL beaker. Melt sample of fat and add slowly with stirring 20 mL of phloroglucinol (0.1 gm in100 mL of diethyl ether, freshly prepared) until sample dissolved. Transfer solution to a separating funnel, add 10 mL concentrated Hydrochloric acid, shake well and allow to separate. Run off acid layer into a 1inch (2.54 mm) Lovibond cell and match the colour using red, yellow and blue glasses. Express result as red Lovibond units. Upto 3 red units indicates incipient rancidity, between 3and 8 units indicates the end of induction period, over 8 units indicates definite rancidity.

(Ref: - Pearsons Composition and Analysis of Foods 9th edn, page 642)

38.2.3 Quantitative:

Shake 5 mL of oil and 5 mL chloroform in a stoppered test tube. Add 10 mL of a 30% solution of trichloroacetic acid in glacial acetic acid and 1 mL of 1 % solution of phloroglucinol in glacial acetic acid. Incubate the test tube at 45 °C for 15 minutes. After incubation, add 4 mL of ethanol and immediately measure the absorbance at 545 nm.Absorbance values below 0.15 indicate no rancidity. Absorbance values greater than 0.2 denote incipient rancidity and absorbance values around 1.0 show that the sample is highly rancid.

(Ref: - Manual Methods of Analysis for Adulterants and Contaminants ICMR (1990) page 16)

38.3 Ultra-violet absorption

Oxidized fatty acids containing conjugated double bonds absorb UV strongly between 230 and 375 nm, dienes absorbing at 234 nm and trienes at 268 nm. Conjugated trienes may be formed by industrial processing, e.g. decolorising with bleaching earths. A secondary absorption by trienes occurs at about 278 nm. In the early stages of oxidation the UV absorption increases somewhat proportionately to the uptake of oxygen and the formation of peroxides. The UV absorption curve forms plateau just before the end of the induction period. The magnitude of UV absorbance is not readily related to the amount of oxidation; so the method is best applicable to detecting relative changes in oxidation of oil in comparison experiments or stability tests.

38.3.1 Procedure:

Weigh accurately into a 25 mL volumetric flask, an amount of the oil sample so that the absorbance of its solution in iso-octane in a 10 mm quartz cell lies between 0.2 and 0.8. Trace the absorption curve against iso-octane between 220 and 320 nm and select the wavelength l max of maximum absorption near 230, 268 and 278 nm, and the absorbance (A) at these points.

The specific absorbance $E_{1\ cm}^{1\%}$ (λ max) = $\frac{A}{c \times d}$

Where,

'c' is the concentration of the sample solution (g/100 mL)

'd' is the cell length in cm

(Ref: - Pearson's Composition and Analysis of Foods 9th edn, page 643/Manual Methods of Analysis for Adulterants and Contaminants ICMR (1990) page 16)

39.0 ISOLATION AND IDENTIFICATION OF OIL SOLUBLE COLOURS

39.1 Principle.

The fat in the unaltered state or extracted from the foodstuff, is dissolved in petroleum ether. The solution is subjected to chromatography on a column of Aluminium oxide and the colouring matters undergo elution by means of several elution solvents The eluates are evaporated to dryness under vacuum and the residues subjected to saponification, if need be, are taken up in diethyl ether and identified using benzene as solvent.

39.2 Apparatus:

(1)Balance

(2) Aluminium dish – diameter 7 cm.

(3) Drying chamber, set at 60°C

(4) Filter papers

(5) Soxhlet apparatus

(6) Graduated Test tubes – 10, 25, 50 100, 250 mL

(7) Chromatography Tube – 20 cm × 1 cm diameter with a tap

(8) Round bottom flask 100 mL with ground glass joint

(9) Rotary evaporator

(10) Development tank for holding TLC plates of 20 × 20 cm

(11) TLC plates – 20 × 20 cm coated with silica gel G to a thickness of 0.25mm/HPTLC plates

(12) Microcapillary pipettes of 2 microlitres or equivalent

(13) Oven – set at 100°C

39.3 Reagents:

(1) Sea sand – washed in hydrochloric acid and calcined

(2) Ethanol 95% (v/v)

(3) Petroleum ether 40 – 60°C

(4) Basic Aluminium Oxide- activated for 1 hour at 400°C

(5) Benzene and Acetone

(6) Mixture of Petroleum Ether and acetone 98:2. Measure exactly by pipetting 2 mL of petroleum ether from a filled 100 mL flask and replace it with 2 mL of acetone

