MANUAL OF METHODS

OF

ANALYSIS OF FOODS

MYCOTOXINS

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GOVERNMENT OF INDIA
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MANUAL FOR METHODS OF ANALYSIS OF MYCOTOXINS

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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 MYCOTOXINS

Mycotoxins are metabolic products of fungi, which are capable of producing acute or chronic toxic effects (e.g carcinogenic, mutagenic, and teratogenic) on animals and probably on men at the levels of exposure.

Toxic syndromes, resulting from the intake of mycotoxins by man and animals, are known as mycotoxicosis. Although mycotoxicosis cause by mould *Claviceps purpurea* have been known for a long time. Mycotoxins remained neglected until the discovery of Aflatoxins in 1960. Mold growth in foods is very common, especially in warm and humid climates. It can occur in fields or in storage after harvest. Mould infection of foods such as grains, seeds and nuts is often localized in pockets, especially in bulk storage and warehouses Currently a few hundred mycotoxins are known, often produced by genre, *Aspergillus, Penicillium* and *Fusarium*. The chemical structures of some important mycotoxins are shown in Figure 1.

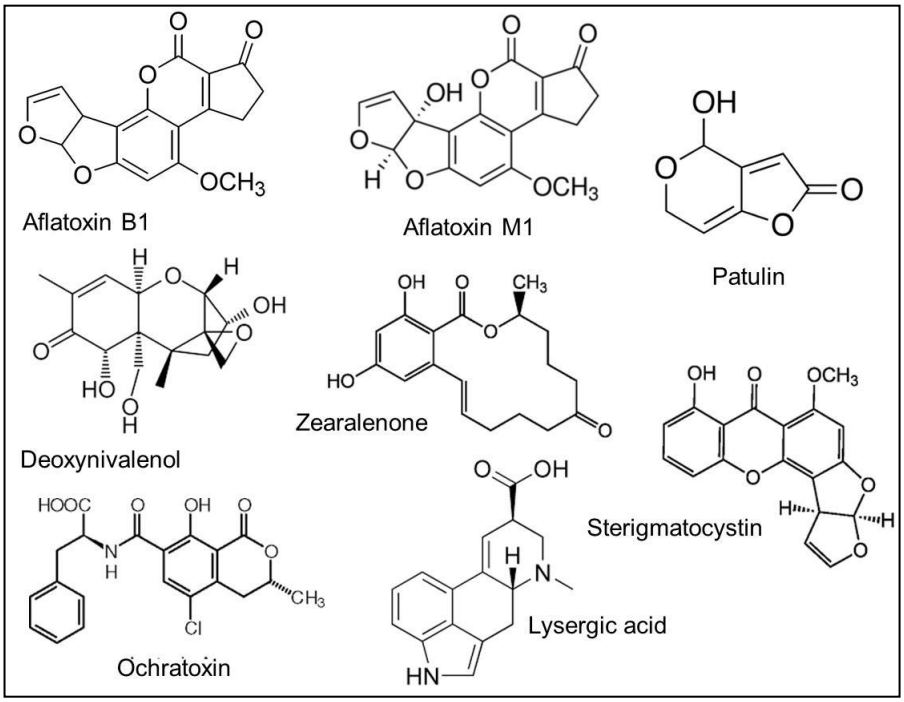

Figure 1 Chemical structures of a few mycotoxins

1.0 AFLATOXINS

Aflatoxin is probably the most common and widely known mycotoxin contaminant. It is produced by the moulds, *Aspergillus flavus* and *Aspergillus parasiticus.* In fact the name is a composite word derived from 'A. flavus toxin'. Foods that are commonly affected include all nuts, especially groundnuts, tree nuts such as pistachio and Brazil nuts, cottonseed, copra, rice, maize, wheat, sorghum, pulses , figs and oilseed cakes. Unrefined vegetable oils made from contaminated seeds or nuts usually contain aflatoxin. However aflatoxin is destroyed in the refining process so that refined oils are safe.

There are six aflatoxins of analytical interest (Figure 2). Four occur in foods and two as metabolites in the milk of animals who have been fed contaminated feed.

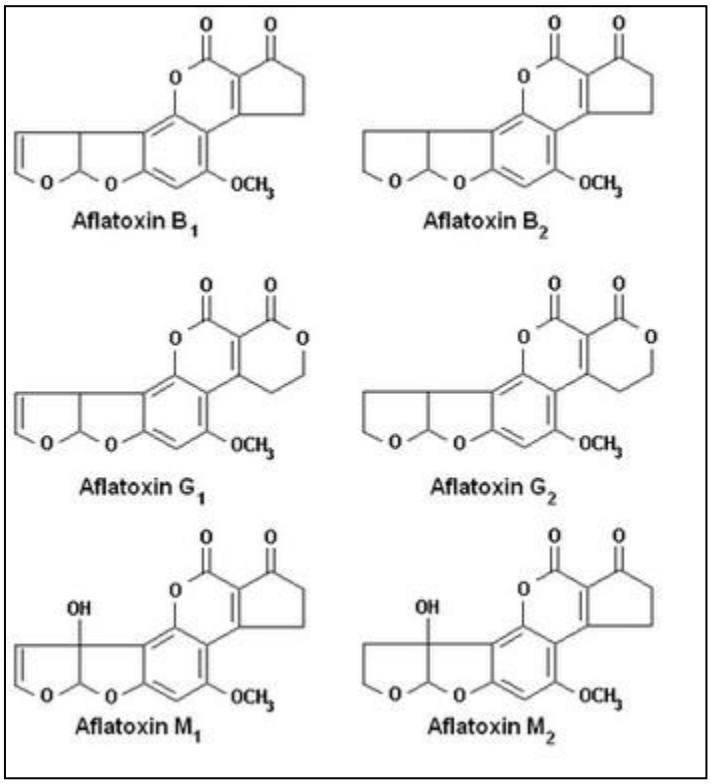

Figure 2: Chemical structure of the six aflatoxins

Aflatoxin B1, B2, G1, and G2 refer to toxins which fluoresce blue (B) or green (G) under ultraviolet light and are separable by thin layer chromatography (TLC). The only structural difference between B and G toxins is the inclusion of an oxygen in the cyclopentanone ring. Aflatoxin M1 and M2 represent the toxin B1 and B2, which have been

metabolized within the body of a lactating animal. Their finding in milk led to their designation as 'M'. The obvious structural difference between B and M is the addition of the hydroxyl group.

The analytical methods for aflatoxin include thin layer chromatography (TLC), High performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELISA). TLC has been most widely used method. Aflatoxins are extremely potent carcinogens in many animals.

Aflatoxins are subject to light degradation. Therefore, all analytical materials must be adequately protected from light and standard aflatoxin solutions stored using amber colored vials or aluminium foil.

1.1 Safety requirements for handling mycotoxins

All food samples suspected of being contaminated with mycotoxins must be handled with care. Use disposable gloves and protective masks if grinding the food creates dust. Aflatoxins are potent carcinogenic substances. While handling pure aflatoxin reference material, extreme precautions must be taken as they are electrostatic. All work must preferably be carried out in a hood. Swab any accidental spill of toxin with 1% sodium hypochlorite bleach (NaOCl), leave 10 minutes and then add 5 % aqueous acetone. Rinse all glassware exposed to aflatoxin with methanol, add 1% sodium hypochlorite solution and after 2 hours add acetone to 5 % of total volume. Let it react for 30 minutes and then wash thoroughly. Use a laboratory coat or apron soaked in 5% sodium hypochlorite solution over night and washed in water.

Reactive vapours i.e. O_2, SO_2, HCl can affect adsorbents used in TLC as well as the stability of adsorbed spots. TLC must, therefore, be performed only in a laboratory free of volatile reagents. Always dry TLC plates thoroughly before exposure to UV light. UV light from sunlight or fluorescent lamps can catalyse changes to compounds being examined when exposed on adsorbent surface, particularly in the presence of solvent. Avoid exposure to UV light of underdeveloped spots and expose developed plates to UV light for the minimum time needed for visualization. Protect analytical material adequately from light and keep aflatoxin standard solutions protected from light by using amber vials or aluminium foil. Put a warning note on the label. Use of non acid washed glassware for

aflatoxin aqueous solutions may cause loss of aflatoxin. Before use soak new glassware in dilute acid (carefully add 105 ml concentrated Sulphuric Acid to water and make upto 1 litre) for several hours, then rinse extensively with distilled water to remove all traces of acid. (Check with pH paper), (FAO Manuals of Food Quality Control 14 /7, 1986, page 185 / AOAC 17th edn, 2000, Chapter 49, subchapter 1 Mycotoxins /Sub chapter 2 Aflatoxins).

1.2 Preparation of sample

(a) Preparation of Lot sample - Mould contamination is by nature non homogeneous and hence the amount of mycotoxin is not uniformly distributed throughout the food stuff. Mycotoxin contamination, particularly in grains and nuts is likely to occur in pockets of high concentration, which may not be randomly distributed. Perform sampling and sample preparation with this factor in mind. Include total laboratory sample in sample preparation. Aim at maximum particle size reduction and the thoroughness of mixing to achieve effective distribution of contaminated portions. One contaminated peanut (ca 0.5gm) can contain enough aflatoxin to result in significant level when mixed with 10,000 peanuts (ca 5 Kg). To obtain 1 piece of contaminated nut in each 50gm portion the single nut must be reduced to 100 pieces and these 100 pieces must be uniformly blended through entire mass.

To achieve this degree of size reduction, nut must be ground to pass through a No 20 sieve, and thorough mixing of sample is needed before taking sample for analysis. When handling large samples coarse grind and mix entire sample, remove about 1/20 and regrind this portion to a finer size. In case of liquids mix thoroughly to obtain a homogeneous sample.

(b) Preparation of Laboratory Sample - Draw with the same precaution as with a lot sample. Wherever practical, divide by riffling or similar random dividing procedure until sub-division is close to the desired analytical sample (AOAC 17th edn, 2000, Official Method 977.16 Sampling of Aflatoxins, Preparation of Sample).

1.3 Preparation of standards for Aflatoxin

(a) For Aflatoxin standards received as dry films or crystals: To containers of dry aflatoxins B1, B2, G1, G2 add the requiredbvolume of one of the following solvents: acetonitrile, benzene–acetonitrile (98+2), methanol or toluene–acetonitrile (9+1), calculated to give a concentration of 8 – 10 µg/mL.

For Aflatoxin M1 use benzene-acetonitrile (9+1). Use label statement of Aflatoxin weight as guide. Vigorously agitate solution for one minute on a vortex shaker and transfer without rinsing to a convenient sized glass flask. Do not transfer dry Aflatoxins for weighing or other purposes unless facilities are available to prevent dissemination to the surroundings because of electrostatic charge on particles.

(b) For Aflatoxins received as solutions

Transfer solution to convenient sized glass stoppered flask. Dilute if necessary to adjust the concentration to 8- 10 µg/mL

(Ref :- AOAC 17th edn , 2000, Official Method 971.22 Standards for Aflatoxins)

1.4 Preparation and storage of working standards

Dilute portions of stock solution to a spotting concentration (0.5 µg/mL). Use same solvent used to prepare aflatoxin standards. Use benzene–acetonitrile (9+1) to dilute Aflatoxin M1 solution. Before storage, weigh flasks to nearest mg and record weight for future reference. Wrap flasks tightly with Aluminium foil and store at 0°C. When the solution is to be used after storage, reweigh flask and record any change.

To avoid incorporation of water by condensation, bring all standards to room temperature before use. Do not remove Aluminium foil until contents have reached room temperature. Standard solutions of aflatoxins B1, B2, G1, G2 are stable for more than one year The criteria of purity of the standards can be checked by determining chromatographic purity and molar absorption. The absorbance close to 350nm is determined and concentration calculated. It is however not necessary if standards are obtained from a recognized source.

(Ref: - AOAC 17th edn 2000, Official Method 971.22 Standards of aflatoxin, sub Para E, Preparation and storage of TLC Standards)

2.0 DETERMINATION OF AFLATOXINS (FOR GROUNDNUTS AND GROUNDNUT PRODUCTS, OILSEEDS AND FOOD GRAINS) – CB METHOD

2.1 Apparatus

(1) Stoppered Conical Flask

(2) Measuring Cylinders – 25, 50, 250 mL

(3) Chromatography column – 25 mm (i.d) × 300 mm length

(4) High speed blender

(5) Funnel – 7.5 cm diameter or Buchner Funnel with Whatman No1 filter paper or equivalent

(6) Wrist action shaker

(7) Rotary evaporator

(8) UV light Chamber

(9) Micropipette– 5 µL – 100 µL, adjustable

(10) Vials, Borosilicate – screw cap lined with foil or Teflon

2.2 Extraction

Fifty gm portion of a powdered representative sample is taken in a 500mL conical flask. 25mL water, 25gm diatomaceous earth (Celite) and 250mL chloroform are added to it. The flask is securely stoppered with masking tape and shaken on a wrist action shaker for 30 minutes to extract the toxin and filtered through a fluted filter paper. If filtration is slow, transfer to a Buchner funnel pre-coated with about 5 mm layer of diatomaceous earth and filter using light vacuum. Collect first 50mL of filtrate.

2.3 Column chromatography

Place a ball of glass wool loosely at the bottom of 22 × 300 mm chromatographic column and add 5gm of anhydrous Sodium sulphate to give base for silica gel. Add Chloroform until the column is about ½ full. Pour 10gm of silica gel made into a slurry with chloroform. Wash sides of the column with about 20mL Chloroform and stir to disperse silica gel. When the rate of settling slows, drain some chloroform to aid settling, leaving 5–7cm above silica gel. Slowly add 15gm anhydrous Sodium sulphate. Drain Chloroform to top of Sodium sulphate. Add 50mL of sample extract to column, elute at maximum flow rate with 150mL hexane followed by 150mL anhydrous ether and discard. Elute Aflatoxin with 150mL methanol–Chloroform (3+97) collecting this fraction from time of addition till flow stops.

