

LAB. MANUAL 7



**MANUAL OF METHODS  
OF  
ANALYSIS OF FOODS**

***MYCOTOXINS***



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MINISTRY OF HEALTH AND FAMILY WELFARE  
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## 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 mycotoxicoses. Although mycotoxicosis cause by mould *Claviceps purpurea* have been known for a long time mycotoxins remained neglected till 1960 when the Aflatoxins were discovered. Mould growth on 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 genera, *Aspergillus*, *Penicillium* and *Fusarium*.

The chemical structures of some important mycotoxins are given below.

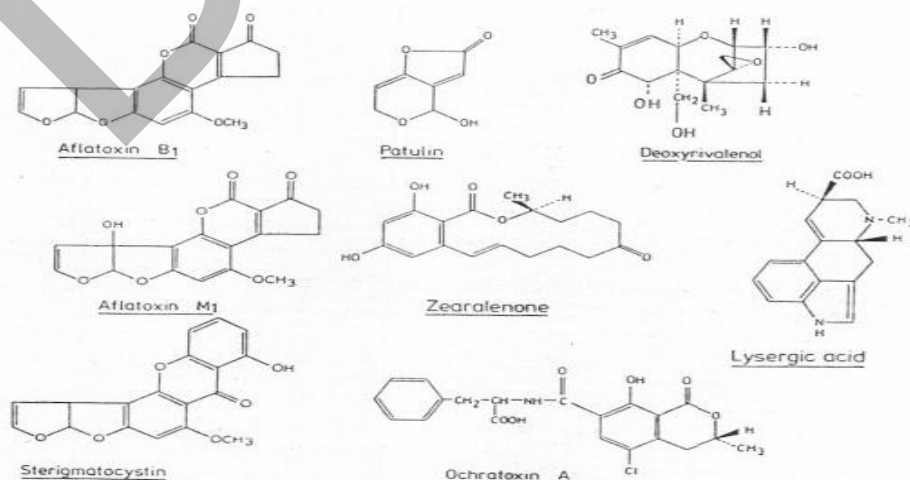


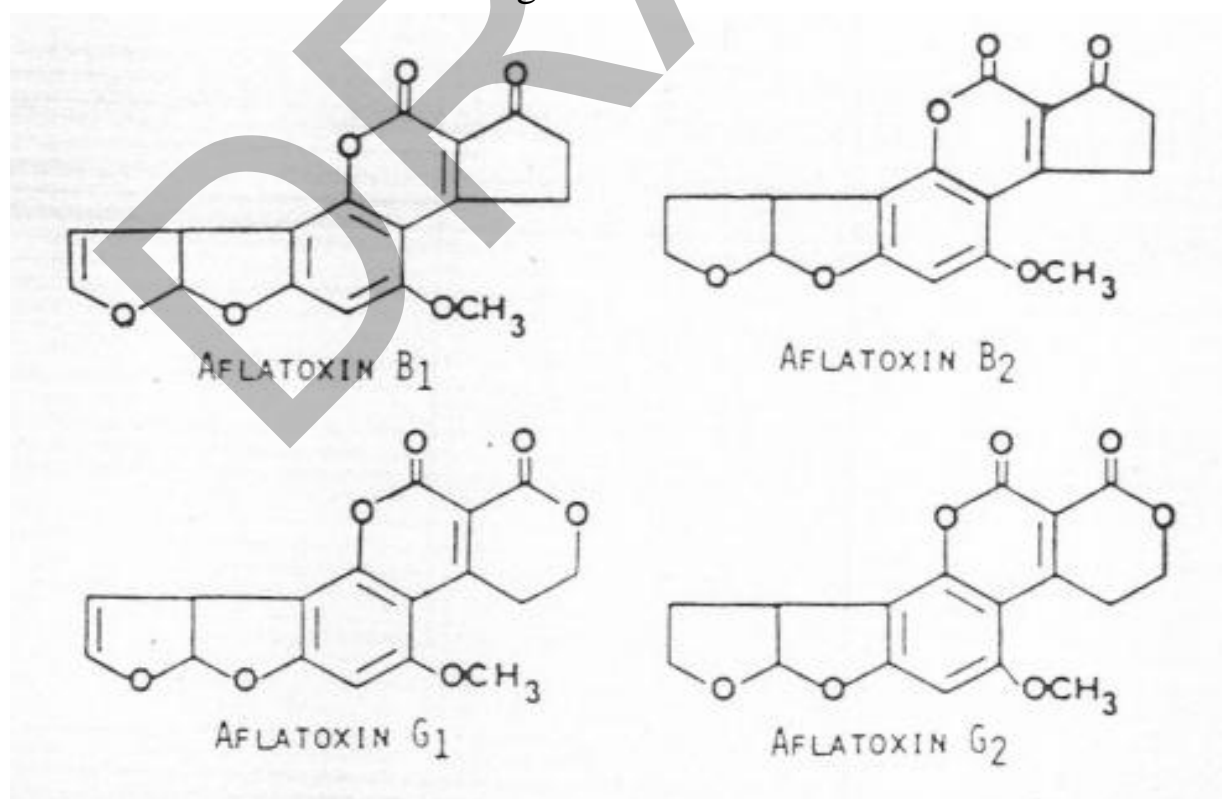
Figure 1. Chemical structures of some important mycotoxins.

