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Food Safety and Standards Authority of India

(A statutory Authority established under the Food Safety and Standards Act, 2006)

(Quality Assurance Division)

FDA Bhawan, Kotla Road, New Delhi – 110002

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ORDER

Subject: Approved methods of analysis of various food products - reg.

This is regarding the methods of analysis of various food products which have been approved by the Scientific Panel on Methods of Sampling and Analysis, Scientific Committee and Food Authority.

2. The food testing laboratories shall use the following methods with immediate effect. However, it would be the responsibility of the respective testing laboratory to confirm that the enclosed method is validated in their laboratory:

(i) Method of Detection of Melamine in Milk and Milk Products
(ii) Method to estimate Total Polar Compounds (TPC) in Edible Oils and Fats
(iii) Method for estimation of Coumarin content in Cinnamon
(iv) Method of analysis of various parameters in Sago
(v) Revised method of detection of acid value in Oils and Fats

Encl: Methods

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Method of Detection of Melamine in Milk and Milk Products

(USFDA Method)

A. Principle

A liquid chromatography triple quadrupole tandem mass spectrometry (LC-MS/MS) method for residues of melamine consists of an initial extraction with 2.5% aqueous formic acid, followed by a series of filtration, centrifugation, and dilution steps. The method is used for detection of both Melamine and Cyanuric Acid using HILIC LC Column. Melamine is detected in positive ion mode and cyanuric acid in negative ion mode. The extracts are analyzed by LC-MS/MS. Analyte concentrations are calculated using external standard calibration with a standard curve prepared in a pre-fortified control matrix which has been carried through the extraction procedure.

B. Chemicals and Reagents

(i) *Melamine (MEL).* CAS #: 108-78-1.
(ii) *Acetonitrile (ACN.)* LC grade.
(iii) *Formic acid.* Reagent grade >95%.
(iv) *Water.* LC grade, or purified by Millipore Milli-Q system to>18 M-ohm resistivity, or equivalent.
(v) *Ammonium Formate.* Purity> 97%.

C. Preparation of Solutions

(i) *0.1% Formic acid in water.* 1mL formic acid is transferred to 1L graduated flask and diluted to volume with LC water.
(ii) *Mobile Phase A.* 0.1% Formic acid in Acetonitrile (5:95 v/v). Mix 50 mL of 0.1% formic acid in water with 950 mL ACN in a 1 L solvent bottle.

(iii) *Mobile Phase B.* 20 mM Ammonium Formate in Acetonitrile (50:50 v/v). Mix 500 mL of 20 mM ammonium formate and 500 mL of acetonitrile in a 1 L solvent bottle.

(iv) *2.5% Formic acid in water.* 25 mL formic acid is transferred to 1 L volumetric flask and diluted to volume with LC grade water.

(v) *20mM Ammonium formate.* 0.63 gm of ammonium formate is weighed and dissolved in 0.5 L LC grade water.

D. Equipment

(i) *Liquid chromatograph.* Binary LC pump is recommended for accurate mixing at low flow rate and rapid response to mobile phase gradient.

(ii) *Liquid chromatography column.* ZIC-HILIC, 2.1 X 150mm, 5µm, 200 A

(iii) *Mass Spectrometer.* Triple quadrupole capable of meeting system suitability.

(iv) *Centrifuge.* Capable of 4000 RPM with 50 mL tubes.

(v) *Microcentrifuge.* Capable of 13,000 RPM with 1.5 or 2 mL tubes.

(vi) *Mixers and shakers*. Single and multi tube vortex mixers (VWR), platform shaker.

(vii) *Utrasonic bath.* Including timer and heater

(viii) *Centrifuge tubes*. 50mL disposable polypropylene with caps, with graducations from 5 to 50 mL and 1.5 mL microcentrifuge tubes.

(ix) *Syringe Filters*. Polyvinylidene fluoride (PVDF), 13mm, 0.22um

(x) *Syringes.* Three mL polypropylene.

E. Procedure

(i) Standard Preparation

Individual stock solutions, Melamine, approximately 100 µg/mL. Weigh approximately 10 mg of standard using a weigh boat to nearest 0.1 mg and transfer to a 100 mL glass volumetric flask. Add 70 mL 0.1% formic acid in

water and sonicate for 10 minutes. Maintain the volume as 100mL with 0.1% formic acid in water and mix thoroughly. Calculate exact concentration, correcting for purity.

Standard mixture dilution, 50 µg/mL is used for fortification and matrix calibration standards. Using volumetric pipets, transfer 5.00 mL of each stock standard into a 20 mL glass scintillation vial.