The elute is evaporated on water bath, preferably under a gentle stream of nitrogen. The residue is quantitatively transferred to a vial, solvent evaporated and redissolved in a known volume of chloroform (0.2-1.0 mL) and kept in a vial for quantification.

2.4 Preliminary TLC

Uncap vial containing the extract, add 200 µL benzene–acetonitrile (98+2) and reseal with a polythene stopper. Shake vigorously to dissolve. Puncture polythene stopper to accommodate the needle of a 10 µL syringe. Under subdued incandescent light and as rapidly as possible spot 2, 5 and 10 µL on an imaginary line 4cm from bottom of the TLC plate. Keep vial for quantitative analysis. On the same plate spot 2.5 and 10 µL of aflatoxin standards. Place 50mL acetone–chloroform (1+9) in trough of unlined developing tank. Use only 1 plate per tank, placing trough to one side to permit maximum exposure of the coated surface to tank volume. Immediately insert plate into the tank and seal tank. The chamber must be saturated with solvent before use.

Develop plate for 40 minutes or until aflatoxins reach an Rf 0.4-0.7. Remove from tank, evaporate solvent at room temperature and view using long wavelength UV lamp in a viewing chamber. Observe pattern of the four fluorescent spots.

2.5 Quantitative TLC

If preliminary TLC shows need for further dilution of test solution, evaporate to dryness on a steam bath and re-dissolve in a calculated volume of benzene–acetonitrile. Spot successively 3.5, 5.0, and 6.5 µL of test solution. All spots should be approximately of the same size and ~ 0.5 cm in diameter. On the same plate spot 3.5, 5.0, 6.5 µL aflatoxin standard. After developing the plate, dry in subdued light. Compare fluorescent intensities of the sample spot with those of the standard aflatoxins and determine which of the sample spot matches the standards. If the spots of the smallest quantity of sample are too intense to match standards, the sample should be further diluted and rechromatographed.

Calculate concentration of Aflatoxin B1 in µg/kg = $\frac{S \times Y \times V}{X \times W}$

Where,

S = µL Aflatoxin standard, which matches the unknown

Y = Concentration of Aflatoxin B1 standard µg/mL

V = µL of final dilution of sample extract.

X = µL of sample extract spotted giving a fluorescent intensity equivalent to S (B1 standard)

W = wt in gm of the sample contained in final extract

(10gm if 50mL Chloroform extract is used)

Calculate Aflatoxin B2 , G 1 , and G 2 similarly

(Ref:- AOAC 17th edn , 2000 Official Method 968.22 Aflatoxins in Peanuts and Peanut Products CB Method)

3.0 DETERMINATION OF AFLATOXINS (PEANUT AND PEANUT PRODUCTS, CEREALS AND PULSES - BF METHOD)

3.1Extraction

Weigh 100gm of peanut meal or powder or 50gm peanut butter into a blender jar. Add: 1) 250mL methanol–water (55+45) and 100mL hexane to peanut butter 2) 500mL methanol–water (55+45), 200mL hexane and 4gm Sodium Chloride to peanut powder. Blend for one minute at high speed. Transfer to 250mL centrifuge bottles and centrifuge for 5 minutes at 2000 rpm. Alternatively let mixture stand undisturbed in blender jar wherein separation will occur within 30 minutes. Pipette 25mL of lower aqueous methanol phase into a separating funnel, add 25mL chloroform, stopper and shake for 30–60 seconds. Let layers separate and drain bottom Chloroform layer through anhydrous Sodium Sulphate into a 250mL beaker. Repeat extraction with two 25mL portions of chloroform.

Evaporate all combined chloroform extracts to between 2mL and just dryness. Do not leave beaker on hot plate after solvent has evaporated. Transfer extract with careful washing to a screw capped borosilicate vial and evaporate to dryness under gentle stream of nitrogen. Dissolve extract in 200 µL benzene–acetonitrile (98+2) and spot on TLC or HPTLC plate along with aflatoxin standards. Develop the plate in chloroform–acetone (9+1) and compare fluorescence intensity under long wave UV lamp as in clause 2.5 above. Calculate as shown in 2.5 above.

(Ref :- AOAC 17th edn , 2000 Official Method 970.45 Aflatoxins in Peanuts and Peanut Products BF Method, AOCS – AOAC method)

4.0 DETERMINATION OF AFLATOXINS - ROMER MINICOLUMN METHOD

4.1Apparatus

(1) High Speed Blender

(2) Ultraviolet light – Long wave UV Lamp with intensity of 430 μ watt/ cm^2 at 15 cm at 365 nm

(3) Minicolumn – Borosilicate standard wall tubing 6mm (i.d) x 150 mm, tapered at 1 end to 2 cm

(4) Minicolumn Support rack- Test tube rack may be used

(5) Rubber bulb – with 7 mm bulb at one end

4.2 Reagents

(1) Solvents – Chloroform and Acetone

(2) Potassium Hydroxide wash solution – 0.02 M KOH with 1% KCl Dissolve 1.12gm KOH pellets and 19gm KCl in 1 litre water

(3) Sodium Hydroxide Solution – 0.02 M – 8.0gm NaOH/litre

(4) 0.03% Sulphuric acid Solution– Add 0.3mL of concentrated Sulphuric acid in 1 litre

(5) Precipitating reagents – (1) Copper carbonate – Basic (2) Ferric Chloride Slurry – Mix 20gm of $FeCl_3$ with 300mL water

(6) Diatomaceous Earth

(7) Column packing (a) Florisil 100– 00 mesh (b) Silica gel 60 for column chromatography (c) Alumina Neutral, 80–200 mesh- activate for 2 hours at 110^0C (d) Calcium Sulphate anhydrous –20–40 mesh. Dry packing material for 1-2 hours at 110^0C. Store all packing materials and packed columns in vapour tight containers.

(8) Aflatoxin solution for spiking - Dilute solutions of B1 and G1 to final concentration of 2 μg/mL

4.3 Preparation of Mini column

Trap a small plug of glass wool into the tapered end of a column. To the column add to the height indicated in the following order: 1) 5-7 mm, Calcium Sulphate, 2) 5-7 mm, Florisil, 3) 18-20 mm, silica gel, 4) 8-10mm, neutral alumina, and 5) 5-7 mm, Calcium Sulphate[4]. Finally trap the column top with a small plug of glass wool. Tap column after each addition to settle packing and maintain uniform interfaces levels as possible. After packing apply pressure to top glass wool plug with a 5 mm dia glass rod.

4.4 Extraction

Weigh 50gm test sample into a blender jar, add 250mL acetone–water (85+15) and blend for three minutes. Alternatively use a 500mL glass stoppered Erlenmeyer flask and shake for 45 minutes on a mechanical shaker. Filter through Whatman filter paper No 4 or equivalent into a 250mL graduated cylinder. Collect 150mL filtrate and transfer to 400mL beaker.

4.5 Purification

To a 600mL beaker quantitatively add 170mL of 0.02N Sodium Hydroxide and 30mL ferric Chloride slurry and mix well. To the filtrate in the 400mL beaker add about three gms basic Copper Carbonate, mix well and add to the mixture in the 600mL beaker. To this add 150mL diatomaceous earth and mix well. Filter using a 160 mm funnel or Buchner funnel using Whatman No 4 filter paper or equivalent. Quantitatively transfer 150mL filtrate to a 500mL separator, add 150mL 0.03% Sulphuric acid and 10mL Chloroform. Shake vigorously for about two minutes and let separate. Transfer lower Chloroform layer (13-14 mL) to 125mL separator. Add 100mL Potassium Hydroxide wash solution swirl gently for 30 seconds and let separate. If emulsion occurs drain emulsion into 10mL glass stoppered flask, add about 1gm anhydrous Sodium Sulphate, stopper shake 30 seconds and let separate (Chloroform phase need not be completely clear). If emulsion is not broken, transfer emulsion to 125mL separator and wash with 50mL 0.03% Sulphuric acid. Collect 3mL of Chloroform layer in a 10mL glass stoppered cylinder for chromatography.

4.6 Chromatography

Transfer 2mL of Chloroform solution to a mini column using a 5mL syringe with 5 inch, 15 gauge needle. Allow to drain by gravity (15 – 30 mins). When solvent reaches top of adsorbent, add 3mL elution solvent (Chloroform – acetone (9+1). Allow to drain by gravity until solvent reaches the top of adsorbent. Do not let columns run dry during determination. Examine columns in darkened room using a UV lamp. Look for a blue fluorescent band at the top of the florisil layer (ca 2.5 cm from bottom of column), which is indicative of aflatoxin. Perform analysis with "clean" test portion and with test portion spiked with known amounts of aflatoxin to obtain comparison standards.

Some uncontaminated products show white, yellow or brown fluorescence at top of florisil in sample column. If band has no definite bluish tint test portion is negative.

(Ref :- AOAC 17th edn, 2000 Official Method 975. 36. Aflatoxins in Food and Feed – AACC-AOAC Method)

5.0 DETERMINATION OF AFLATOXINS IN CORN AND PEANUT POWDER / BUTTER - LIQUID CHROMATOGRAPHIC METHOD

5.1 Principle

Aflatoxins are extracted, purified, derivatised with trifluoroacetic acid and then separated by reverse phase liquid chromatography and detected by fluorescence. Method can measure 0.1 ng of aflatoxin B1, B2, G1, and G2. Detection limit is about 0.3 ng/gm.

5.2 Apparatus

(a) Liquid chromatograph – with Rheodyne septumless injector, Fluorichrom fluorescence detector, 7.54 and 7.60 excitation filters (360nm) 3-73 and 4-76 glass emission filters (440nm) fitted with flow cell integrator or recorder, 0.5 cm/min chart speed. Flow rate 1.0 mL/min. Set up detector preferably with tungsten source, using low lamp, high gain attenuation 20 or adjust range to give minimum half scale deflection with 1.25 ng aflatoxin B1 or G1. For optimum performance detector should be left on continuously.

(b) Column – 15 cm × 4.6 mm i.d. Supercosil LC -18 or equivalent

Note: - New LC columns or those that have been stored in methanol for extended periods require conditioning with concentrated standards in order to achieve optimum resolution and sensitivity to aflatoxin B1 and G1.

(c) Clean Up Column – 20 cm × 1cm i.d with Teflon stopcock and coarse frit bed support, detachable glass solvent reservoir with 24/40 fitting

(d) Adjustable autopipettes – 10-100 and100–200 µL with disposable tips

(e) Filter tube – glass 15 × 2.5 cm i.d with coarse frit bed support (glass wool not recommended)

5.3 Reagents

(a) Solvents – Glass distilled or HPLC grade: methanol, hexane, methylene chloride, benzene, acetone, acetonitrile. Anhydrous ethyl ether stored in metallic container (Glass bottled ether forms peroxides soon after opening which degrades aflatoxins)

(b) LC elution solvents – Water: acetonitrile: methanol (700:170:170). Adjust ratio of water to obtain baseline resolution of aflatoxin B2 and G2

(c) Silica gel for Column chromatography – Silica gel 60, (0.063-0.2 mm). activatd by drying at 100°C. Cool to room temperature. Weigh desired quantity (100gm) into glass stoppered container. Add one ml water in small increments, agitate silica gel between additions. Shake or tumble mechanically 4-6 hours. Let stand 16 hours

(d) Trifluoroacetic acid (TFA) – Assay by titration– equal to or more than 98.5%. Transfer 1-2mL TFA to a 1 dram vial with a Teflon lined cap. Keep in freezer when not in use. Discard if discoloration appears.

(e) Sodium sulphate, anhydrous. Sift out fines to obtain 20 – 40 mesh. Heat for 2-3 hours at 600°C to remove organic impurities.

(f) Aflatoxin standard solutions –

(1) Aflatoxin stock solution – 10 µg/mL. Prepare individual stock solution in benzene-acetonitrile (98+2) and determine concentration of each by measuring UV absorption if desired.

(2) Working standard solutions - Use an autopipette (Pipetman) to transfer an appropriate quantity stock solution to each 4 dram vial to obtain the final concentrations of aflatoxins in each vial as indicated below.

Vial	B1and G1 ng	B2 and G2 ng
1	250	125
2	500	250
3	1000	500
4	2000	1000

Evaporate solutions to dryness under a gentle stream of nitrogen (drying may be facilitated by warming to 40°C). Using Eppendorf pipette add 200µL hexane and 50µL of trifluoroacetic acid to each vial, cap and vortex for 30 seconds. Let solutions stand 5 minutes, then add 10mL water: acetonitrile (9+1) and vortex for 30 seconds. Let layers separate for 5 -10 min or centrifuge at 1000 rpm for 30 seconds. Final concentration of aflatoxins shall be:

Vial	B1and G1ng/10.05 ml	B2and G2 ng/ 10.05 ml
1	0.25	0.125
2	0.5	0.25
3	1.0	0.50
4	2.0	1.0

5.4 Extraction and partition

Transfer 50gm prepared corn, or peanut powder or peanut butter to a one litre blender jar, add 200mL of methanol followed by 50mL of 0.1 M hydrochloric acid and blend for three minutes at high speed. Filter through 24 cm Whatman No 1 filter paper or equivalent. Filtrate may not be completely clear. Collect 50mL filtrate. Transfer to 250mL separatory funnel. Add 50mL 10% Sodium chloride solution, swirl, add 50mL hexane and shake gently for about 30 seconds. Let phases separate then drain lower aqueous layer into another 250mL separator funnel. Discard hexane layer. Add 25mL methylene chloride and shake moderately for 30 seconds. If emulsion occurs break up with clean pipette. Let phases separate then drain lower methylene chloride layer through coarse granular anhydrous sodium sulphate in glass filter tube. Collect elute in a 250mL beaker. Evaporate elute, on steam bath under a gentle stream of nitrogen, to 2 -3mL.