(7) Mixture of Petroleum ether and acetone 1:1 (v/ v). Measure 25 mL of pet. ether and 25 mL of acetone and mix

(8) Mixture of ethanol and acetone 4: 1 (v/v). Measure 40 mL of acetone and 10 mL of ethanol and mix

(9) Mixture of ethanol and ammonia 2: 1 (v/v). Measure 40 mL of ethanol and 20 mL of ammonia 0.910 and mix

(10) Ammonia 25 % (m/m), density - 0.910

(11) Ethanolic Potassium. Hydroxide – 0.5 M Weigh 14 gm Pot hydroxide and dissolve in 500 mL ethanol. Keep in dark

(12) Solutions of reference colours – 0.5 % in ethanol or Chloroform. Dissolve 50 mg of each reference colour in 10 mL of ethanol except carotene which must be dissolved in chloroform

(13) Mixture of n – hexane and ethyl acetate, 9:1 (v/v)

(14) Carr – Price reagent – Dissolve 25 gm of antimony trichloride in 75 mL of chloroform in a glass stoppered conical flask

39.4 Procedure:

(a) Exraction from foodstuff: - Weigh 5 – 10 gm sample in an aluminium dish containing sand, add 5 - 10 mL ethanol and leave mixture in oven overnight.

Transfer contents of dish to a thimble or filter paper and extract for 4 hours in a soxhlet. Evaporate the solvent from the extract and take residue in 10 mL of petroleum ether in a beaker.

(b) Extraction from oil – Dissolve 0.5 - 1 gm oil in10 mL petroleum ether.

Place a plug of cotton wool in the chromatography tube and push this down to just above the tap. Fill the tube with a suspension of aluminium oxide in benzene so as to obtain a column of 10 cm in height. Run off benzene taking care that the column does not become dry. Rinse the column with 50 mL of petroleum ether or until all benzene has been removed. Pour the petroleum ether extract of the colour obtained above on to the column and regulate the speed of the flow to about 1 mL/minute. Rinse the column with 100 mL of Petroleum ether. Do not allow the column to become dry. Discard the eluate.

c) Eluate Carotenes with 50 mL of mixture of petroleum ether/acetone. Collect eluate in a 100 mL round bottom flask. Evaporate under partial vacuum using a rotary evaporator or a current of nitrogen with the flask over a water bath. Take up residue in 1 mL diethyl ether.

d) Eluate the amino- aniline colours with 50 mL of mixture of petroleum ether /acetone 1:1, collect eluate in 100 mL flask, evaporate under partial vacuum using rotary evaporator or by current of nitrogen with flask over a waterbath. Take up residue in 1 mL diethyl ether.

e) Elute the hydroxyl aniline colour with 50 mL of acetone / ethanol mixture. Collect eluate in a 100 mL flask; evaporate to dryness under vacuum using rotary evaporator or on a water bath in a current of nitrogen. Take up residue in 1 mL of diethyl ether.

f) Elute the bixin and the hydroxyl aniline colours which may still remain on the column with 50 mL of the mixture of ethanol / ammonia 2:1. Collect the eluate in a 100 mL round bottomed flask. Evaporate under partial vacuum using a rotary evaporator or in a current of nitrogen with the flask on a water-bath. Take up residue in 1 mL of diethyl ether. Change of colour of the aluminium column to a red violet shade after the ethanol / ammonia mixture has been added indicates presence of curcumin in the sample.

The presence of residual oil or fat in the eluted colours can hinder identification and it is desirable to saponify the lipids present. Add 50 mL of ethanolic Potassium Hydroxide solution and some fragments of pumice stones.

Boil for 45 minutes under reflux. Cool and transfer solution to a separating funnel using 100 mL water. Carefully extract the aqueous phase, if it does not contain bixin once with 50 mL and twice with 25 mL diethyl ether. Then wash the ethereal extracts three times using 25 mL water each time. If it contains bixin acidify with sulphuric acid 4 M and extract once with 50 mL and twice with 25 mL diethyl ether. Wash ethereal extracts 3 times with 25 mL water each time.

Dry the ether phase with anhydrous magnesium sulphate; evaporate under partial vacuum in a rotary evaporator or in a current of nitrogen over a water bath. Take up residue for identification.