(ii) Sample Preparation

1. Sample powder (2.0 ± 0.1gm) is weighed in a 50 mL polypropylene centrifuge tube.
2. Pre-fortify control and matrix calibration standards.
3. 14 mL of 2.5% Formic acid in water is added to samples. Tube is tightly sealed. Dissolve sample by shaking for 15-30 seconds (vortex as needed), then sonicate in ultrasonic bath and mix on multi vortex mixer for 30 minutes each.
4. Centrifuge at 4000 rpm (3750 gm) for 10 minutes at room temperature.
5. Approximately 1.4 mL of the supernatant is transferred into a 1.5 mL micro centrifuge tube.
6. Centrifuge at 13,200 rpm (16100 gm) for 30 minutes.
7. Load aqueous extract into a plastic 3 mL syringe and force through a 13mm, 0.22um PVDF filter into a micro centrifuge tube. (Note: some formulations may require some force, or two filtration steps to obtain a clear solution before the next step.). Possible stopping point: aqueous extracts can be stored at 5-10°C for future dilutions.
8. Vortex mix for 30 seconds and centrifuge at 13200 rpm (16100gm) for 30 minutes.
9. Supernatant is transferred to a 2 mL autosampler vial, avoiding the precipitate.

F. Instrumental Analysis

The column is equilibrated in Mobile Phase A at 0.4 mL/min for 30-60 min.

It is necessary to evaluate system suitability, solvent blank (1x) and mixed standard are injected at 7.0 ng/mL (3-4x).
Data should meet the signal-to-noise and ion ratio criteria before continuing.

It is recommended to inject the standards and sample in following sequence: (i) solvent blank (Mobile Phase A), (ii) extracted matrix standards from 0.25 to 5 µg/g, (iii) solvent blank, (iv) control extracts, (v) post-fortified extracts and solvent standards for calculation of recoveries and matrix effects, (vi) solvent blank, (vii) unknown samples, and (viii) continuous calibration standards (an extracted matrix standard as well as solvent standard at 7 ng/mL), to verify that instrument response was maintained during the run.

G. Calculations

Use external standard calibration. The calibration curve should not include the origin, but does include a matrix blank with a concentration of 0. Export the processed data into Microsoft Excel or equivalent spreadsheet program for further calculations:

Recovery (%) = calculated from extracted calibration curve
Matrix effect (%) = 100 x Post-fortified sample / solvent standard (same cone)

The limit of quantification (LOQ) for each analyte is defined as the concentration of the lowest calibration standard used, or the lowest calibration standard which shows > 10-fold higher response than background signals in negative control sample.

H. Calculations for Confirmatory Analysis

Calculate ion ratios as percent relative abundances. The Melamine ion ratio is m/z 68/85.

REVISED METHOD FOR DETERMINATION OF ACID VALUE IN OILS AND FATS

(CLAUSE 11 OF FSSAI MANUAL OF METHODS OF ANALYSIS OF FOODS – OILS & FATS)

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

b) Alkali Blue 6B indicator solution: When testing rice bran oil or rice bran oil based blended 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.

Preparation: (2%) Extract 2gm of alkali blue 6B with rectified spirit in a Soxhlet apparatus at reflux temperature. Filter the solution if necessary and dilute to 100Ml with rectified spirit. Alkali blue 6B indicator to be stored in closed Ambered colored bottle to avoid oxidation of dye.

c) Ethyl alcohol:

1) Ninety-five percent alcohol or rectified spirit neutral to phenolphthalein indicator.

2) Ninety-five percent alcohol or rectified spirit neutral to Alkali blue 6B indicator in case of rice bran oil or rice bran oil based blended oil or fats.

d) 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

a) Weigh accurately appropriate amount of the cooled oil sample as mentioned in the above table in a 250 mL conical flask.
b) Add 50 mL of freshly neutralised hot ethyl alcohol and about one ml of phenolphthalein indicator solution. In case of rice bran oil or RBO based blends, add about 1mL of Alkali blue indicator.
c) Heat the mixture for about fifteen minutes in water bath (75-80°C)
In case of Rice bran oil or RBO based blended oils or fats, add 1mL of Alkali blue indicator after heating.

d) Titrate while hot against standard alkali solution shaking vigorously during the titration.

e) End point using phenolphthalein indicator shall be from colourless to light pink (Persisting for 15 sec.)

f) End point using Alkali blue 6B indicator shall be disappearance of blue colour which developed during addition of indicator.

Note: Noting burette reading after "obtaining dark pink colour OR Orangish red" as end point should be avoided as it will lead to erroneous result

g) 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 10mL.