5.5 Column Chromatography

Make a slurry of 2gm silica gel with about 10mL ether–hexane (3+1) in a 30mL beaker. Pour slurry into a clean up column and wash beaker with additional 5mL ether–hexane solvent to effect complete transfer. Keep stop cock closed and let silica gel settle without tamping. Wash sides of column with 2-3mL ether–hexane using squeeze bottle. After gel settles, open stop cock and while column drains add about 1 cm anhydrous sodium sulphate. Transfer eluate collected after extraction to column. Wash beaker with about 2mL of methylene chloride and add wash to column. Do not use more than 5- 6mL methylene chloride to transfer eluate to column. With stop cock fully open, add 25mL benzene–acetic acid (9+1) and the 30mL ether–hexane (3+1) to column, draining each wash to top of sodium sulphate. Discard washes. Elute aflatoxin with 100mL methylene chloride – acetone (90+10) and collect elute in 250mL beaker. Evaporate elute on steam bath under a gentle stream of nitrogen to about 6mL. Quantitatively transfer to 3 dram vial.

Evaporate elute to dryness using a steam bath or an aluminium block under a gentle stream of nitrogen. Evaporate remaining 200µL just to dryness under a gentle stream of nitrogen by holding vial in palm of hand and slowly rotating vial.

5.6 Derivatisation

Add 200µL hexane to the residue obtained above. Then add 50µL of TFA using Eppendorf pipette, cap the vial and vortex vigorously for 30 seconds. This procedure must be followed closely to ensure consistent reaction yields. Let mixture stand 5 minutes. Using Eppendorf pipette add 1.950mL water-acetonitrile (9+1).Vortex vigorously for exactly 30 seconds and let layers separate 10 mm. Concentration is 10 gm/2mL aqueous acetonitrile.

[Note: Post column derivitization using Kobra Cell may also be used]

5.7 Determination

Using instrument parameters mentioned under apparatus successively inject 25µL of derivatised standard solution. Prepare standard curve to check linearity of responses. Inject 25µL of trfluoroacetic acid treated test solution (lower aqueous phase). If test peaks are outside the linear range, dilute aliquot of trfluoroacetic acid treated test solution to suitable volume with water –acetonitrile, remix on vortex mixer and inject another 25µL portion.

Calculate individual aflatoxin concentration as follows:

Use responses of standard containing 500 ng B1and G1 and 250 ng B2 and G2 for calculation

Aflatoxin ng/gm = (P/ P') x C x (2 /10) x1000 x D

Where,

P and P' = peak areas or heights for test solution and standard per 25µL injection

C = concentration of individual aflatoxin in standard solution(0.5 or 0.25 µg/10 .05mL

D = dilution factor if 2mL test solution for injection is diluted

(Ref:- AOAC 17th edn, 2000 Official Method 990.33 Aflatoxins in Corn and Peanut Butter, Liquid Chromatographic Method)

6.0 DETERMINATION OF AFLATOXIN M1 IN MILK, CHEESE AND KHOYA

Aflatoxin M1 is the hydroxylated metabolite of Aflatoxin B1 secreted in the milk of animals receiving aflatoxin B1. It is a potential hepatocarcinogen. Handle with same care as other aflatoxins.

6.1 Apparatus

(1) Separating funnel 250mL

(2) Centrifuge

(3) Chromatographic column – 22 mm i.d × 300 mm length with a tap

(4) TLC/HPTLC plates

(5) Whatman Filter paper No1 or equivalent

(6) Micropipettes 5- 100µL, adjustable

(7) Borosilicate vials, screw capped with Al or Teflon lining

6.2 Reagents

(1) Solvents – Glass distilled acetic acid, acetone, acetonitrile, chloroform, ether – peroxide free, ethyl alcohol, hexane , isopropanol, toluene

(2) Sodium chloride solution – Saturated solution, about 40 gm/100 mL

(3) Silica gel for column chromatography- Suitable silica gel or equivalent. Stir 1hr in methanol, filter, treat similarly with Chloroform Activate by drying 1 hour at 105°C. Add water 1 mL/100 gm, shake until thoroughly mixed and store 15 hours in air tight container

(4) Sodium Sulphate , anhydrous, granular

(5) Diatomaceous earth – Celite

(6) Aflatoxin M1 reference standard – Make standards by dilution of stock solution with benzene – acetonitrile (9+ 1)

(7) Densitometer

6.3 Extraction

Shake 50mL milk, 10mL of saturated salt solution (40gm Sodium chloride/100mL water), and 120mL chloroform at 30°C in a 250mL separating funnel and allow to separate for 2 minutes. For milk powder reconstitute 5gm with 50mL water, for cheese and Khoya blend 15gm sample with 1mL saturated salt solution, 5gm diatomaceous earth or celite and 100mL chloroform for 60 seconds in a blender jar.

Drain lower Chloroform layer into 125mL erlenmeyer flask .Centrifuge if layers do not separate (15 minutes at 2000 rpm). Add 10gm anhydrous Sodium Sulphate to Chloroform with stirring. Filter into 100mL graduated cylinder. Save filtrate for column chromatography.

6.4 Column chromatography

Fill column half full with Chloroform. Add 2gm silica gel slurry made with Chloroform. Add 2gm Sodium Sulphate above silica gel. Drain off excess Chloroform and rinse silica off column sides with Chloroform Add sample extract and drain entire solution through column by gravity if flow rate slows stir Sodium Sulphate gently. Rinse graduated cylinder with Chloroform and add rinsing to column. Wash column with 25mL toluene – acetic acid (9 + 1) to remove coloured compounds. Wash with 25mL of hexane – ether – acetonitrile (5 + 3 + 2) to remove fat. Elute Aflatoxin M1 with 40mL Chloroform – acetone (4 + 1).

Evaporate to dryness and use for TLC or HPTLC as desired.

6.5 Thin Layer chromatography

Dissolve sample residue in 100µL of benzene – acetonitrile (9 + 1) , mix well and spot on TLC or HPTLC plates. Spot 20µL of test solution and 2 , 4 , 6 , 8 , and 10µL M1 standard

(0.25 µg/mL). Develop plate in chloroform - actone – isopropanol (87 + 10 + 3) and calculate Aflatoxin M1 in µg/kg or ppb as follows:

$$\text{Aflatoxin M1 (µg/ g)} = \frac{S \times Y \times V}{X \times W}$$

Where,

S = volume M1 standard which matches with sample

Y = Concentration of M1 standard in µg/mL

V = Volume final dilution of sample extract in µL

X = volume of sample extract spotted which matches with florescence intensity equivalent to M1 standard (S)

W = Volume in mL of sample contained in final extract

(W = Original volume or wt test portion x filterate volume/120)

(Ref:- AOAC 17th edn , 2000 Official Method 980.21 Aflatoxin M1 in Milk and Cheese Thin Layer Chromatography Method)

7.0 DETERMINATION OF AFLATOXINS B1, B2, G1&G2, IN SPICES, TEA, COFFEE, NUTMEG AND RICE BY HPLC-MS/MS

7.1 Principle of LC/MS

LC/MS is a hyphenated technique, combining the separation power of HPLC, with the detection power of mass spectrometry. Even with a very sophisticated MS instrument, HPLC is still useful to remove the interferences from the sample that would impact the ionisation. Interface that will eliminate the solvent and generate gas phase ions, then transferred to the optics of the mass spectrometer.

7.2 Instruments & Apparatus

Centrifuge, Sonicator, Blender, Horizontal shaker, HPLC complete setup with a Mass Spectrometry detector (triple Quadrupole), variable volume micropipette, 150mL beaker,

glass funnels, 10mL pipettes, 50mL graduated cylinders with ground glass stoppers, 50mL voulmetric Flask, Parafilm, Aflatest pump stand, fluted filter papers and glass fiber filter paper and Immuno affinity/Aflatest columns.

7.3 Chemicals/reagents

Sodium chloride AR/GR grade, Methanol (HPLC grade), Tween-20, Acetonitrile (HPLC grade), formic acid AR grade, Ammonium acetate (AR/GR grade).

7.4 Reference Standards

- Aflatoxin mixed std.(B1,B2,G1,G2) of 20ppm concentration from Fluka
- Intermediate standard solution of 120 ppb and the working standards solutions of 1.0, 2.5, 5.0, 7.5, 10, and 12.5 ppb concentration are prepared as follows;
- Aflatoxin Standard dilution procedure:

    -Stock Solution (1 mL) in Acetonitrile- 20 mg/mL (20ppm)

    -Add 250µL of the stock diluted to 50mL (50:50 v/v, methanol:water)

    Volumetric flask – 100ppb (Afla-Mix)

The following dilutions should be done in Methanol: Water mix for calibration dilutions.

-10µL of 100ppb (Afla-mix) +990µL of methanol:water (50:50) – 1.0ppb

-25µL of 100ppb (Afla-mix) + 975µL of methanol:water (50:50) - 2.5ppb

-50µL of 100ppb (Afla-Mix) +950µL of methanol:water (50:50) – 5.0ppb

-75µL of 100ppb (Afla-Mix) +925µL of methanol:water (50:50) - 7.5ppb

-100µL of 100ppb (Afla-Mix) +900µL of MeOH: H2O (50:50) – 10ppb

-125µl of 100ppb (Afla-Mix) +875µl of methanol:water (50:50) – 12.5ppb

The stability of Stock, intermediate & working standards have to be ensured in the lab before they are put to use.

7.5 Instrumental condition

HPLC Mobile phase

A. 10mM Ammonium acetate with 0.1% formic acid

B. Methanol and Acetonitrile (v/v 50:50) with 0.1% formic acid

Injection volume- 10 µL

Mobile phase Gradient:

Time (minutes)	Mobile phase-A (%)	Mobile phase-B (%)
0	50	50
4	10	90
7	10	90
7.2	50	50
9	50	50

i) Instrument:

Triple quadrupole HPLC-MSMS and Analytical Column RP-18 end-capped, 150 × 4.6 mm, 5 µm particle size or its equivalent, Flow rate: 0.3-1.0 mL per min

Depending on column ID & length Run time: 9-12 min

ii) MS/MS Conditions:

ES -- Positive

MRM of

Aflatoxin- G2- 331> 285 & 245

Aflatoxin- G1- 329> 283 & 243

Aflatoxin- B2- 315> 287 & 259

Aflatoxin- B1- 313> 285 & 241

The resolution, Dwell time, Collision energy etc those are specific to model and make of MS system need to be standardized by the analyst.

7.6 Test Procedure

Sample Preparation:

Weigh 25gm of sample into a 250mL conical flask, to this add 5gm of AR/GR grade Sodium Chloride and 100mL of Aflatoxin extraction solution (methanol : water 70: 30). In case of rice and in case of coffee & oleoresin the extraction solution shall be 80% methanol & 20% water mix). Cap & seal the conical flask with Parafilm, shake at about 140 rpm on a horizontal shaker for 30 minutes. After shaking keep the flasks standing for 5 minutes for settling of suspended particles if any. Filter all the extraction solution through a fluted filter into a 150mL beaker. From the beaker take 15mL of the filtrate into a 50mL graduated measuring cylinder and to this add 30mL of H_2O.

If the sample is nutmeg, oregano or black pepper, from the 150mL beaker take 10mL of the filtrate into 50mL graduated measuring cylinder and add 40mL of 20% Tween-20 solution. If the sample is not one of the above products mentioned, add 40mL of de-ionized water to the cylinder and mix.

Filter the contents of the graduated cylinder through a glass fiber filter into a 150mL beaker. This filtrate will be used in the Immuno Affinity column /Aflatest column.

7.7 Immuno-Affinity Column Clean-up

Attach an Aflatest-column to the pump stand. Pipette 10mL of filtrate on the column and allow it to absorb on column. Once the entire filtrate has passed through the column, rinse the column with 10mL of de-ionized water. Repeat de-ionized water rinse. Place a 2 or 4mL vial under the tip of the column and add 1mL of methanol to the column collect the elute and again add 1mL water to the column and collect the eluate in the same 2 or 4mL vial. This sample is now ready for injection into the HPLC-MSMS.

7.8 Injection Sequence

(a) Inject calibration standard(s)

(b) Inject the recovery sample

(c) Inject the blank sample and verify the absence of analytes above 5% of the recovery or sample concentration(s).

(d) Inject sample extract(s).

(e) Re-inject the calibration standard at the appropriate level at least after every 20 injections and at the end of the run to verify instrument response.

Note: If significant carryover is detected, inject wash solution as needed until it is reduced to an acceptable level.

7.9 Calculations

1. For Quantitation of each compound of interest:

a. Review the chromatograms to verify that the analyte peaks are within the retention time windows and that the peaks are integrated correctly.

b. Calculate the normalized peak for each component of interest by dividing the component response by the internal standard response, if any:

$$\text{Normalized Response Component 1} = \frac{\text{Response of Component 1}}{\text{Response of Internal Standard}}$$

c. Generate a linear curve fit to each analyte in standard curve using normalized response to concentration in tissue (µg/gm or ppm).

d. Standard curve must have a correlation coefficient greater than or equal to 0.995.

e. Blank must exhibit a response of less than 5% of the recovery used contemporaneously in the set.