39.4.1 Identification by TLC/HPTLC:

Spot 4 microlitres or more of each of the solutions using a microlitre pipette about 2.5 cm away from the edge of the plate. Space the spots at an interval of 2 cm. In the same way spot 2 microlitres of solutions of reference colours.

Develop plate with benzene in a developing tank saturated with the vapours of the solvent, allow to migrate over a distance of 17 cm. allow the plate to dry in air. Develop again with benzene if necessary. To separate Sudan I from Sudan II develop with mixture of n – hexane / ethyl acetate. Examine the plate and identify the colours comparing the Rf values of spots of extracts with R_f values of the reference colours.

After examination, place the plate in a tank containing enough Carr–Price reagent to saturate the tank with its vapour until the plate becomes visibly wet.

A blue stain appearing in the fraction obtained with ethanol /ammonia 2:1 indicates presence of bixin. Heat the plate for 10 minutes at 100˚C. The blue stain turns reddish brown.

(Ref :- FAO Manuals of Food Quality Control 14/ 2 , page 69 / PearsonsComposition and Analysis of Foods, 9th edn , 1991 , page 107)

39.5. Alternate Test for presence of Synthetic Oil Soluble Colours

Oil soluble colours are natural as well as synthetic which are soluble in oils and fats.

39.5.1 Hydrochloric acid test

39.5.1 Principle:

The petroleum ether solution of oil sample gives different shades of colour with different concentrations of hydrochloric acid in presence of coal tar synthetic oil soluble colour in the oil/fat.

39.5. 2 Reagents:

i) Concentrated hydrochloric acid – Prepare 4:1, 3:1, 2:1 and 1:1 hydrochloric acid: water mixture

ii) Petroleum ether

39.5.3 Procedure:

To 5 mL of oil sample in separate test tubes add 15 mL of petroleum ether followed by 5 mL of hydrochloric acid of different concentrations to different tubes. Observe for the change in the colour indicating the presence of synthetic oil soluble colour in the sample.

39.6 Thin Layer chromatography method for Isolation and confirmation of oil soluble colours

39.6.1 Principle:

The oil sample in hexane is treated with silica gel to absorb the colours. After eluting the oil with hexane the colour absorbed by silica gel is recovered by eluting with diethyl ether. Identification of colours is made by silica gel G thin-layer chromatography.

39.6.2 Apparatus:

a) Glass stoppered conical flasks - 250 mL capacity

b) Beakers - 100 mL capacity

c) Glass plates of 20 x 20 cm

d) Applicator and board

e) Developing tank

39.6.3 Reagents:

i) Silica gel - G for TLC/HPTLC plate

ii) Silica gel (column chromatography grade)

iii) Hexane

iv)Diethyl ether

v) Solvent mixture of benzene, hexane and acetic acid in 60:40:1 (v/v.)

vi)Standard solutions of known oil soluble colours (0.1% solution in oil)

39.6.4 Procedure:

Take about 5 mL of oil sample in a glass stoppered conical flask. Add 25 mL of hexane followed by 10 gm silica gel (column chromatography grade) and 2 gm anhydrous sodium sulphate. Stir the mixture well and keep aside for 5 minutes Decant off the solvent. Add once again 25 mL of hexane and stir well and decant the solvent. Likewise add hexane 25 mL 3 - 4 times to the flask and draining out the solvent each time to remove almost all the oil leaving behind the silica gel in the flask.

Elute the colouring matter absorbed by silica gel in the flask by shaking with diethyl ether 2 - 3 times using 20 mL each time. Collect the diethyl ether extract in a beaker. Evaporate the solvent on a hot water bath. Spot the concentrated ether extract using capillary tube on an activated plate and develop the plate in a tank containing solvent mixture. Remove the plate when the solvent layer has reached 12 to 15 cm height and dry at room temperature. On heating the plate at 100°C in an oven for 1 hour; natural colours like carotenes would fade away leaving oil soluble coal tar colours. Compare the spots with spots of known oil soluble colours spotted side by side.

(Ref: Manual Methods of Analysis for adulterants and contaminants in Foods ICMR (1990) page 16)

40.0 TEST FOR PRESENCE OF BEEF FAT IN LARD (PORK FAT)

40.1 Principle:

The presence of beef fat, tallows and similar fats as well hydrogenated and interesterified pork fat in lard is detected by determining difference between m.p of crystallized glycerides and the melting point of fatty acids derived from these glycerides. The value is large for pure pork fat and small for beef fat.