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

Method for estimation of Coumarin content in Cinnamon

(Method by Spices Board, Cochin)

Objective: To estimate the Coumarin content in Cinnamon and Cassia by HPLC

A. Apparatus:

1. Measuring Cylinders, 50 mL, 100 mL capacity
2. Conical Flask, 250 mL capacity
3. HAPC system with accessories as mentioned under Instrument conditions
4. Micro litre syringe capable of injecting 1-20 mL
5. Balance, readable to 0.001gm
6. Whatman No. 1 filter paper (90 mm)/ syringe filter 0.45 mm.
7. Sample powdering mill or equivalent

B. Reagents:

1. Methanol HPLC grade
2. Acetonitrile HPLC grade
3. Water HPLC grade
4. Acetic and HPLC grade.
5. Ammonium acetate
6. Coumarin Standard (>90%)

Standard stock solution:

1. Weight accurately 0.1 gm of the above standard and dissolve and make upto 100 mL with HPLC Methanol.
2. Keep this solution as stock solution (1000 ppm) in standard flask wrapped in black cover. Shelf life is one year under refrigeration.
3. Working standard 10 ppm- From the stock solution pipette 1 mL to the 100mL standard flask and make up to the mark with HPLC Methanol.

4. Keep under refrigeration in standard flask wrapped in black cover. Shelf life is six months under refrigeration.

C. Procedure:

Sample preparation:

Whole Cassia & Cinnamon: After mixing & quartering, powder 100gm of the sample and pass through the sieve ASTM No. 20. (850 mm)

Ground Cassia & Cinnamon: Take a subsample of 100 gm by mixing and quartering of the entire sample

1. Weight accurately 1.0gm of the above sample in duplicate into 250mL conical flask.
2. Add 50 mL 90 % (v/v) Methanol using Calibrated measuring cylinder.
3. Shake for 30 minutes.
4. Filter 3 to 4 mL through whatman no.1 filter paper or 0.45mm syringe filter into stoppered test tubes.

D. Instrumentation Conditions

HPLC System – HPLC System with UV-Detector

HPLC Column – 5mm C18 (4.6X250 mm) steel column.

Mobile phase A: Water, 5mm Ammonium acetate buffer with 0.2% (v/v) acetic acid.

Mobile phase B: Acetonitrile: Methanol 1: 2 (v/v).

All solvent should be HPLC grade.

Flow rate: 0.8 mL/minute in a gradient program. The gradient program is as follows.

Time	Conc of B in A
14’	22%
16’	70%

22’	70%
25’	30%
30’	Stop

UV Absorbance- 279.8 nm

Volume for injection – 5 to 20 µL

E. Calculations :

Coumarin Contect is calculated as follows:

$$\text{Coumarin contect (mg/Kg)} = \frac{\text{x}(nG)\ X\ 50mL}{5\mu L\ X\ 1g} = \text{x X 10 mg/Kg}$$

F. Result and Reporting :

Report Coumarin content to an accuracy of 0.0 mg/kg.

G. Environmental aspects:

Coumarin is harmful. Handle with care.

METHOD OF ANALYSIS OF VARIOUS PARAMETERS IN SAGO

Methods as given in IS 899: 1971 (Reaffirmed 2017), Specification for Tapioca Sago (Saboodana), for analysis of parameters as under:

S. No.	Parameter
(i)	Moisture
(ii)	Total Ash (on dry basis)
(iii)	Acid insoluble ash (on dry basis)
(iv)	Starch (on dry basis)
(v)	Protein (on dry basis)
(vi)	Crude fibre (on dry basis)
(vii)	pH of aqueous extract
(viii)	Colour of gelatinized alkaline paste in the porcelain cuvette on the Lovibond Scale not deeper than
(ix)	Sulphur Dioxide content
(x)	Hydrocyanic acid

Method to estimate Total Polar Compounds in Edible Oils and Fats

(AOAC Official Method 982.27)

A. Principle

The method determines the extent to which fats and oils deteriorate when used for frying. These fats and oils can be separated by the process of Silica Gel based column chromatography into polar and non polar components.

Note: Polar components include polar substances such as monoglyerides, diglycerides, free fatty fatty acids that occur in unused fats, as well as polar transformation products formed during frying of foodstuffs and/or during heating and these components of fats can be determined by column chromatography under specified conditions. Nonpolar components are mostly unaltered triglycerides.

B. Equipment

(a) Column - Glass, 2.1 cm id x 45 cm. with Teflon Stopcock and ground-glass joint

(b) TLC plates – Pre coated silica gel (without fluorescence indicator), 20 x 20 cm, layer thickness = 0.25 mm

C. Chemicals & Reagents

(a) Silica gel 60 (Adsorbent) - particle size 0.063-0.200 mm (70-230 mesh ASTM), adjust to H_2O content of 5% as follow: Dry silica gel≥4 h in porcelain dish in 160˚C oven; cool in desiccator to room temperature. Adjust H_2O content to 5%, e.g., weigh 152gm silica gel and 8gm H_2O in 500mL round-bottom flask with ground-glass stopped and mechanically shake 1 h.