2. For Confirmation:

a. Choose a standard or recovery containing the analyte of interest.

b. Identify 2 product ion peaks in the sample and verify that their peaks are present with a signal to noise ratio ≥ 3. Auxiliary ions may be used if necessary.

c. Identify the retention time of the two product ion peaks in the standard or recovery and in the sample of interest. The sample peak retention times must be within ± 5% of the standard or recovery retention times.

d. Calculate the ratio of the response of product ion #2 to product ion #1 in the standard or recovery for the analyte of interest:

   Ratio = Product ion#2/ Product Ion #1 Note: Ion ratio should be less than 1. If not, then invert the ratio.

e. Ion ratios determined for each analyte shall be within tolerance limits as described in the EU document 2002/657/EC incase of positive samples. Suggested tolerances are based on EU guidelines and range from ± 20% for peaks greater than 50% of the base peak and to ± 50% for those less than or equal to 10% of the base peak.

Software provided in the instrument can be used for auto Quantitation by using linear regression (y=mx+b), where y=peak area/ height, x= Analyte concentration in ppb/ µg/kg, m=slope of curve, & b= intercept of y) for samples taking in to account dilution factor, if any.

References:

1. Official Analytical Methods of the ASTA, 4th Edition, 1997 Method no. 24.2
2. Commission Decision 2002/657/EC of 12 August 2002, implementing Council directive 96/23/EC concerning the performance of analytical methods and interpretation of results.
3. AOAC Official Methods of Analysis (2005), Ch.49.2.18 Method, 991.31

[Note: AOAC method 991.31/49.2.18 by LCMS/HPLC-FLD may also be used for quantitation of all mycotoxins in cereals (Rice, Wheat etc.) and ASTA Analytical Method –ASTA 4th edition method 24.2 by LCMS/HPLC-FLD may be particularly included for quantitation of all mycotoxins in tea, coffee & Spices etc.]

8.0 DETERMINATION OF AFLATOXINS B1, B2, AND G1 IN CORN, COTTONSEED, PEANUTS, AND PEANUT BUTTER BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

8.1 Principle

Antibiotics specific to aflatoxins B1, B2, and G1 are immobilized on a filter, and toxin (aflatoxin B1) is labeled with an enzyme (horseradish peroxidase). Binding of toxin-enzyme conjugate by immobilized antibodies is inhibited by addition of free toxin present in test sample. Since fixed number of antibody reaction sites are available, enzyme activity is proportional to amount of bound toxin-enzyme conjugate. Antibody-toxin-enzyme complex concentration is inversely proportional to concentration of free toxin added. Bound enzyme catalyzes oxidation of substrate to form blue complex. Development of color indicates that test sample contains aflatoxins at <20 ng/gm; no color development indicates that test sample contains aflatoxins at ≥ 20 ng/gm.

8.2 Specificity of Antibodies

Antibodies have specific ability to bind structurally related compounds, namely, aflatoxins B1, B2, and G1. Determine specificity of purified rabbit anti-aflatoxin B1 polyclonal antibodies by direct competitive ELISA method. Coat serially diluted antibodies on microtiter plates. Prepare standard solutions of aflatoxins B1, B2, G1, G2, and M1; zearalenone; T-2 toxin; and deoxynivalenol, and add to individual microtiter wells. Then add solution of aflatoxin B1 conjugated to horseradish peroxidase to each well. Add substrate solution of tetramethylbenzidine and hydrogen peroxide, and measure development of color with scanner. Least color development indicates highest reactivity of toxin-antibody reaction. Cross-reactivity to aflatoxin B1 for antibody used in collaborative study of this method was 100, 70, 75, and <10% for aflatoxins B1, B2 and G1 and G2, respectively. All other toxins tested showed no cross-reactivity.

A. Sensitivity of ELISA Reagent

(a) Negative control test sample – is used to ensure all reagents are working properly. Negative control test sample should develop blue color

(b) Threshold-level standard – Used to define lower limit of determination. Dispense 100 µL working standard and perform ELISA. Threshold standard should show no color development

(c) Positive control test sample – Use working standard solution; follow procedure for ELISA. Positive control test sample should have no color development

B. Reagents

Reagents from commercial suppliers can be used provided requirements listed below are met.

(a) Antibody-coated solid support – Antibody-coated filter material attached to analytical cup made of porous polyethylene (3.2 cm diameter, 2.5 cm high, capacity 4 mL). Coated cup is specified by manufacturer to be stable for 6 months stored at 4-8°C. Coated 8/12/96 well strips or plates may be used.

(b) Aflatoxin-enzyme conjugate- Aflatoxin B1- horseradish peroxidase conjugate at toxin-enzyme molar ratio of 10-15:1. Conjugate is specified by manufacturer to be stable for 6 months at 4-8°C.

(c) Wash solution- Phosphate-buffer saline solution. Dissolve 0.23gm $NaH_2PO_4.H_2O$, 1.95gm $K_2HPO_4.3H_2O$, 8.70gm Sodium chloride, 0.125mL Tween 20 (polyoxyethylene[20]sorbitan monolaurate), and 10mg thimerosal (ehtymercurithiosalicylic acid, sodium salt), in 900mL H_2O adjust pH to 7.2, and dilute to 1 L.

(d) Buffer – 0.1% bovine serum albumin in phosphate buffered saline solution containing 0.05% thimerosal.

(e) Substrate solution A – Tetramethylbenzidine (TMB) (0.4 gm/L H_2O), pH 8.3.

(f) Substrate solution B – Hydrogen peroxide (0.02% H_2O_2 in 0.13% aqueous citric acid solution), pH 3.0.

(g) Methanol, hexane, and chloroform – Reagent grade.

(h) Standard aflatoxin B1 – Approximately 28 µg as dry film.

8.3 Apparatus

a) High- speed blender – With 500mL jar

b) Micropipette and tips- recommended range 100-1000 µL; use with disposable polypropylene tips.

c) Glass culture (test) tubes- 10×75 mm; 3mL.

d) Filters- Whatman No. 4 or equivalent.

e) Timer- Graduated in 1s intervals.

f) Carborundum boiling chips.

8.4 General Instructions

Store all kit components at 4-8°C. Do not freeze. Before use, allow one hour for cups (ELISA plate) and reagents to reach room temperature (23-29°C). use separate disposable pipet tips for each solution to avoid cross contamination. Include one negative control with each group (20 cups/wells) of test samples. Negative control must be functioning properly (must develop blue colour in center of cup/wells) for test to be valid. Positive standard is provided for periodic checking or for use with each group of test portions (must show no colour in the center of the cup/well). Threshold level standard should also be used and must show no colour development. If colour develops, repeat the test. Colour development in more than 2 tests indicates a defective kit.

Reagents are stable for 6 hours at room temperature. To ensure shelf life of kit components promptly return reagents to refrigerator after use.

Because of the difficulty in monitoring one minute intervals, run 1 cup at a time. As proficiency is gained, analysts can run 3 cups successively spaced at convenient time intervals for making observations.

8.5 Extraction of test portion

(a) Corn, raw peanuts, and whole cottonseed- weigh 50gm test portion into blender jar. Add 100mL methanol-water (8+2). Blend for three minutes at high speed. filter mixture and recover filtrate. Alternatively, let mixture stand 10-15 minutes and recover supernatant liquid. Dilute extract in ratio 1:1 with extraction solvent.

(b) Peanut butter- Weigh 50gm test portion into blender jar. Add 100mL hexane and 250mL methanol-water (55+45). Blend for three minutes at high speed. Filter mixture and transfer filtrate to separator funnel. Let layers separate for 10 minutes.

Place 20mL lower layer in 150mL beaker. Add minimum of 15 boiling chips and heat in steam bath or on hot plate. Boil for 3 minutes and let cool.

8.6 Preparation of Aflatoxin B_1 Standard Solutions

(a) Stock solution- Add 3mL Chloroform to vial containing 28µL aflatoxin B_1 standard (ca 9 ng/µL). Cap vial, mix contents, and store vial in refrigerator.

(b) Working solution- Dispense 300 µL stock solution into vial. Add 2400 µL methanol (1 ng/µL), mix and store solution in refrigerator. Prepare daily. Dispense 10 µL diluted standard (1 ng/µL) into test tube. Add 300 µL methanol and 700 µL buffer, Prepare ≤2h before use. Proceed as for diluted test extract.

8.7 Enzyme Immunoassay

(a) Corn, raw peanuts and whole cottonseed-

i. Allow 1 hour for all reagents to reach room temperature (23-29°C).

ii. Prepare fresh substrate in a small culture (test) tube by mixing 500 µL (10 drops) substrate solution A with 500 µL (10 drops) substrate solution B for each cup/well being used. Do not combine substrate solution A with Substrate solution B more than 15 min before use.

[**Note:** Run 1 negative control and 1 positive standard contrl each day to ensure that all reagents are functional. Threshold-level standard should be run with each set of new reagents. Negative control should be run by using 100 µL buffer. For positive standard contrl, using working

iii. Add 200 µL test extract to 400 µL buffer (600 µL total).

iv. Thoroughly mix diluted test extract and apply one 150 µL aliquot to cup/well. Using timer, wait one minute and then add second 150 µL aliquot of diluted test extract. Using timer, wait additional 1 min before proceeding to next step.

v. Apply 100 µL enzyme solution to center of cup. Using time, wait one minute.

vi. Wash with 1.5mL wash solution added drop wise. If more than 1 cup is being used, wash successively with 500 µL per cup 3 times.

vii. Add entire contents of substrate solution 1.0mL from each test tube to each cup. (Start time as soon as substrate mixture is added to cup.) Wait one minute and immediately observe the disk (center of cup) for blue colour development (negative) or no colour development (positive) (see K).

(b) Peanut butter-

(1) Allow 1 hour for all reagents to reach room temperature (23-29°C).

(2) Prepare fresh substrate solution in small culture (test) tube by mixing 500 µL (10 drops) substrate solution A with 500 µL (10 drops) substrate solution B for each cup being used. Do not combine substrate solution A with substrate solution B more than 15 min before use.

(3) Add 500 µL test extract to 500 µL buffer, E(d), (1000 µL total).

(4) Thoroughly mix diluted test extract and apply one 200 µL aliquot to center of cup. Using timer, wait one minute and add second 200 µL aliquot of diluted test extract. Using timer, wait additional one minute and then add third 200 µL aliquot of diluted test extract. Using timer, wait additional one minute and then add third 200 µL aliquot of diluted test extract before proceeding to next step. Proceed as for corn, etc., steps (5)-(7) above.

8.8 Interpretation of Results

Observe well/cup for blue colour or no colour development at exactly 1 min after adding substrate A and B mixture.

Negative- If it turns light blue or darker, test sample contains total aflatoxin B_1, B_2 and G_1 at < 20 ng/g (cottonseed, butter).

Positive- If no colour is observed in disk (center of cup) and disk remains completely white (no colour change) for at least 1 min, test sample contains total aflatoxin B_1, B_2 and G_1 at < 20 ng/g.

Negative control- Negative control cup must develop blue colour in center of cup.

Positive control standard- Positive standard cup must remain completely white (no colour change) for at least 1 min.

Threshold-level standard- Cup must remain completely white (no colour change) for 1 min.

References:

AOAC Official Methods of Analysis (2000), Ch.49.2.07 Method, 990.34

[Note: AOAC method 970.45/49.2.09 by LCMS/HPLC-FLD may be particularly included for quantitation of all mycotoxins in Cashew nuts and peanuts etc.]

Note: ELISA kit are meant for primary screening purposes and results obtained from the use of ELISA kit are to be verified with other analytical methods. Since various manufacturers have different protocol for using these kits, labs desiring to use the ELISA kit may follow the protocols of the relevant manufacturer. It would be the responsibility of the lab to confirm whether the ELISA kit results are validated and the kit gives proper result in their laboratory .

9.0 DETERMINATION OF TOTAL AFLATOXINS (B_1, B_2, AND G1) IN CORN BY ENZYME-LINKED IMMUNOSORBENT ASSAY METHOD

9.1 Sample extraction and analysis

The ground sample is thoroughly mixed using a highspeed laboratory blender. 10gm of ground sample was weighed to nearest 0.01gm on a piece of aluminium foil and transferred into a 50mL beaker. Then 50mL of methanol/water mixture (50:50) and 10mL hexane was added and mixed thoroughly for 30 minutes using a magnetic stirrer. 10mL of the mixture was centrifuged at 1500 K for 10 minutes. 3mL of the lower methanol/water layer was recovered and mixed thoroughly on a vortex. 400 µL sample extract was pipette into a mixture of 1600 µL PBS (Phosphate Buffer Saline) and 2000 µL methanol: water (10:90) in a mixing vial.

9.2 Enzyme linked immunosorbent assay (ELISA)

Validated ELISA Kits from reputed manufacturers may be used. It is the responsibility of the laboratory to follow the manufacturer's instructions for both assay and storage of the reagents. Further each laboratory must validate the performance of the kit (LOD and LOQ).

Aflatoxin standards (0 ppb, 5 ppb, 10 ppb, 20ppb and 30 ppb) prepared by serial dilution of a calibrated standard provided either in the kit or prepared in the laboratory must be used.

References:

AOAC Official Methods of Analysis (2000), Ch.49.2.13 Method, 993.16

Note: ELISA kit are meant for primary screening purposes and results obtained from the use of ELISA kit are to be verified with other analytical methods. Since various manufacturers have different protocol for using these kits, labs desiring to use the ELISA kit may follow the protocols of the relevant manufacturer. It would be the responsibility of the lab to confirm whether the ELISA kit results are validated and the kit gives proper result in their laboratory .