40.2 Procedure:

Weigh 5 gm of melted and filtered lard into glass stoppered cylinder and add 20 mL warm acetone. Mix well, taking care that solution is clear and above 30°C. Let stand 16-18 hours at constant temperature of 30°C. Fine mass of crystals not less than 3 mL should be found at the bottom of the cylinder. If volume of crystals exceed 3 mL take smaller amounts of lard (3 - 4 gm) for new test. If crystals obtained from 5 gm lard are insufficient increase weight of lard and volume of acetone proportionately.

Decant supernatant acetone solution from crystallized glycerides. Add three 5 mL portions of warm acetone (30 – 35°C) taking care not to breakup deposit in washing and decant first 2 portions. Actively agitate third portion and by quick movement transfer crystals to small filter paper. Using wash bottle wash crystals with 5 successive small portions of warm acetone. Spread out paper and contents breaking up any large lumps and air dry at room temperature. Thoroughly comminute mass and determine mp of crystals in closed 1 mm tube.

Melting point is reached when fused substance becomes perfectly clear and transparent. When mp of glycerides is less than 63°C presence of beef fat or other fat should be suspected. Confirm presence of foreign fat by taking up mp of fatty acids prepared from glycerides.

Transfer crystallized glycerides to 50 mL beaker, add 25 mL of appox 0.5 N alcoholic KOH and heat on steam bath until saponification is complete. Pour solution into separator containing 200 mL water, acidify, add 75 mL ether shake and let stand. Drain aqueous acid layer and wash ether solution 3 times with water.

Transfer ether solution to dry 50 mL beaker, evaporate ether on steam bath and finally dry acids at 100°C. Let acids remain at room temperature for 2 hours and determine melting point. If mp of glycerides plus twice difference between mp of glycerides and mp of fatty acids is less than 73°C lard is regarded as adulterated.

(Ref: - AOAC 17th edn, 2000, Official Method 920. 163 Fats (Foreign) containing tristearin in lard / Pearsons Composition and Analysis of Foods 9th edn, page 611)

41.0 DETERMINATION OF RESIDUAL HEXANE IN OILS AND FATS

41.1 Definition and Scope:

The residual hexane content is the quantity of volatile hydrocarbons remaining in the fats and oils following processing involving the use of solvents. The volatile hydrocarbons are desorbed by heating the sample at 80°C in a closed vessel after addition of an internal standard. After determination of a calibration factor, hydrocarbons in the head space are determined by gas chromatography using packed or capillary columns. Results are expressed as hexane in mg/kilogram (mg/kg, or ppm). The method is applicable to the determination of 'free' volatile hydrocarbons expressed in terms of hexane remaining in animal and vegetable fats and oils after extraction with hydrocarbon based solvents. It is suitable for determination of quantities of hexane between 10 and 1500 mg/kg in fats and oils.

41.2 Apparatus:

(1) Gas Chromatograph having

(a) Thermostatic column capable of maintaining the desired column temperature within ±1 °C,

(b) Sample inlet system, separately thermostated which can be maintained at a minimum temperature of 100°C. If a capillary column is used, the inlet system must be capable of a 1/100 split injection. For serial analysis a headspace gas chromatograph with automatic sample injection and tempering bath is satisfactory

(c) Flame ionization detector which can be separately thermostated and maintained at a minimum of 100°C

(2) Recorder - If a recorder trace is to be used for calculating the composition of the samples analysed, an electronic recorder of high precision is required or

(3) Electronic Integrator (preferred) which permits rapid and accurate calculations.

(4) Chromatographic Column – Either packed or capillary column with the following minimum requirements

(a) Packed Column - stainless steel or glass, approx 2 metres long and 1 / 8 inch internal diameter with acid washed and silanised diatomaceous earth,150- 180 µ particle size (80 - 100 mesh Chromosorb WAW is suitable), stationery phase – squalene consisting of 10% of packing

(b) Capillary column – glass or fused silica approx 30 metres long and 0.3 mm internal diameter

Stationery phase – Methyl polysiloxane (film thickness 0. 2 µ)

(5) Syringe – 1 µL, 10 µL, 1000 µL capacity, gas tight.