## 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 oil made from contaminated seeds or nuts usually contains aflatoxin. However aflatoxin is destroyed in the refining process so that refined oils are safe.

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

Their chemical structures are given below.



Aflatoxin B1, B2, G1, and G2 refers to toxins which fluoresce blue (B) or green (G) under ultraviolet light and are separable by thin layer chromatography. The only structural difference between B and G toxins is the inclusion of an oxygen in the cyclopentanone ring. Aflatoxin M 1 and M 2 represent the toxin B 1 and B 2 which have been metabolized within the body of a lactating animal. Their finding in milk led to their M designation. The obvious structural difference between B and M is the addition of the hydroxyl group.

(Ref: - F.A.O Manuals of Food Quality Control 14 / 7, 1986, page 285)

The analytical methods for aflatoxin include thin layer chromatography (TLC), High performance liquid chromatography (HPLC), and enzyme-linked immunosorbent assay (ELIA). TLC has been most widely used method. Aflatoxins are extremely potent carcinogens to many animals. Neither effects of aflatoxins on man nor possible routes of entry are presently known.

Aflatoxins are subject to light degradation. Protect analytical materials adequately from daylight and keep aflatoxin standard solutions protected from light by using amber vials or aluminium foil.

Use of acid-washed glassware for aflatoxin aqueous solutions may cause loss of aflatoxin. Before use, soak new glassware in dilute acid (e.g., sulfuric acid, 2M; carefully add 105 ml H<sub>2</sub>SO<sub>4</sub> to water to remove all traces of acid.

(Ref: - AOAC Official Methods of Analysis (2000), Ch.33)

## 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 are to be taken as they are electrostatic. Work preferably in a hood. Swab any accidental spill of toxin with 1% Sod. Hypochlorite bleach (NaOCl), leave 10 minutes and then add 5 % aqueous acetone. Rinse all glassware exposed to aflatoxin with methanol, add 1% Sod 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% Sod. Hypochlorite solution overnight and washed in water.

Reactive vapours i.e O<sub>2</sub>, SO<sub>2</sub>, HCl can affect adsorbents as well as stability of adsorbed spots. Perform TLC only in laboratory free of volatile reagents. Always dry 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 presence of solvent. Avoid exposure to UV light of under developed spots and expose developed plates to UV light for minimum time needed for visualization Aflatoxins are subject to light degradation. Protect analytical material adequately from daylight 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 H<sub>2</sub>SO<sub>4</sub> 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).

(Ref: - F.A.O Manuals of Food Quality Control 14 /7, 1986, page 185 / A.O.A.C 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 thoroughness of mixing to achieve effective distribution of contaminated portions. One contaminated peanut (ca 0.5 gm) 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 50 gm 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 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 finer size. In case of liquids mix thoroughly to obtain homogeneous sample.

(b) Preparation of Laboratory Sample Draw with same precaution as with lot sample. Wherever practical, divide by riffing or similar random dividing procedure until sub-division is close to the desired analytical sample.