10.0 DETERMINATION OF AFLATOXIN IN CORN AND PEANUTS BY THIN LAYER CHROMATOGRAPHIC METHOD

Applicable to determination of 5-50 ng B1/g corn, 3-15 ng B2/g corn, 10-50 ng G1/g corn, 3-15 ng G2/g corn, 5-25 ng B1/g raw peanuts and 1.5-7.5 ng B2/g raw peanuts by densitometry; 10-50 ng B1/g corn, 10-25 ng B1/g peanuts, 7.5 ng B2/g raw peanuts, and 10-25 ng G1/g raw peanuts by visual comparison.

10.1 Principle

Aflatoxins are extracted from samples with methanol-water. Filtrate is diluted with Sodium chloride solution and defatted with hexane. Aflatoxins are partitioned into chloroform which is then removed by evaporation. Aflatoxins are purified by chromatography on 0.5gm silica gel column, and quantitated by TLC/HPTLC on Silica gel 60 plate with densitometry or visual estimation.

10.2 Apparatus

(a) Wrist-action shaker.- Capable of holding four to eight 250mL flasks.

(b) Silica gel column.- 6 mL disposable column, packed with 40µm (60Å) silica gel.

(c) Vacuum apparatus.- Equipped with vacuum gauge/flow controller and manifold fitted with 10 female Luer connectors.

(d) Vials.- 2 dram (8mL), with foil or Teflon-lined screw caps.

(e) TLC/HPTLC plate.- 20×20 cm glass plate coated with 0.25 mm thick gel without fluorescent indicator (precoated silica gel 60 plates).

(f) Viewing cabinet.- 270×270 mm base minimum, equipped with 15 W long wave ultraviolet (UV) lamp.

(g) Fluorodensitometer (TLC/HPTLC scanner).- Capable of scanning in reflectance mode by fluorescence, equipped with high-pressure Hg lamp, monochromater for adjustment to excitation 366 nm, and emission cutoff filter 420 nm.

10.3 Reagents

(a) Solvents- Methanol, hexane, chloroform, anhydrous ethyl ether (100%), dichloromethane, acetone and isopropanol.

(b) Aflatoxin standard solution.- Prepared in benzene-acetonitrile (98+2) to contain 0.5 µg/mL each B1 and G1 and 0.15 µg/mL each B2 and G2.

10.4 Extraction and Partition

Weight 50gm (ground to pass No. 20 sieve) corn or peanuts into 500mL glass-stoppered Erlenmeyer flask. Add 200mL methanol-H_2O (85+15) and secure stopper with masking tape. Shake vigorously by hand until samples show no clumps. Shake 30 minutes on wrist-action shaker and filter mixture through medium fluted paper. Collect 40mL filtrate in 50mL graduated cylinder. Transfer filtrate to 125mL separatory funnel. Add 40mL 10% Sodium chloride solutions, mix, and add 25mL hexane. Shake 1 minute. Let the phases separate, drain lower (aqueous) phase into second 125mL separatory funnel, and discard upper phase.

Extracts aflatoxins from aqueous phase with two 25mL portions Chloroform; shake one minute each time. Combine Chloroform fractions in 125mL Erlenmeyer flask and evaporate to dryness on steam bath.

10.5 Silica Gel Column Chromatography

Attach silica gel column, to extraction system, (or clamp to stand if using gravity flow only). Condition the column by washing with 3mL hexane, followed by 3mL dichloromethane using vacuum (flow rate 6 mL/min), or let drip freely unassisted by suction.

Check column suitability by adding aflatoxin B_1 standard (3mL dichloromethane containing 100 ng aflatoxin B_1) to 0.5gm silica gel column. Recovery must be $\geq$90% by this method.

Dissolve residue, from D, in 3 mL dichloromethane and add to column. Let drip freely (flow rate ca 3 mL/min, apply vacuum if needed). Rinse residue container with two 1mL portions of dichloromethane and add rinses to column. Wash column with 3mL hexane, 3mL anhydrous ethyl ether, and then 3mL dichloromethane. (Use vacuum, flow rate 6 mL/min, or use syringe and adapter to apply pressure to increase solvent flow if necessary. Do not pull up syringe plunger while it is still attached to column.) Turn off vacuum, remove extraction system cover, and place vial, under each column (test tube rack can be used to hold vials).

Elute aflatoxins (without vacuum) with two to four 3mL portions (according to results of column suitability test) of chloroform-acetone (9+1). Evaporate eluate to dryness on steam bath under stream of nitrogen.

10.6 Thin-Layer Chromatography-Flurodensitometry Determination

Dissolve residue from E in 250 μL $CHCl_3$. Spot plate, B(e), with 5 μL chloroform test solution in duplicate and 2, 5, 10, and 20 μL aflatoxin standard solution, Randomize standard and test solution spots across plate so duplicate test solution spots are not next to each other and standard spots are dispersed evenly. To avoid errors, prepare spoting plan, either on plate or in notebook, prior to spotting.

Develop plate 1 hour with chloroform-acetone (9 + 1). Evaporate solvent for five minutes in fume hood followed by 2 minutes at 50°C forced draft oven. Examine plate under long wave UV light to determine presence or absence of aflatoxins. Quantitate by fluorodensitometric measurement. Scan test and aflatoxin reference spots (transmission or reflectance mode,

excitation 365 nm and emission cutoff 430 nm). At end of plate scan, rescan 1st or 2nd lane. Scans of test spots should be within ±5%; if not, rescan entire plate.

10.7 Calculation

Calculate concentration of aflatoxin B_1 in test portion, using following formula:

$$B_1, \text{ ng/g} = \frac{250 \times R_u}{5 \times R_s \times 10}$$

Where, 250=μL test solution volume; R_u = average densitometer response for B_1 spots of test solution duplicates; 5 = μL test solution spotted; R_s = calculated average densitometer response/ng for 4 B_1 standard spots; 10 = g corn or peanut represented by extract.

Calculate concentrations of aflatoxins B_2, G_1, and G_2 similarly.

References

AOAC Official Methods of Analysis (2000), Ch.49.2.15 Method, 993.17

11.0 DETERMINATION OF AFLATOXINS M1 AND M_2 IN FLUID MILK BY LIQUID CHROMATOGRAPHIC METHOD

11.1 Principle

Aflatoxins M1and M2 are extracted from milk on a CI8 catridge, eluted with ether onto silica column, eluted with methylene chloride-alcohol, and M1 is derivatized with trifluoracetic acid. Liquid chromatographic peaks are detected fluorometrically compared with standard-TFA derivatives.

11.2 Regents

(a) *Solvents.* - Distilled in glass acetonitrile, methylene chloride, isopropyle alcohol; reagent grade alcohol, ether (0.01% ethyl alcohol preservative), hexane, methanol, trifluoroacetic acid, and H_2O (deionized, filtered through 0.45μm filter).

(b) *Water-acetonitrile wash solution.*—95+5.

(c) *Methylene chloride*—alcohol elution solution.—95+5.

(d) *Mobile phase.*—prepare H_2O-isopropyl alcohol—acetonitrile (80+ 12+ 8). Degas in ultrasonic bath, or equivalent. Alternative solvent proportions may be used to give optimum resolutions (i.e., 84 + 11 + 5).

(e) *Aflatoxin standard solutions.*— Aflatoxins M1 and M2 (Sigma Chemical Co., or other suitable source). Prepare stock solutions (ca 200 µg M1/mL and 100 µg M2/mL) in acetonitrile and determine concentrations spectrophotometrically using extinction coefficients of 19850 and 21400 for M1 and M2, respectively, in acetoitrile. Make working standard solution containing 0.50 µg M1 and 0.10 µg M2/mL in acetonitrile.—benezene (1+9) for use in preparing M1—TFA derivative.

(f) *Dichlorodimethylsilane (DDS).* - 5% in toluene. Add 5 mL DDS (99%) to toluene and dilute to 100 mL. Store in glass-stoppered flask in cold. (Caution: DDS is a lachrymator and is flammable.)

11.3 Apparatus

(a) *Silica gel cleanup columns.*—0.8 × 4.0 cm polypropylene Econo-Column with Luer tip, 35 µg porous polypropylene bed support disk, and 10 mL reservoir (Bio-Rad Laboratories, Cat. No. 731-1550, or equivalent).

(b) *Silica gel cleanup columns packing and preparation.*—Dry silica gel 60, particle size 0.40-0.063 mm in 105°C oven for 1 hour. Cool and add 1% H_2O by weight. Shake in sealed container and equilibrate overnight before use. Assemble polypropylene column and 25mL vacuum flask fitted with 1-hole stopper**.** Fill column to ca 2mL mark with silica gel (ca l gm). Pull gentle vacuum to pack bed and add ca 1 gm anhydrous Sodium Sulphate to top of silica gel bed.

(c) *Extraction catridges.*—C18 Sep-Pak sample preparation cartridges (Waters Associates, Inc.).

(d) *Disposable pipet tips.*—50 and 200µL Eppendorf or equivalent.

(e) *Liquid chromatograph.*—Any pulse-free or pulse-dampened liquid chromatographic system which includes pump(s), injector, and compatible recorder.

(f) *Fluorescence detector.*-Any fluorescence detector capable of providing 365nm excitation and ≥400 nm emission wavelengths and sensitivity of 50-100% full-scale response for 1 ng M1-TFA derivative.

(g) *LC analytical column.*—Any 0.4×25 cm column containing spherical 5µm particle size C18 bonded silica gel (e.g. DuPont ODS [MAC-MOD Analytical, Inc., Chads Ford, PA 19317, USA], Spherisorb 5 ODS 2 [Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Clwyd, UK]).

(h) *Vaccum regulator.*—Any commercial or custom device capable of creating and controlling partial and full vacuum with side arm vacuum flask.

(i) *Silylated vials for aflatoxin standard solutions.*-Fill 1 or $1^1/_2$ dram (4or 6mL) glass vials nearly full with 5% DDS and heat ca 40 min at 45°-55°C. Discard solution, and rinse vials 3 times with toluene and then 3 times with methanol. Heat vials in oven at 75°C for 20-30 min to evaporate methanol. Cap vials (with Teflon liners) and store for aflatoxin standard solutions.

11.4 Extraction

Attach intel (longer) stem of C18 cartridge to Luer tip of 30-50mL syringe. Assemble syringe, cartridge, and vacuum flask**.** Adjust vacuum to pull solvents through cartridge in fast drop wise manner (ca 5 mm Hg). Prime cartridge by adding 5mL methanol, then 5mL water in stem). Discontinue vacuum and move cartridge-syringe assembly from stopper to prevent loss of priming solution.

Warm milk (test sample) to room temperature. Gently invert test sample ≥ 10times to evenly distribute cream. Transfer 20mL milk to graduated test tube containing 20 mL hot (ca 80°C) water. If necessary, more hot water may be used to thin milk solution.

Replace cartridge-syringe assembly in stopper. Pour entire 40mL warm diluted milk into syringe and gently pull liquid through cartridge at flow rate ca 30mL/min (very fast drops). (Caution: Too fast a flow will not allow sufficient time for aflatoxin to adsorb, resulting in low recoveries). Add 10mL water-acetonitrile wash solution to syringe and pull through. Plug syringe barrel with stopper and pull hard vacuum on cartridge for ca 30 seconds to remove as much wash solution as possible from packing. Remove cartridge and dry inside of both stems with cotton swab or tissue paper to eliminate any remaining wash solution.

Reprime cartridge by adding 150 µL acetonitrile to inlet bed support disk and let solvent soak into packing for 30 seconds. Attach cartridge to dry glass or plastic 10mL Leur tip syringe, retaining same stem as inlet.

Insert silica gel cleanup column into 250mL vacuum flask fitted with one-hole rubber stopper. Wash column with five mL ether. Add seven mL ether to syringe cartridge positioned above silica gel cleanup column. With plunger, slowly force through cartridge (in portions), collecting eluate in column reservoir. Pull ether slowly through silica cleanup column, using vacuum to maintain flow rate ca 10mL/min (fast drops). Rinse silica column with 2mL additional ether, continuing to use vacuum. Discard ether.

Remove column and stopper from flask and place 16 × 125 mm collection tube in flask to catch eluate from column. Add 7mL elution solvent (Methylene chloride-alcohol) to column reservoir. Pull solvent through column with vacuum at ca 10mL/min flow rate, collecting eluate in tube.

Discontinue vacuum and remove collection tube from assembly. Evaporate eluate just to dryness under nitrogen stream, using heat to keep collection tube near room temperature or under vacuum at ≤35°C.

Transfer residue to one dram vial with Methylene chloride and evaporate to dryness under nitrogen on steam bath or in heating block ≤50°C (Do not overheat dry residue). Save for derivative preparation.

11.5 Liquid Chromatography

Prepare derivative of residue from above by adding 200 µL hexane and 200 µL trifluoroaceticacid to dry residue in vial. Shake on vortex mixer ca 5-10 seconds. Let mixture sit for 10 minutes at 40°C, in heating block or bath; then evaporate to dryness under nitrogen on steam bath or heating block (<50°C). Add 2mL water-acetonitrile (75 + 25) to vial to dissolve residue and mix well using Vortex mixer for LC analysis.