(6) Septum vial -20 mL capacity

(7) Septa and Aluminium caps suitable for septum vials together with crimping pliers. The septa must be resistant to oils and solvents (butyl rubber or red rubber is recommended.)

(8) Tongs suitable for holding septum vials

(9) Heating bath with clamps for holding septum vials, thermostatically regulated and capable of maintaining a temperature of 80°C. For continuous operation glycerol is recommended as heating liquid

(10) Shaking machine.

41.3 Reagents:

(1) Gases

(a) Carrier – Helium (preferred for better resolution) or Nitrogen 99.99 % pure, dried and containing a maximum of 10 mg O_2/kg

(b) Flame Ionization Detector – Hydrogen, minimum purity 99.95 %, Air or Oxygen, dry, hydrocarbon free (less than 2 ppm hydrocarbon equivalent to CH_4)

(2) Technical Hexane or light petroleum with a composition similar to that used in industrial extraction or failing this n–hexane. For calibration, technical extraction hexane is preferred

(3) n- Heptane (internal standard) analytical reagent grade

(4) Vegetable Oil -, solvent free, freshly refined and deodorized. The oil is to be used for calibration and should be of a similar nature as the sample. It should be free from extraction solvent (less than 0. 01%)

41. 4 Sampling and sample storage:

It is essential that loss of solvent from the sample be prevented. The laboratory sample should be in a completely sealed condition and stored at 4°C. Plastic containers should not be used. Sample analysis should be carried out immediately when the sample container is opened.

41.5 GC Operating Conditions:

Carrier gas flow depends on the carrier gas and the type of column being used for analysis and should be optimized accordingly. The flow of hydrogen and air or oxygen to the FID should be optimized according to the manufacturer's recommendation. Injector and detector temperatures should be set at about 250°C. The column should be maintained at 40°C.

41.6 Procedure:

(1) Determination of the calibration factor - Weigh to the nearest 0.01 gm, 5 gm of solvent free vegetable oil (reagent 4) into each of the 7 septum vials. Seal each vial with a septum and cap. By means of a syringe add technical Hexane to 6 of the seven vials (the vial with no added solvent is the blank) according to the following table:

µL / 5 gm	0.5	1	2	4	7	10
mg/100 gm	67	134	268	536	938	1340

One vial remains without the addition of solvent.

If n–hexane is used for calibration the following table applies

µL / 5 gm	0.5	1	2	4	7	10
mg/100 gm	66	132	264	528	924	1320

Shake the 6 vials containing the solvent in the shaking machine vigorously for 1 hour. Using the syringe add 5 µL of internal standard (reagent 3, n-heptane) to each of the 7 vials. Successively immerse the vials upto the neck in the heating bath set at 80°C at intervals of approx 15 minutes. This time interval depends on the duration of the GC analysis, which is complete on the elution of the internal standard (n – heptane). The samples must be placed in the heating unit at intervals such that each sample is tempered for exactly 60 minutes.

Warm the gas tight syringe to 60°C. After tempering at 80°C for exactly 60 minutes and without removing the vial from the heating bath, use the gas tight

syringe and withdraw through the septum 1000 µL (1 mL) of the head space above the oil. Inject immediately into the gas chromatograph. For each of the vial containing added solvent a calibration factor F may be determined by the formula.

$$F = \frac{C_s \times A_1}{(A_H - A_B - A_1) \times C_1}$$

Where,

A_H = Total peak area of solvent hydrocarbons including the area of internal standard present in the spiked oil. For identification purposes a typical choursomatogram of solvent composition should be obtained. Hydrocarbons which usually make up the technical hexane are 2-Methyl pentane, 3-Methyl pentane. Methyl cyclopentane, cyclohexane, etc. Do not include peaks due to oxidation products which may be present in significant amounts

A_B = Peak area of the solvent hydrocarbons present in the oil to which solvent has not been added (blank) less the peak area of the internal standard

A_1 = Peak area corresponding to the internal standard in the spiked samples

C_1 = Quantity of the internal standard added expressed in mg/kg of the oil

C_S = Quantity of technical hexane added to the oil present in the vial expressed in mg/kg of the oil

Express the results to the third decimal place.

Calibration factors of the six standards should be approximately the same. The mean calibration factor should be 0.45 if n–heptane is used and 0.57 if cyclohexane is used.