(Ref :- A.O.A.C 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 container of dry aflatoxins B1, B2, G1, G2 add volume 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  $\mu\text{g} / \text{ml}$ .

For Aflatoxin M 1 use benzene acetonitrile (9+ 1). Use label statement of Aflatoxin weight as guide. Vigorously agitate solution 1 minute on vortex shaker and transfer without rinsing to convenient sized glass flask. Do not transfer dry Aflatoxins for weighing or other purposes unless facilities are available to prevent dissemination to 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 concentration to 8- 10  $\mu\text{g} / \text{ml}$

(Ref :- A.O.A.C 17th edn , 2000, Official Method 971.22 Standards for Aflatoxins)

### 1.4 Preparation and storage of working standards

Dilute portions of stock solution to spotting concentration (0.5  $\mu\text{g} / \text{ml}$ ). Use same solvent used to prepare aflatoxin standards. Use benzene – acetonitrile (9 + 1) to dilute Aflatoxin M 1 solution. Before storage weigh flasks to nearest mg and record weight for future reference. Wrap flasks tightly in Aluminium foil and store at 0 °C. When 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 Al 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: - A.O.A.C 17th edn 2000, Official Method 971.22 Standards of aflatoxin, subpara 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) X 300 mm length.
- (4) High speed Blender
- (5) Funnel – 7.5 cm dia or Buchner Funnel with Whatman No1 filter paper
- (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

50 gm of powdered representative sample is taken in a 500 ml conical flask and 25ml water, 25 gm diatomaceous earth (Celite) and 250 ml 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. Filter through 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 50 ml of filtrate.

## 2.3 Column Chromatography

Place ball of glass wool loosely in bottom of 22 x 300 mm chromatographic column and add 5 gm of anhydrous Sod Sulphate to give base for silica gel. Add Chloroform until tube is about  $\frac{1}{2}$  full. Pour 10 gm of silica gel made into a slurry with chloroform. Wash sides of the tube with about 20 ml Chloroform and stir to disperse silica gel. When rate of settling slows, drain some  $\text{CHCl}_3$  to aid settling, leaving 5 – 7 cm above silica gel. Slowly add 15 gm anhydrous Sod sulphate. Drain  $\text{CHCl}_3$  to top of Sod. Sulphate. Add 50 ml of sample extract to column, elute at maximum flow rate with 150 ml hexane followed by 150 ml anhydrous ether and discard. Elute Aflatoxin with 150 ml 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 extract residue, add 200  $\mu$ l benzene – acetonitrile (98+ 2) and reseal with polythene stopper. Shake vigorously to dissolve. Puncture polythene stopper to accommodate needle of 10  $\mu$ l syringe. In subdued incandescent light and as rapidly as possible spot 2, 5 and 10  $\mu$ l spots on imaginary line 4cm from bottom of TLC plate. Keep vial for quantitative analysis. On same plate spot 2.5 and 10  $\mu$ l of aflatoxin standards Place 50 ml acetone –  $\text{CHCl}_3$  (1+9) in trough of unlined developing tank. Use only 1 plate per tank, placing trough to one side to permit maximum exposure of coated surface to tank volume. Immediately insert plate into tank and seal tank. The chamber must be saturated with solvent before use.

Develop plate 40 minutes or until aflatoxins reach  $R_f$  0.4-0.7. remove from tank, evaporate solvent at room temperature and view under long wave UV lamp in a viewing chamber. Observe pattern of 4 florescent spots

## 2.5 Quantitative TLC

If preliminary TLC shows need for further dilution of test solution, evaporate to dryness on a steam bath and redissolve in calculated amount of benzene – acetonitrile. Spot successively 3.5, 5.0, and 6.5  $\mu$ l of test solution. All spots should be approx same size and equal to 0.5 cm diameter. On the same plate spot 3.5, 5.0, 6.5  $\mu$ l aflatoxin standards. After development of the plate, dry the plate in subdued light. Compare florescent intensities of the sample spot with those of the standard spots and determine which of the sample spot matches one of the standards. If the

spots of the smallest quantity of sample are too intense to match standards, the sample should be diluted and rechromatographed.