For derivatizing of standard M1, add 200 µL hexane and 50 µL trifluoroacetic acid to silylated vial and mix. Add 50µL M1-M2 working standard solution directly into hexane-trifluoroacetic acid mixture and mix using vortex mixer 5-10 seconds. Treat as described for test solution. Stabilize instrument and detector for suitable period at flow rate of 1.0 mL/min with water-isopropanol-acetonitrile (80 + 12 + 8). Adjust detector attenuator so that 50-100µL injection of standard (0.625-1.25 ng M1, 0.125-0.25 ng $M_{2)}$ gives 50-70% full-scale recorder pen deflection for aflatoxin M1. Inject LC standard 2-3 times until peak heights are constant. Prepare standard curve from either peak heights or peak areas to ensure linear relationship. Inject test solutions (typically 50-100µL) with standard injections interspersed to ensure accurate quantitation. Retention times M1 (as trifluoroacetic acid derivative) and M_2 are ca 4-5 min and ca 7 min, respectively. Calculate aflatoxin concentration.

$$\text{ppb (M1 or } M_2) = \frac{H \times C' \times VI' \times V}{H' \times VI \times W}$$

Where, H and H' = peak height or area of injected test solution and LC standard, respectively; C'=concentration of standard (ng/µL); VI' and VI = volume injected of standard and test solution, respectively; V = final total test solution volume (µL); and W= volume of milk represented by test solution (typically 20mL). Separately calculate concentration for M1and M_2.

References

AOAC Official Methods of Analysis (2005), Ch.49.3.06 Method, 986.16

12.0 DETERMINATION OF AFLATOXINM1 IN LIQUID MILK BY IMMUNOAFFINITY COLUMN CHROMATOGRAPHY FOLLOWED BY LIQUID CHROMATOGRAPHY

(Applicable to determine aflatoxin M1 in raw liquid milk at >0.02 ng/mL)

Caution: This method requires the use of solutions of aflatoxin M1., which are carcinogenic to humans. Aflatoxins are subject to light degradation. Protect all analytical work procedures from light and store aflatoxin standard solutions in amber colored vials or covered with aluminum foil. The use of non acid-washed glassware (e.g., vials, tubes, flasks) for aflatoxin aqueous solutions may cause a loss of aflatoxin. Special attention should be taken with new glassware. Thus, before use, soak glassware in dilute acid (e.g. sulfuric acid, 110 mL/L) for several hours; then rinse extensively with distilled water to remove all traces of acid (check with pH paper).

12.1 Principle

The test portion is extracted and cleaned up by passing through an immunoaffinity column containing specific antibodies bound onto a solid support. Antibodies selectively bind aflatoxin M1 (antigen) in the extract, to give an antibody-antigen complex. Other components of matrix that are unbound are washed off the column with water. AflatoxinM1 bound to the column is eluted with acetonitrile and concentrated. The amount of aflatoxin M1 is determined by LC and fluorometric detection.

12.2 Performance Standards for Immunoaffinity Columns

The immune-affinity column shall contain antibodies against aflatoxin M1 with a capacity of binding not less than 100 ng aflatoxin M_+ (which corresponds to 2 ng/mL when 50mL test portion is applied). Recovery of not less than 80% must be obtained for aflatoxin M1 when a calibrant solution containing 4 ng toxin is applied (which corresponds to 80 ng/L for a load volume of 50mL).

Any immune-affinity column meeting the above specifications can be used. Check the performance of the column regularly, at least once for every batch of columns.

12.3 Apparatus

(a) Disposable syringe barrels.—To be used as reservoirs (10 and 50mL capacity).

(b) Vacuum system.—For use with immunoassay columns.

(c) Centrifuge.—To produce a radical acceleration of at least 2000gm.

(d) Volumetric pipets

(e) Microsyringes.—100, 250, and 500 µL (Hamilton, or equivalent).

(f) Glass beakers.

(g) Volumetric flasks.—50 mL.

(h) Water bath.- 37°±2°C.

(i) Filter paper.— Whatman No. 4, or equivalent.

(j) Conical glass tubes- 5 and 10mL, stoppered.

(k) Spectrophotometer.—Wavelength 200-400nm, with quartz cells of optical length 1 cm.

(l) Liquid Chromatography equipment.—With pump delivering a steady flow rate of 0.8 mL/min; loop injection system of 50-200µL capacity; flourescent detection with 365 nm excitation and 435 nm emission; and recorder, integrator, or computer-based processing system.

(m) Reversed-phase LC analytical column— The suitable columns may be used

(n) Mobile phases- Water –acetonitrile (75+25) or (67+33); water-acetonitrile-methanol (65+25+10);' or water-isopropanol-acetonitrile (80 + 12 + 8). Degas before use.

12.4 Reagents

(a) Chloroform,--Stabilized with 0.5-1.0% ethanol.

(b) Nitrogen.

(c) Aflatoxin M1 standard solutions.—

(1) Stock standard solution.—1 µg/mL. Suspend a lyophilized film of reference standard aflatoxin M1 in acetonitrile to obtain the required concentration. Determine the concentration of aflatoxin M1 by measuring its absorbance at the maximum (ca 365 nm) in a calibrated spectrophotometer against acetonitrile as a blank between 200-400 nm. Check purity by nothing an undistorted shape of the recorded peak. Calculate the mass concentration (C, µg/mL) from the equation:

$$C = \frac{100AM}{\varepsilon}$$

Where, A is the measured absorbance at the maximum wavelength, M is the molecular mass of aflatoxin M1 (328 g/mol), and ε is the absorption coefficient of aflatoxin M1 in acetonitrile (1985m^2/mol). Store this stock solution in a tightly stoppered amber vial below 4°C. This is stable ca 1 year.

(2) Working Standard Solution.—0.1 µg/mL. Transfer by means of a syringe 50µL of the standard stock solution, into an amber vial and evaporate to dryness under a steady stream of Nitrogen. Dissolve the residue in 500 µL acetonitrile by vigorously vortexing in a Vortex mixer. Store this solution in a tightly stoppered amber vial below 4°C. Solution is stable ca 1 month.

(3) Calibrant standard solutions.- Prepare on the day of use. Bring working standard solution, to ambient temperature. Prepare a series of standard solutions in the mobile phase, of concentrations that depend upon the volume of the injection loop in order to inject, e.g. 0.05-1.0 ng aflatoxin M1.

12.5 Preparation of Test Solution

Warm milk before analysis to ca 37°C in a water bath, and then gently stir with magnetic stirrer to disperse the fat layer. Centrifuge liquid milk at 2000 × g to separate the fat and discard thin upper fat layer. Filter through one or more paper filters, collecting at least 50mL. Let immuno- affinity column reach room temperature. Attach syringe barrel to the top of immuno affinity cartridge. Transfer 50mL (V_s) of prepared test portion using a volumetric flask or volumetric pipet into syringe barrel and let it pass through immuno affinity column at slow steady flow rate of ca 2-3mL/min. Gravity or vacuum system can be used to control flow rate.

Remove syringe barrel and replace with a clean one. Wash column with 20mL water at steady flow rate. After washing completely, blow column to dryness with nitrogen steam. Put another dry clean barrel on the cartridge. Slowly elute aflatoxin M1 from column with

4mL pure acetonitrile. Allow acetonitrile to be in contact with column at least 60 seconds. Keep a steady slow flow rate. Collect eluate in conical tube. Evaporate eluate to dryness using gentle stream of nitrogen. Dilute to volume V_f with mobile phase, i.e., 200µL (for 50µL injections) to 1000µL (for 250µL injections).

12.6 LC Determination with Fluorescence Detection

Equilibrate the LC column with the mobile phase at a constant flow rate. Depending on the kind of column, the acetonitrile-water ratio and flow rate of the mobile phase may be adjusted to ensure optimal separation of aflatoxin M1 from other extract components. As a guideline for conventional columns (with a length of 250 mm and id of 4.6 mm), a flow rate of ca 0.8mL/min gives optimal results. Check optimal conditions with aflatoxin M1 calibrant solution and spiked milk before analyzing test materials. Check linearity of injection calibrant solutions and stability of chromatographic system. Repeatedly inject a fixed amount of aflatoxin M1 calibrant solution until stable peak areas or heights are obtained. Peak areas or heights corresponding to consecutive injections must be within ±5%. Retention times of aflatoxin M1 can vary as a function of temperature and must be monitored by injecting a fixed amount of aflatoxin M1 calibrant solution at regular intervals.

(1) Calibration curve of aflatoxin M1.—Inject in sequence suitable volumes V_i depending on the injection loop, aflatoxin M1 standard solutions containing from 0.05 to1 ng. Prepare a calibration graph by plotting the peak area or peak height against the mass of injected aflatoxin M1.

(2) Analysis of purified extracts and injections scheme.—Inject suitable volume V_i (equivalent to at least 12.5mL milk) of eluate into LC apparatus through injection loop. Using the same conditions as for calibrant solutions, inject calibrants and test extracts according to stipulated injection scheme. Inject an aflatoxin M1 calibrant with every 10 injections. Determine aflatoxin M1 peak area or height corresponding to the analyte, and calculate aflatoxin M1 amount W_a in test material for the calibration graph, in ng. If aflatoxin M1 peak area or height corresponding to test material is greater than the highest calibrant solution, dilute the eluate

quantitatively with mobile phase and re-inject the diluted extract into the LC apparatus.

12.7 Calculations

Calculate aflatoxin M1 mass concentration of the test sample, using the following equation:

$W_m = W_a \times (V_f / V_i) \times (1/ V_5)$

Where W_m =the numerical value of aflatoxin M1 in the test sample in ng/mL (or µg/L); W_a = the numerical value of the amount of aflatoxin M1 corresponding to the area or height of the aflatoxin M1 peak of the test extract (ng); V_f = the numerical value of the final volume of redissolved eluate (µL);

V_i = the numerical value of the volume of injected eluate (µL)

V_s = the numerical value of volume of prepared test portion passing through the column (mL).

Express the results to 3 significant figures.

References

AOAC Official Methods of Analysis (2005), Ch.49.3.07 Method, 2000.08

13.0 DEOXYNIVALENOL (DON) IN WHEAT

Deoxynivalenol (DON) is one of the 150 related compounds. Known as trichothecenes that are formed by a number of species of *Fusarium*. The main commodities affected are cereals such as wheat, rice, barley, oats and maize etc.

13.1 Determination of Deoxynivalenol

13.1.1 Apparatus

(1) Grinder

(2) Chromatographic tube – polypropylene (10 mm i.d ×50 mm)

(3) Filter flask – 125mL fitted with a rubber stopper having a hole to hold chromatographic tube

(4) TLC/HPTLC Plates – Precoated 20 × 20 cm silica gel plates. Dip plates in 15% aluminium chloride solution prepared by dissolving 1.5gm $AlCl_3.6H_2O$ in 15mL water and 85mL alcohol. Let stand in vertical position for 5 minutes to drain.

Remove residual aluminium chloride from back of plate with wet paper. Air dry for 2 hours and activate for 1 hour at 105°C. store in dust tight cabinet.

(5) Viewing cabinet fitted with long wavelength UV lamp or Densitometer

13.1.2 Reagents

(1) Activated Charcoal

(2) Alumina , neutral- 80 – 200 mesh

(3) Diatomaceous Earth – acid washed Celite 545

(4) Aluminium Chloride solution – spray reagent – 20gm $AlCl_3.6H_2O$ in 100 mL alcohol

(5) Deoxynivanelol Standard solution

(a) Stock Solution – 0.5 mg/mL. Weigh 5.0 mg DON into a 10mL glass stoppered volumetric flask, dilute to volume with ethyl acetate – methanol (19 + 1) and shake to dissolve.

(b) TLC/HPTLC working standard – 20 µg/mL – Pipette 1mL of DON stock solution into a 25mL volumetric flask and dilute to volume with ethyl acetate– methanol (19 +1) and shake to dissolve.

13.1.3 Preparation of sample

Grind large sample (2 – 4 Kg) to pass through 20 mesh sieve

13.1.4 Extraction

Weigh 50gm prepared sample into 500mL glass stoppered conical flask. Add 200mL acetonitrile- water (84 + 16) mixture, secure stopper with tape and vigorously shake for30 minutes on shaker. Filter and collect 29mL filtrate in 250mL graduated cylinder.

13.1.5 Column chromatography

Secure chromatographic tube to a 125mL filter flask. Place a small plug of glass wool at the bottom of the tube and add about 0.1gm Celite. Weigh 0.7gm charcoal, 0.5gm alumina, and 0.3gm Celite. Place in 50mL beaker and mix with a spatula. Add mixture to chromatographic tube. Tap tube lightly to settle packing. Apply suction and place a ball of glass wool on top. Apply 20mL of the extract (filtrate) to column and apply vacuum. Flow rate should be 2-3 mL/min wit 20 cm Hg vacuum. As solution reaches top of packed bed rinse cylinder with 10mL acetonitrile – water and add rinse to column and continue aspiration till flow stops. Do not let column go dry between addition of extract and rinse. Cover vacuum nipple with Aluminium foil and evaporate solvent slowly to dryness on steam bath. Do not contaminate sample with water from condensing steam. It is essential that no water droplets remain in flask on cooling. Add 3 mL of ethyl acetate to residue and heat to boiling on steam bath and gently swirl to dissolve DON.

Transfer solution to small vial, rinse with three 1.5mL portions of ethyl acetate. Evaporate to dryness and retain dry residue for TLC/HPTLC. Final extract, represents 5gm sample.

13.1.6 Thin Layer Chromatography

Dissolve residue in vial in 100 µL chloroform – acetonitrile (4 + 1). Apply 5 and 10 µL aliquots side by side 1, 2 , 5 . 10 and 20 µL working standard solution (20 µg DON/mL) Develop plate with chloroform – acetone – Isopropanol (8 + 1 + 1) in unequilibrated tank (development time is about 1 hour). Remove plate, let solvent evaporate completely at room temperature. Residual solvent can result in fading of DON spots. Heat plate for 7 min in upright position at 120°C. Place plate on cool surface in dark1 minute. Observe DON as blue florescent spot under long wave UV light at Rf about 0.6. Quantify DON by comparing florescence intensity of test spots with those of standard DON spots visually or by densitometer.