(b) Petroleum ether (bp 40°-60°C) - ether (87+13) [Eluting solvent mixture]
(c) Sea-sand - Analytical reagent grade; purified by acid and calcined.
(d) Spray reagent- Molybdophosphoric acid, 10% in alcohol.

D. Procedure

(i) Preparation of Sample

Semi liquid and solid fats are warmed to temperature slightly above melting point and mix thoroughly such as to avoid overheating. Visible impurities are removed by filtration. Hydrophobic filter are to be used, if water is present.

(ii) Preparation of Column

(a) Column is to be prepared using 30 mL (approx.) of petroleum ether-ether (87+13). Also place wad of cotton wool in bottom of column and remove air by pressing with glass rod.
(b) Prepare slurry of 25gm silica gel and approx 80 mL petroleum ether-ether (87+13) in 100 mL glass beaker. Pour the slurry into column using 8 cm glass funnel. Beaker, funnel and sides of column are to be rinsed with same solvent. Open stopcock and drain solvent to 10cm above silica gel. Silica gel is leveled by tapping the column.
(c) Approx 4gm of sea-sand is added through funnel into column. Solvent is drained to sand layer.

(iii) Chromatography

Only nonpolar fraction is used to determine polar components by difference. However, if separation is controlled by TLC, both polar and nonpolar fractions are required. Separation may also be controlled by checking recovery of analytes. But for products containing substantial

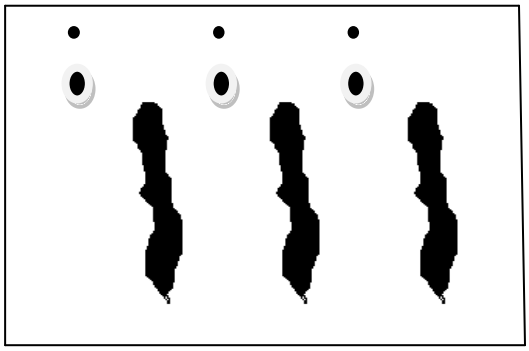
amounts of polar material, recovery may be incomplete because small amounts of highly polar material, generally 1-2%, are not eluted under conditions specified.

2.5±0.1 gm (to 0.001 gm) test portion is accurately weighed into 50 mL volumetric flask, and dissolved in approximately 20 mL petroleum ether-ether (87+13) while warming slightly. Let it cool to room temperature and dilute to volume with same solvent. 20 mL aliquot is transferred to column using volumetric pipet, without disturbing surface.

Two 250 mL round-bottom flasks are dried in 103° ± 2°C oven, cool to room temperature, and accurately weigh to 0.001 gm. One flask is placed under column, stopcock is opened, and solution is drained to level of sand layer. Nonpolar components are eluted with 150 mL petroleum ether-ether (87+13) contained in 250 mL dropping funnel. Flow rate is adjusted such that 150 mL passes through column within 60-70 min. After elution, wash any substance adhering to outlet of column into round-bottom flask with petroleum ether-ether (87 + 13).

In same manner, polar components are eluted into second 250 mL round-bottom flask with 150 mL ether. Silica gel is discarded.

Solvent is removed from each fraction with a rotary evaporator and 560°C water bath or with N_2 Stream in 250 mL flask on steam bath.



1 2 1 2 1 2

FRACTION

Figure1. Evaluation of efficiency of fractionation by TLC separation of polar and nonpolar fraction:
Fraction 1 contains nonpolar components, and
Fraction 2 contains polar components.

Avoid losses due to foaming. If rotary evaporator is used, shortly before end of evaporation, introduce N_2 into system. Cool residue to ambient temperature and introduce N_2 into flask. Weigh flasks.

(iv) Calculations

Calculate polar components, as percent (w/v) with formula:

Polar components, % $=\frac{E-A}{A}x100$

Where A = nonpolar fraction (in gm); E= test portion (in gm) in 20 mL aliquot (ca 1 g). Report result to one decimal place.

(v) Check of Column Chromatography Efficiency by Thin-Layer Chromatography

Dilute polar and nonpolar fraction (1+9) in $CHCI_3$. Apply 2µL spots using capillary dispensing pipet. Develop plate with petroleum ether-ether-CH_3COOH (70+30+2) in tank lined with filter paper for approximately 35 minutes (ca 17 cm). Remove plate and let solvent evaporate.

Spray plate with 10% molybdophosphoric acid. After evaporation of alcohol, heat plate in 120˚-130˚C drying oven. Fraction 1 (nonpolar) should be free of polar substances (see Figure 1).