The factor (F) so evaluated can be used for determining vial quantities of hexane less than 60 mg/kg. If the value of F found for the vial containing 0.5 µL of

hexane is significantly below the mean value, this deviation is probably due to difficulty in introducing exactly 0.5 µL and this determination must be either eliminated or repeated. For quantities of hexane between 10 and 20 mg/kg it is better to prepare calibration standards by adding 2 µl of internal standard instead of 0.5 µL.

41 7 Sample Analysis:

Weigh to the nearest 0.01 gm, 5 gm of the test sample into a septum vial as quickly as possible and close immediately with a septum and cap. Using a syringe add through the septum exactly 5 µL of the internal standard. Shake vigorously by hand for about 1 minute and then immerse the vial up to the neck in the heating bath at 80°C for exactly 60 minutes. Warm the gas tight syringe to 60°C. After tempering at 80°C for exactly 60 minutes use the gas tight syringe and take from the vial without removing it from the bath 1000 µL (1 mL) of the head space above the sample. Immediately inject into the gas chromatograph. Carry out two determinations in rapid succession on each sample

41.8 Calculation:

The residual solvent expressed in mg / kg (ppm) is given by the formula:

$$W = \frac{(A_H - A_1) \times F \times C_1}{A_1}$$

Where,

A_H = Total peak area of solvent hydrocarbons including the area of internal standard. Hydrocarbons which usually make up the technical solvents are 2 methyl pentane, 3 methyl pentane, methyl cyclopentane, cyclohexane etc. Do not include peaks due to the oxidation products. Some of these products may be present in significant amount.

A_1 = Peak area corresponding to internal standard in the sample

C_1 = Quantity of the internal standard added in mg/kg

Note: - For an addition of 5 µL of heptane / 5 gm of sample C1 = 680 mg/kg and C1 = 750 mg/kg if cyclohexane is used

F = Calibration factor obtained in procedure

Report the final result as a mean of two determinations.

(Ref: - AOCS (1989) Official Method Ca 3b. - 87)

42.0 DETERMINATION OF TRANS FATTY ACIDS HYDROGENATED VEGETABLE OIL

42.1 Principle:

Triglyceride, triundecanoin (C11:0), is added as internal standard. Fat is extracted into ether, then methylated to fatty acid methyl esters (FAMEs) using BF3 in methanol. FAMEs are quantitatively measured by capillary gas chromatography (GC) against C 11:0 internal standard. Total transfat is calculated as sum of individual transfatty acids expressed as triglyceride equivalents.

42.2 Apparatus:

a) Gas chromatograph (GC)-Equipped with hydrogen flame ionization detector, capillary column, split mode injector, oven temperature programming sufficient to implement a hold ramp hold sequence. Operating conditions: temperature (°C): injector, 225; detector, 285; initial temp, 100 (hold 4 min); ramp, 3°C/min; final temperature 240; hold 15 min; carrier gas, helium; flow rate, 0.75 mL/ min; linear velocity, 18 cm/s; split ratio, 200:1.

b) Capillary Column — Separating the FAME pair of adjacent peaks of C18:3 and C20:1 and the FAME trio of adjacent peaks of C22:1, C20:3, and C20:4 with a resolution of 1.0 or greater. SP2560 100 m × 0.25 mm with 0.20 µm film is suitable.

c) Water Bath — With nitrogen stream supply, maintaining 40 ± 5°C.

d) Gravity Convection Oven — Maintaining 100 ± 2°C

e) Three Dram Vials — About 11 mL

f) Teflon/Silicone Septa — To fit vials

42.3 Reagents:

a) Chloroform

b) Diethyl ether

c) Toluene Nanograde

d) Sodium Sulfate — Anhydrous.

e) Boron Trifluoride Reagent — 7% BF3 (w/w) in methanol, made from commercially available 14% BF solution. Prepare in the hood.

f) Triglyceride Internal Standard Solution — C11:0-triundecanoin; 5.00 mg/mL in Chloroform. Accurately weigh 2.50 gm C11:0-triundecanoin into 500 mL volumetric flask. Add ca 400 mL Chloroform and mix until dissolved. Dilute to volume with Chloroform. Invert flask at least 10 additional times. Triglyceride internal standard solution is stable up to 1 month when stored in refrigerator (2 - 8°C).