Calculate DON as follows:

DON ng/ gm = S x (C / X) x (V / W)

Where,

S = µL standard equal to test spot

C = concentration of standard solution (20 µg/mL)

X = µL test solution that has florescence intensity equal to standard spot

V = Final volume of test solution (µL)

W = amount of test portion represented by final test solution

(Ref :- AOAC 17th edn, 2000 Official Method 986,17 Deoxynivalenol in Wheat – Thin Layer Chromatographic Method)

14.0 PATULIN

Patulin is produced by certain fungal species of *Penicillium*, *Aspergillus* and *Byssochlamys* growing on fruits including apples, pears. grapes and other fruits. The risk arises when unsound fruit is used for production of juices and other products

14. 1 Determination of Patulin

14.1.1 Principle

Patulin is extracted with ethyl acetate and extract cleaned up on a silica gel column. Patulin is detected in the eluate after concentration by TLC/HPTLC, by spraying with 3-methyl -2 – benzothiazolinone hydrazone hydrochloride –HCl reagent. Limit of detection is 20 µg/L

14.1.2 Apparatus

(1) Chromatographic tubes -22 × 300 mm with Teflon stopcock, reservoir type (250mL) for 50gm sample.

(2) Hollow polyethylene stoppers – 13 mm top diameter, 7 mm bottom diameter.

(3) Rotary evaporator

(4) Tube shaking machine - vortex or eqvt.

(5) Vials , borosilicate glass –1 , 2 , and 4 drams (4 , 8 and 15 ml) screw cap (foil or Teflon lined)

(6) Cellulose purified

(7) Beakers 250mL and 25mL.

(8) TLC/HPTLC apparatus and a spray bottle of 125mL to produce a fine even spray. and sample streaker for preparative TLC

14.1.3 Reagents

(1) Solvents benzene, ethyl acetate – redistilled

(2) Sodium Sulphate anhydrous

(3) Silica gel 60 (0.063 -0.2 mm) or equivalent.

(4) Patulin standard solution –

a) Stock standard solution – weigh 0.5mg pure crystalline patulin to nearest 0.001mg in a 5mL volumetric flask and dissolve in Chloroform to prepare 100 µg/mL stock solution

b) Working standard solution - Introduce 1mL of stock solution into a 10mL volumetric flask and dilute with Chloroform to make 10 µg/mL solution

(5) 3-methyl–2 benzothiazolinone hydrazone (MBTH) hydrochloride solution – Dissolve 0. 5gm MBTH in 100mL water. Store in refrigerator and prepare fresh every 3 days.

If necessary determine concentration of patulin as follows:-

Using a precision syringe withdraw 5mL of working solution (10 µg/mL) into a 4 dram vial and evaporate to dryness under nitrogen. Immediately add 5mL absolute alcohol. Record UV spectrum of patulin solution from 350 to 250 nm against absolute alcohol in reference cell. Determine concentration of patulin solution from Absorbance at wavelength of maximum absorption(about 275 nm) using following equation

$$\mu g \text{ patulin / mL} = (A \times MW \times 1000 \times CF)/ \varepsilon.$$

Where CF is correction factor, MW = 154 and $\varepsilon = 14600$

Store standard and stock solution at 0°C in glass stoppered volumetric flasks wrapped lightly in Al foil. Bring working standard to room temperature before use. Do not remove Aluminium foil from flask until contents have reached room temperature.

14.1.4 Extraction

Analyze apple juice immediately after opening can or bottle. Vigorously extract 50mL juice with three 50mL portions of ethyl acetate in 250mL separatory funnel. Dry combined extracts (upper phase) about 30 minutes over anhydrous sodium sulphate. Decant into graduated 250mL beaker. Wash sodium sulphate with two 25mL portions of ethyl acetate and add to extract. Evaporate to less than 25mL on steam bath under gentle stream of nitrogen (Do not evaporate to dryness). Let cool to room temp, adjust volume to 25mL mark with ethyl acetate and dilute to 100mL with benzene.

14.1.5 Column chromatography

Place glass wool plug firmly in bottom of chromatography column containing about 10mL benzene and add a slurry of 15gm silica gel in benzene. Wash sides of the tube with benzene, let silica gel settle and drain solvent to top of adsorbent. Carefully add sample extract to column, drain to top of silica gel bedand discard eluate. Elute patulin with 200mL benzene – ethyl acetate (75+25) at about 10 mL/min. Evaporate eluate to near dryness under Nitrogen on steam bath. Quantitatively transfer residue to 4 dram vial with chloroform and evaporate to dryness under Nitrogen on steam bath. Immediately dissolve residue in 500 µL chloroform with the aid of vortex mixer. Seal with polyethylene stopper if TLC/HPTLC is not performed on the same day, store test solution in freezer to avoid evaporation of solvent.

14.1.6 Thin layer Chromatography

(a) Preparation of plates – weigh 30gm silica gel into 300mL glass stoppered flask, add water as recommended, shake vigorously and pour into applicator. Immediately coat five 20 × 20 cm plates with 0.25 mm thickness, let plates rest until gelled. Dry coated plates for 1 hr at 110°C and store in dessicating cabinet. Alternatively use precoated plates.

(b) Preliminary TLC/HPLC – Using a 10 µL syringe spot two 5 µL spots and one 10 µL spot of test solution from above and 1, 3 ,5, 7 ,and 10 µL of working standard on imaginary line 4 cm from the bottom edge of plate. Spot 5 µL of standard solution on top of the5 µL test solution spot.

Develop plate with toluene–ethyl acetate – 90% formic acid (5+4+1) contained in V shaped metal trough inside unlined equilibrated tank with silica gel facing maximum tank volume. When solvent front reaches 4 cm from top of plate, remove plate and dry in air, preferably in the fumehood.Spray plate with 5% MBTH solution until layer appears wet, then heat for about 15 minutes at 130°C. Examine plate in transmitted and reflected long wave UV light. Patulin appears as a yellow brown florescent spot at Rf about 0.5. 1 µL standard solution should be just detectable.

Patulin may also be seen as a yellow spot under visible light if concentration is more than 0.05µg. Examine TLC/HPTLCpatterns. Fluorescent spot from test solution thought to be patulin must have an Rf value and colour identical to patulin standard spot when test solution and internal standard spots are super imposed. The spot from internal standard and test solution should be more intense than either test solution spot or standard alone. Visually compare fluorescence intensity of patulin spots from test solution and internal standard. If intensity of the test spot is between two standard spots interpolate or rechromatograph spotting appropriate quantity of test solution and standard solution to obtain closer estimate. If weakest test spot is too intense to match standards, dilute test solution and re chromatograph.

14.1.7 Calculation

$$\mu g \text{ Patulin} = \frac{S \times Y \times V}{50 \times X}$$

Where, S = µL standard equal to test solution

Y = Concentration of standard in µg/mL

V = Volume of test solution

X = µL test solution spotted giving fluorescent intensity equal to S (standard) Sprayed TLC plate shall slowly turn to blue on standing in air for few hours unless covered by second glass plate.

(Ref:- AOAC 17th edn 2000 Official Method 974.18 Patulin in Apple Juice)

Note:- Patulin can also be determined by Liquid Chromatographic method. AOAC 17th edn 2000, Official Method 995.10

15.0 DETERMINATION OF OCHRATOXIN IN BARLEY

Ochratoxin A (OTA) causes kidney and liver damage and is carcinogenic in some animals. Observe precautions while handling standards and material

15.1 Principle

OTA acids and esters are extracted from barley by using chloroform and aqueous phosphoric Acid. The acids are entrapped on an aqueous diatomaceous s earth column. Esters and fat are removed wth hexane and chloroform, and acids are eluted with formic acid–chloroform. Esters are isolated by entrapment on methanol–aqueous sodium bicarbonate-diatomaceous earth column, fats are removed with hexane-benzene, and esters are eluted with formic acid-hexane-benzene. Compounds are determined from fluorescence after TLC. All glassware must be free of alkaline soap or detergent residues to avoid loss of toxins.

15.2 Apparatus

a) Chromatographic coulmns with stop cocks: 700 × 17 mm and 350 × 25 mm

b) Wrist-action shaker

c) Büchner funnels.—Glass, 9 cm diameter fitted with Whatman GF/B glass fiber paper, or equivalent; 24 cm diameter fitted with Whatman No. I paper, or equivalent.

d) Thin-layer chromatographic apparatus

e) Densitometer

15.3 Reagents

a) Diatomaceous earth.—Soak ~ 900gm acid-washed Celite 545 overnight in methanol. Filter through double thickness Whatman No. I paper or equivalent over Buchner and wash with 8 L water, and dry at 150°C for 12 hours

b) Silica gel for thin-layer chromatography: Test adsorbent for resolution and fading of ochratoxins. Ochratoxins on occasion fade rapidly on some silica gel plates, especially when exposed to ≥60% humidity. Protect plate from humidity during spotting by placing in chamber under nitrogen or under stream of warm air from hair dryer, or by covering with clean glass plate. After development, dry plate at 50°C for 15 minutes and immediately cover with clean glass plate, using tape on sides as spacers, for protection during scanning densitometry.

c) Solvents: Chloroform, hexane, acetic acid, methanol, formic acid (90%), and phosphoric acid (85%).

d) Methanolic sodium bicarbonate solution.—Dissolve 0.3gm sodium bicarbonate in 30 mL water and add 70 mL methanol.

e) Alcoholic sodium bicarbonate solution.—Dissolve 6.0gm sodium bicarbonate solution in 100mL water and add 20mL alcohol.

f) Formic acid–benzene–hexane solution.—Shake 100mL benzene–hexane (20:80) with 10mL of water–methanol (30 + 70), let layers separate. Discard lower layer. Shake upper layer with 5mL formic acid, let separate, and discard lower layer.

g) Boron trifluoride 14% (w/v): Bubble gaseous BF3 into chilled alcohol. (*Caution: Perform in hood. Avoid contact with skin, eyes, and respiratory tract.*)

h) Developing solvents.--(1) Benzene–methanol–acetic acid (18:1:1).--Combine 2 volumes methanol–CH3COOH (1 + I) with 18 volumes benzene. Adjust benzene (methanol–CH3COOH) ratio, if necessary, to produce required resolution. Decrease benzene to increase Rf.

i) Hexane–acetone–acetic acid (18: 2: 1).—Combine 3 volumes acetone–CH3COOH (2 + 1) with 18 volumes hexane. Adjust hexane: (acetone–CH3COOH) ratio, if necessary, to produce required resolution Decrease hexane to increase Rf.

j) Ochratoxin standard solutions.— Prepare original solutions, each ca 40 µg/mL, in acetic acid-benzene (1:99) Determine concentration using the table of Molecular weights and absorptivities of ochratoxins given below. Dilute to required concentration (1-5 µg/mL) using benzene.

k) Purified cotton: Wash 50gm absorbent cotton in beaker with 1 L of chloroform. Decant solution, evaporate residual solvent, and store cotton in closed container.

Table of Molecular weight and absorption of ochratoxins

Ochratoxin	λ max (nm)	Molecular Weight	Molar absortion coefficient (ε)
A	333	403	5550
B	320	369	6000
A Ethyl ester	333	431	6200
B ethyl ester	320	397	6500

15.4 Preparation of Test Sample and Extraction

Weigh 50gm of sample into a 500mL glass-stoppered Erlenmeyer flask and add 25mL 0.1M Phosphoric acid and 250mL Chloroform, and secure stopper with masking tape. Shake for 30 minutes using a wrist-action shaker and filter through glass fiber paper, covered with ca 10gm diatomaceous earth, using a 9 cm Büchner funnel.

15.5 Separation of Ochratoxins

(a) ***Removal of esters***: Plug a 700 x 17 mm chromatographic tube with the purified cotton. Mix 2.0 gm diatomaceous earth with 1mL 1.25% sodium bicarbonate solution in 50mL beaker. Add to chromatographic tube and tap firmly. Mix 50mL filtrate with 40mL hexane, and add to column. Reserve remainder of filtrate for confirmation of identity (Step 15.8). Elute at maximum flow rate; then elute with 75mL chloroform. Combine eluates, evaporate to dryness on steam bath, and reserve for ochratoxin ester separation.

(b) ***Removal of acids***.-Elute Ochratoxins A and B with 75mL freshly prepared formic acid-chloroform (1 + 99), and collect in 250mL Erlenmeyer. Immediately add 2 boiling chips and evaporate nearly to dryness on steam bath, and quantitatively transfer residue to 15mL conical centrifuge tube with chloroform. Evaporate to dryness under gentle stream of nitrogen on steam bath. *(Note: Delay in evaporation of solvent may result in loss of ochratoxins.*) Reserve residue for TLC.

15.6 Separation of Ochratoxin Esters

Prepare column as described above using 350 x 25 mm chromatographic tube, 2.5mL methanolic sodium bicarbonate solution, and 4gm diatomaceous earth. Dissolve residue (**from 15.5a**) in 50mL hexane and add to column. Rinse extraction vessel with each subsequent solvent in turn; add rinses to column. Force eluting solvents through column at convenient rate with compressed gas at 1-2 psi (6.9-13.8 kPa). Do not let liquid fall below top of column. Elute with 50mL benzene-hexane (1 + 9) previously equilibrated with 2.5mL methanolic sodium bi carbonate solution (discard). Then elute with 100mL formic acid-benzene-hexane mixture. Immediately evaporate eluate to dryness, quantitatively transfer to conical centrifuge tube with chloroform, evaporate to dryness under gentle stream of nitrogen on steam bath, and reserve for TLC.