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

Where,

S =  $\mu\text{l}$  aflatoxin standard which matches the unknown

Y = Concentration of Aflatoxin B1 standard  $\mu\text{g} / \text{ml}$

V =  $\mu\text{l}$  of final dilution of sample extract.

X =  $\mu\text{l}$  of sample extract spotted giving florescent intensity equivalent to S (B1 standard)

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

(10 gm if 50 ml  $\text{CHCl}_3$  extract is used)

Calculate Aflatoxin B2 , G 1 , and G 2 similarly.

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

### **3.0 Determination of Aflatoxins (For Peanut and Peanut Products, Cereals and Pulses - BF Method)**

#### **3.1 Extraction**

Weigh 100 gm of peanut meal or powder or 50 gm peanut butter into a blender jar. Add 250 ml methanol – water ( 55 + 45) and 100 ml hexane to peanut butter and 500 ml methanol – water (55 + 45) 200 ml hexane and 4 gm Sod. Chloride to peanut powder. Blend 1 minute at high speed.. Transfer to 250 ml centrifuge bottle and centrifuge 5 minutes at 2000 rpm, otherwise let mixture stand undisturbed in blender jar when separation will occur within 30 minutes. Pipette 25 ml of lower aqueous methanol phase in a separating funnel, add 25 ml chloroform stopper and shake 30 – 60 seconds. Let layers separate and drain bottom  $\text{CHCl}_3$  layer through

anhydrous sod. sulphate into a 250 ml beaker. Repeat extraction with two 25 ml portions of chloroform.

Collect in a beaker and evaporate combined chloroform extract to between 2 ml and just dryness. Do not leave beaker on hot plate after solvent has evaporated. Transfer extract with careful washing to screw capped borosilicate vial and evaporate to dryness under gentle stream of nitrogen, dissolve extract in 200  $\mu$ l benzene – acetonitrile (98 + 2), spot on TLC or HPTLC along with known 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 :- A,O,A,C 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**

##### **4.1 Apparatus**

- (1) High Speed Blender
- (2) Ultraviolet light – Long wave UV with intensity of 430 u watt/ cm<sup>2</sup>. at 15 cm at 365 nm
- (3) Minicolumn – Borosilicate standard wall tubing 6 ( 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) Pot. Hydroxide wash solution – 0.02 M KOH with 1% KCL Dissolve 1.12 gm KOH pellets and 19 gm KCL in 1 litre water
- (3) Sod. Hydroxide Solution – 0.02 M – 8.0 gm NaOH / litre
- (4) Sulphuric acid Solution 0.03% – Dissolve 0.3 ml H<sub>2</sub>SO<sub>4</sub> in 1 litre
- (5) Precipitating reagents – (1) Copper carbonate – Basic (2) Ferric Chloride Slurry – Mix 20 gm of FeCl<sub>3</sub> with 300 ml water.
- (6) Diatomaceous Earth
- (7) Column packing (a) Florisil 100 – 200 mesh (b) Silica gel 60 for column chromatography (c) Alumina Neutral – 80 – 200 mesh - activate 2 hrs at 110<sup>0</sup>C (d) Calcium Sulphate anhydrous – 20 – 40 mesh. Dry packing material 1- 2 hrs at 110<sup>0</sup>C. 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 Minicolumn

Trap small plug of glass wool into tapered end of column. To column add to height indicated in the following order – 5-7 mm CaSO<sub>4</sub>, 5-7 mm Florisil, 18-20 mm silica gel, 8-10 neutral alumina and 5-7 mm CaSO<sub>4</sub>. Tamp small plug of glass wool on top of column. Tamp column after each addition to settle packing and maintain interfaces as level as possible. After packing apply pressure to top glass wool plug with 5 mm dia glass rod.

#### 4.4 Extraction

Weigh 50 gm test sample into the blender jar, add 250 ml acetone – water (85+ 15) and blend 3 minutes. Alternatively use 500 ml glass stoppered Erlenmeyer flask and shake 45 minutes on mechanical shaker. Filter through Whatman filter paper No 4 into 250 ml graduated cylinder. Collect 150 ml filtrate and transfer to 400 ml beaker.