g) Mixed FAMEs Standard Solution — Reference mixture containing series of FAMEs, including C18:1 cis and trans (available commercially, or equivalent). To prepare mixed FAMEs standard solution break top of glass vial, open and carefully transfer contents of to 3-dram glass vial. Wash original vial with hexane to ensure complete transfer and add washings to 3-dram glass vial. Dilute to ca 3 ml with hexane.

h) Individual FAME Standard Solutions — Standard FAMEs solutions of several *cis* and *trans* fatty acids are available commercially and the required trans fatty acid standards can be used. Prepare individual FAME standard solutions as follows: Break top of glass vial open and carefully transfer contents to 3 dram glass vial. Wash original vial with hexane to ensure complete transfer and add washings to 3 dram glass vial. Add 1.0 mL C11:0 FAME standard

solution dilute to total volume of ca 3.0 mL with hexane. Individual FAME standard solutions are stable up to 1 week when stored in refrigerator (2 - 8°C).

42.4 Methylation to FAMEs:

Accurately weigh ca 100-200 mg of vanaspati or hydrogenated fat Dissolve in 2-3 mL chloroform and 2-3 mL diethyl ether. Transfer mixture to 3 dram glass vial and then evaporate to dryness in 40°C water bath under nitrogen stream. Add 2.0 mL 7% BF, reagent and 1.0 mL toluene. Seal vial with screw cap top with Teflon/silicone septum. Heat vial in an oven for 45 minutes at 100°C. Gently shake vial ca every 10 minutes.

NOTE — Evaporation of liquid from vials indicates inadequate seals: if this occurs, discard solution and repeat the entire procedure.

Allow vial to cool to room temperature (20 - 25°C). Add 5.0 mL water, 1.0 mL, hexane, and ca 1.0 gm anhydrous sodium sulphate. Cap vial and shake for 1 minute. Allow layers to separate and then carefully transfer top layer to another vial containing ca 1.0 gm anhydrous sodium sulphate.

NOTE —Top layer contains FAMEs including FAME of triglyceride internal standard solution.

42.5 GC of FAMEs:

Relative retention time (vs FAME of triglyceride internal standard solution) and response factors of individual FAMEs can be obtained by GC analysis of individual FAME standard solution and mixed FAME standard solution. Inject ca 2 µL each of individual FAME standard solutions and 2 µL of mixed FAMEs standard solution. Use mixed FAMEs standard solution to optimize chromatographic response before

injecting any test solutions. After all chromatographic conditions have been optimized, inject test solutions.

NOTE — With matrixes of unknown composition, it may be necessary to analyze test portion without addition of internal standard to ensure against interferences. Should interfering peak be found, the area of C11 internal standard peak must be corrected before performing calculations. Use 2.0 mL chloroform instead of internal standard solution.

42.6 Calculations:

Calculate retention times for each FAME in individual FAMEs standard solutions (D-3.13.3), by subtracting retention time of C11:0 peak from retention time of fatty acid peak. Use these retention times to identify FAMEs in mixed FAMEs standard solution. Use additional FAME solutions (from the same supplier) when necessary for complete FAME identity verification.

Calculate percent of trans fat in test sample [w/w; expressed as sum of only trans fatty acids (C14:1,Trans Myristelaidic + C16:1, Trans Palmitelaidic + C18:1,Trans 6 Petroselenic + C18:1,Trans Elaidic + C18:1,Trans 11 Vaccenic + C18:2, Trans Linolelaidic + C18:2, Trans 9- Linolelaidic + C18:2, Trans 12-Linolelaidic + C18:3, Trans Linolenic + C20:1, Eicosenic Trans 11)] as follows:

$$\text{Transfat, (\%)} = (\sum \text{transfat } W_i / W_{test} \text{ portion}) \times 100$$

Wi= weight of individual trans FAME in mixed FAMEs standard solution

W_{test}=weight of individual trans FAME in test sample

NOTE — Test samples containing hydrogenated fat will yield complicated chromatograms due to large number of isomers formed during hydrogenation process. One general indication of hydrogenation is presence of C18:1 trans peak(s). trans peaks elute prior to cis, therefore, include all peaks between C18:1 cis and C18:2 cis,cis in calculation of C18:2 peak area. Often C18:1 trans "peak" consists of broad series of peaks [due to positional isomers from hydrogenation]; include all of these in C18:1 trans peak area

42.7 Reference:

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