15. 7 Thin Laye Chromatography

a) Visual analysis: Use appropriate silica gel and dissolve residue from 15,5b in 750 µL acetic acid-benzene (1 + 99). Spot 3, 5, 7.5, and 10 µL on same plate, spot 10 µL L extract superimposed with 10 ng each ochratoxin A and B standard solutions as internal standard. Also spot 5, 7.5, and 10 µL, ochratoxin A and B standard solutions. Develop plate to solvent stop line, but <90 min, with benzene-methanol-acetic acid (18 + 1 + 1) in unlined, unequilibrated tank. Remove plate, let solvent evaporate at room temperature, and view in dark under long- and shortwave UV lamp. The Rf of Ochratoxins A and B should be in the range of 0.4-0.8. Ochratoxin A is above B, typically at 0.65 and 0.5, respectively. Ochratoxin A fluoresces most intensely under long wave UV, while ochratoxin B is brightest under shortwave light. Examine the pattern of test solution for fluorescent spots for spots with Rf close to those of standards and with similar appearance.Compare fluorescence intensities of test solution spots with those of standard spots, and determine standard and test spot that match most closely, interpolating, if necessary. If, concentration of test spots is outside range of standards, concentrate or dilute, solution and rechromatograph. Calculate concentration ochratoxin A in µg/kg. Spray plate with alcoholic sodium bicarbonate and dry at room temperature. View spots in dark under long wavelength light. Fluorescence should

have changed from greenish blue to blue and increased in intensity. Again estimate ochratoxin .A in tea, In case of disagreement, use estimate obtained before spraying.

Perform TLC of ochratoxin A and B esters from 15.6 on plate in same manner as above for acids, and develop plate in hexane-acetone-acetic acid (18 + 2 + 1). Rf value of Ochratoxin A ester is ca 0.5, above ochratoxin B ester.

b) Densitometry:

Prepare and develop as described for visual analysis. In separate channels spot about 4-5 spots with increasing amounts standard ochratoxin A in range 3-10 ng/spot. Scan the plate in a densitometer or scanner following the manufacturer's instructions. Optimum spectral settings for ochratoxin A are excitation at 310-340 nm and emission, 440-475 nm. Plot standard curve from instrument response for linearity and system performance. Dissolve residue from 15.5(b) in 0.5mL acetic acid-benzene and spot replicates of at least two test extracts and standard of ≥ 3µL each. The test extract must have ochratoxin within the standard concentration range.

15.8 Confirmation of Identity of Ochratoxins A and B by formation of Ethyl Esters

Dissolve residue from 15.5 (b), containing equivalent of ≥ 10gm test sample, in 5mL chloroform in a 25mL Erlenmeyer. Into separate 25mL Erlentheymeyer add 10 ng of ochratoxin A and B standard solution. (This step may be omitted when Ochratoxin A and B ester standards are available.) Add 10mL of 14% boron trifluoride. Heat to boiling point and hold on steam bath 5 minutes. Transfer to a separator funnel containing 30 mL water. Extract with three 10mL portions of chloroform. Combine extracts, wash with three 10mL portions of water and evaporate to dryness. Quantitatively transfer to a centrifuge tube with chloroform, and evaporate to dryness under a gentle stream of nitrogen. Dissolve residue in 250 µL acetic acid-chlroform, (1 + 99) and do TLC as described under 15.7a, with the following modifications:

Spot 10 µL each of 1) underivatized test solution, 2) esterified (derivatized) test solution, 3) standard Ochratoxin esters, and 4) esterified test extract plus 10 µL standard esters. Develop plate with benzene-methanol. Examine plate under long- and short UV. Ochratoxin

A ester has a greater than Rf than that of Ochratoxin A ester, typically 0.8 and 0.7, respectively. The Rf of the Ethyl esters is greater than ochratoxins A and B, but have the same fluorescence intensity. For positive confirmation, the Ochratoxin A or B spots should be absent after esterification.

Reference

AOAC 17th edition , 2000 Official Method 973. 37 Ochratoxins in Barley.

JAOAC, 56, 817, 822(1973).

16.0 DETERMINATION OF AFLATOXINS B1, B2, G1, AND G2 IN FOODSTUFFS OTHER THAN DESCRIBED ABOVE

16.1 Chemicals and Reagents

Hexane, diethylether, peroxide-free, dried petroleum ether, toluene, dichloromethane, chloroform, acetone, acetonitrile, methanol, water, distilled, acetic acid, trifluoroacetic acid, sodium chloride, sodium sulfate, paraffin oil, RP-18 cartridge, 6 mL/1g sorbent, silica gel cartridge, 3 mL/0.5g sorbent, standard: aflatoxin B1, B2, G1, and G2, (Aflatoxin standard Kit, 1 mg each).

16.2 Sample Preparation for Spices

1. Grind or homogenize sample and mix 5.6gm with 100mL methanol for 3 min.
2. Add 40mL water, mix for 4 minutes, leave to stand for 10 minutes and then filter.
3. Shake 20mL of filtrate with 20mL Sodium chloride solution (10%) and 20mL petroleum ether for 2 minutes and leave to separate for 10 minutes (extraction of matrix in petroleum ether).
4. Shake aqueous phase with 50mL dichloromethane for 1 minute and leave to separate (extraction of aflatoxins in dichloromethane).
5. Dry, dichloromethane phase with 5gm sodium sulfate, filter and evaporate to dryness.

6. Dissolve residue in 0.5mL toluene–acetonitrile (98:2). Use extract (= 0.8gm sample) for application to the HPTLC layer.

For some critical matrices such as paprika, it is advisable to dissolve the residue in 2 mL toluene–acetonitrile (98:2) and perform further purification:

1. Purification of the extract on a silica gel cartridge: Rinse the sorbent with 6mL toluene–acetonitrile (98:2) (Do not let the sorbent run dry). Elute extract and rinse remaining matrix with 20mL toluene-acetic acid (9:1) and 20mL hexane–diethylether–acetonitrile (6:3:1) (dry the sorbent between and in the end). Elute the aflatoxins fraction with 7 and 4 ml dichloromethane–acetone (3:1) directly into a pear shaped flask (dry sorbent between and in the end).

2. Evaporate eluate to dryness and take up the residue in 0.5mL methanol.

3. Purification of the extract on an RP-18 cartridge: Rinse cartridge with 2mL methanol, dry and condition with 4mL methanol–water (2:8) and 2mL water (Do not let the cartridge run dry). Load the extract and rinse remaining matrix with 5mL methanol–water (2:8), dry for 1 min. Elute the aflatoxins with 4 × 2.5mL methanol –water (5:5) direct in a pear shape flask (dry sorbent between and in the end).

4. Shake aqueous phase for 1 min with 20mL Sodium chloride solution (10%) and 18mL dichloromethane and leave to separate for 5min (extraction of aflatoxins in dichloromethane). Separate dichloromethane phase. Repeat extraction of the aqueous phase with 2mL dichloromethane.

5. Evaporate eluate to dryness and take up the residue in 0.5mL toluene–acetonitrile (98:2).

6. Use extract (= 0.8 gm sample) for application to the HPTLC layer.

16.3 Sample Preparation for Other Commodities

Use a higher weighted amount (e.g. 80gm for nuts) if necessary and adjust the amounts of solvent, etc. accordingly.

16.4 Standard Solution

Make up a standard mixture of aflatoxins B1, B2, G1, and G2 in toluene–acetonitrile (98:2) containing 200 pg/L each of aflatoxins B1 and G1 and 100 pg/L each of G2 and B2.

16.5 TLC/HPTLC plates

HPTLC plates or sheets silica gel, 20 × 10 cm or 20 × 20 cm.

16.6 Sample Application

Apply bandwise, distance from lower edge of sheet 10 cm (for plates 6 cm), band length 8 mm, distance between tracks 4 mm, distance from left edge 15 mm.

16.7 Application Pattern

S1 U U U U S1 U U U S1 (S1 = standard mixture 5 µL each, U = sample of 100µL each)

16.8 Chromatography

2-Dimensional development (in opposing direction) in twin-trough chambers.

1. For the first dimension, which removes the matrix from the start zone, fill the first chamber to a depth of 5cm with peroxide-free, dried diethyl ether and place the sheet or plate (6 cm free side downwards) in the chamber: migration distance 50 mm (sheet) and 40 mm (plate), respectively. View sheet or plate under UV 366 nm; the fluorescent aflatoxins should have migrated little or not at all from the start zone. Cut off the top 85-90 mm (sheet) and 25-30 mm (plate), respectively and turn the plate or sheet through 180°.

2. For the second dimension, which separates the aflatoxins, charge the second chamber normally (to a depth of about 8 mm) with chloroform:acetone:water (140:20:0.3) and insert plate or sheet; migration distance 80 mm (sheet) and 60 mm (plate), respectively.

16.9 Densitometric Evaluation

TLC/HPTLC scanner with CATS software, fluorescence measurement at 366/>400 nm, single level calibration via peak height confirmed by a multilevel calibration (Fig. 17.16 (B).

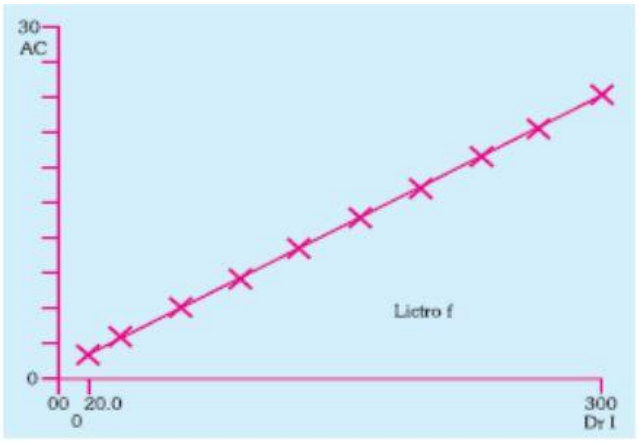

Figure 2a: Calibration function of aflatoxin B1 (Peak height) after dipping in paraffin oil-n hexane

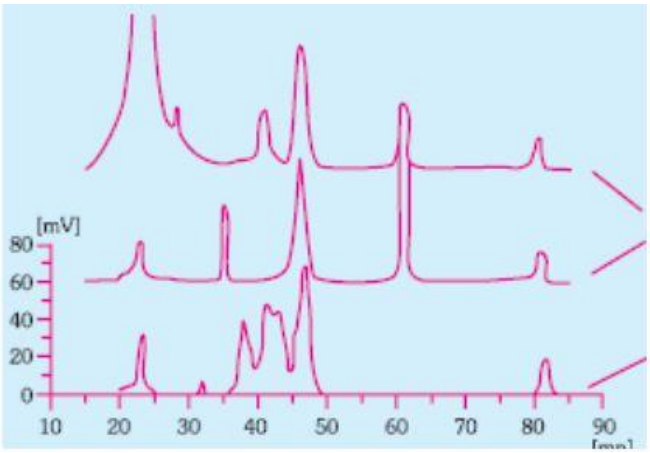

Figure 3b: TLC chromatogram with standard mixture and aflatoxin extracts from different types of paprika (extract additional purified)

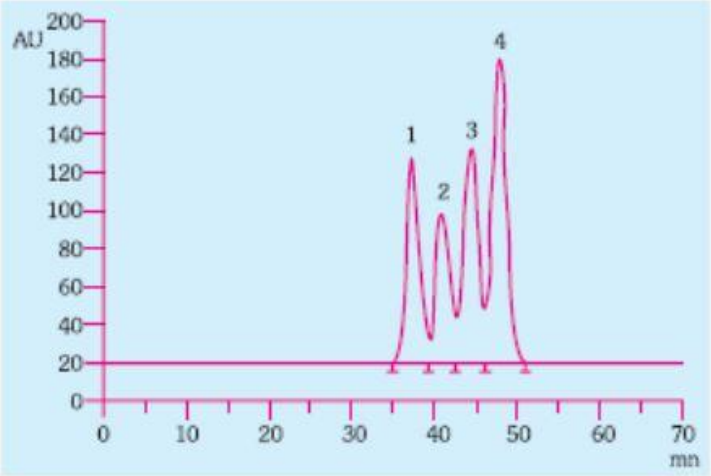

Figure 3c: HPTLC chromatogram with standard mixture (1 = G2; 2 = G1; 3 = B2; 4 = B1)

16.10 Discussion

Recovery is between 70 and 100%. The detection limit is 10 pg for aflatoxins B1 and G1 and 5 pg for B2 and G2. This can be improved 2 to 3-fold by dipping in paraffin oil-n-hexane (2:3).

Positive results can be confirmed by development after pre-chromatographic derivatization. For this purpose, additionally apply 5mL trifluoroacetic acid to the start zones, leave for 5 min, then heat for 2 min at 35-40°C on a plate heater. The derivatives of aflatoxins B1 and G1 are now polar and stay behind at the start; B2 and G2 lie in the medium Rf range.

16.11 Precautions

1. Avoid contact of aflatoxins with your skin.
2. Aflatoxins are sensitive to light and oxidation. Store chromatographed HPTLC plates or sheets, standards, extracts, etc. in the dark at about 5°C.
3. Verify the concentration of aflatoxin stock or standard solutions regularly by photometry.
4. Place contaminated materials at least for 30 min in 6% Javel water.
5. Aflatoxins are able to accumulate at synthetic material and falsify results. Avoid contact with such materials, e.g. plastic tip of an Eppendorf pipette.

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