#### 4.5 Purification

To a 600 ml beaker quantitatively add 170 ml of 0.02N Sod Hydroxide and 30 ml  $\text{FeCl}_3$  slurry and mix well. To the filtrate in 400 ml beaker add about 3 gm basic Copper carbonate, mix well and add to the mixture in 600 ml beaker. To this add 150 ml diatomaceous earth and mix well. Filter using 160 mm funnel or Buchner funnel using Whatman filter paper No 4. Quantitatively transfer 150 ml filtrate to 500 ml separator, add 150 ml 0.03% Sulphuric acid and 10 ml Chloroform. Shake vigorously for about 2 minutes and let separate. Transfer lower  $\text{CHCl}_3$  layer (13-14 ml) to 125 ml separator. Add 100 ml KOH wash solution, swirl gently 30 seconds and let separate. If emulsion occurs drain emulsion into 10 ml glass stoppered flask, add about 1 gm anhydrous Sod Sulphate, stopper shake 30 seconds and let separate ( $\text{CHCl}_3$  phase need not be completely clear). If emulsion is not broken, transfer emulsion to 125 ml separator and wash with 50 ml 0.03%  $\text{H}_2\text{SO}_4$ . Collect 3 ml Chloroform layer in 10 ml glass stoppered cylinder for chromatography.

## 4.6 Chromatography

Transfer 2 ml of  $\text{CHCl}_3$  solution to minicolumn using 5 ml syringe with 5 in, 15 gauge needle. Let drain by gravity (15 – 30 mins). When solvent reaches top of adsorbent, add 3 ml elution solvent ( $\text{CHCl}_3$  – acetone (9 + 1)). Let drain by gravity until solvent again reaches top of adsorbent. Do not let columns run dry during determination. Examine columns in darkened room under UV lamp. Look for blue fluorescent band at the top of florisil layer (ca 2.5 cm from bottom of column) 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 :- A.O.A.C 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 and purified, derivatised with trifluoroacetic acid (aflatoxin B1 and G1 to B2 and G2 respectively) 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) LC column – 15 cm x 4.6 mm i.d. Supercosil LC -18 No5 – 8230
- 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 x 1cm i.d with Teflon stopcock and coarse frit bed support, detachable glass solvent reservoir with 24/40 fitting
- (d) Adjustable pipettes – 10-100 and 100 – 200 µl with disposable tips
- (e) Filter tube – glass 15 x 2.5 cm i.d with coarse frit bed support ( glass wool not recommended)

## 5.3 Reagents

- (a) Solvents – distilled – in – glass 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 – H<sub>2</sub>O - CH<sub>3</sub>CN - CH<sub>3</sub>OH (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, (0.063-0.2mm). activate by drying at 100°C. Cool to room temperature. Weigh desired quantity (100 gm) into glass stoppered container. Add 1ml water in small increments, agitate silica gel between additions. Shake or tumble mechanically 4 -6 hrs. Let stand 16 hrs
- (d) Trifluoroacetic acid (TFA) – Assay by titration – equal to or more than 98.5%. Transfer 1-2 ml TFA to a 1 dram vial with 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 2-3 hrs at 600°C to remove organic impurities.
- (f) Aflatoxin standard solutions –
- (1) Aflatoxin stock solution – 10 µg / ml. Prepare individual stock solution in benzene-CH<sub>3</sub>CN(98+2) and determine concentration of each by measuring UV absorption if desired.
  - (2) Working standard solutions - Use Eppendorf pipette to transfer appropriate quantity stock solution to each 4 dram vial to obtain final concentrations of aflatoxins in each vial.

Vial	B1 and G1 ng	B2 and G2 ng
1	250	125
2	500	250
3	1000	500
4	2000	1000

Evaporate solutions to dryness under gentle stream of nitrogen (drying may be facilitated by warming to 40 °C). Using Eppendorf pipette add 200 µl hexane and 50 µl of TFA to each vial cap and vortex mix 30 seconds. Let solutions stand 5 minutes, then add 10 ml H<sub>2</sub>O – CH<sub>3</sub>CN (9+1) and vortex mix 30 seconds. Let layers separate 5 -10 min or centrifuge at 1000 rpm for 30 sec. Final concentration of aflatoxins shall be:

Vial	B1and G1ng/10.05ml	B2and G2 ng/ 10.05ml
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 50 gm prepared corn, or peanut powder or peanut butter to 1 litre blender jar, add 200 ml of methanol followed by 50 ml of 0.1M HCl and blend 3 minutes at high speed. Filter through 24 cm whatman No 1 filter paper. Filtrate may not be completely clear. Collect 50 ml filtrate. Transfer to 250 ml separatory funnel. Add 50 ml 10% NaCl solution, swirl, add 50 ml hexane and shake gently about 30 seconds. Let phases separate and drain lower aqueous layer into another 250 ml separatory funnel. Discard hexane layer. Add 25 ml CH<sub>2</sub>Cl<sub>2</sub> and shake moderately 30 seconds. If emulsion occurs break up with clean pipette. Let phases separate and drain lower CH<sub>2</sub>Cl<sub>2</sub> layer through coarse granular anhydrous sod. sulphate in glass filter tube.. Collect elute in 250 ml beaker. Evaporate elute on steam bath under gentle stream of nitrogen to 2 -3 ml.

### 5.5 Column Chromatography

Slurry 2 gm silica gel with about 10 ml ether – hexane (3+1) in 30 ml beaker, pour slurry into clean up column and wash beaker with additional 5 ml ether – hexane solvent to effect transfer.. Keep stop cock closed and let silica gel settle without tamping. Wash sides of column with 2-3 ml ether – hexane using wash bottle. After gel settles, open stop cock and while column drains add about 1 cm anhydrous sod. sulphate. Transfer eluate collected after extraction to column. Wash beaker with about 2 ml of  $\text{CH}_2\text{Cl}_2$  and add wash to column. Do not use more than 5- 6 ml  $\text{CH}_2\text{Cl}_2$  to transfer eluate to column. With stop cock fully open, add 25 ml benzene – acetic acid (9+1) and the 30 ml ether – hexane (3+1) to column draining each wash to top of sod. sulphate. Discard washes. Elute aflatoxin with 100 ml  $\text{CH}_2\text{Cl}_2$  – acetone (90+ 10) and collect elute in 250 ml beaker. Evaporate elute on steam bath under gentle stream of nitrogen to about 6 ml. Quantitatively transfer to 3 dram vial.

Evaporate elute to dryness to steam bath in an aluminium block under gentle stream of nitrogen. Evaporate remaining 200  $\mu\text{l}$  just to dryness under gentle stream of nitrogen by holding vial in palm of hand and slowly rotating vial.

### 5.6 Derivatisation

Add 200  $\mu\text{l}$  hexane to the residue obtained above. Then add 50  $\mu\text{l}$  of TFA using Eppendorf pipette, cap vial and vortex mix vigorously for 30 seconds. This procedure must be followed closely to ensure consistent reaction yields. Let mixture stand 5 minutes. Using Eppendorf pipette add 1.950 ml  $\text{H}_2\text{O}$  - $\text{CH}_3\text{CN}$  (9+1). Vortex mix vigorously for exactly 30 seconds and let layers separate 10 mm. Concentration is 10 gm / 2 ml aqueous  $\text{CH}_3\text{CN}$ .

## 5.7 Determination

Using instrument parameters mentioned under apparatus successively inject 25 µl of derivatised standard solution. Prepare standard curve to check linearity of responses. Inject 25 µl of TFA treated test solution (lower aqueous phase). If test peaks are outside the linear range, dilute aliquot of TFA treated test solution to suitable volume with water – CH<sub>3</sub>CN, remix on vortex mixer and inject another 25 µl portion.

Calculate individual aflatoxin concentration as follows:

Use responses of standard containing 500 ng B1 and G1 and 250 ng B2 and G2 for calculation

$$\text{Aflatoxin ng/ gm} = (P/P') \times C \times (2/10) \times 1000 \times D$$

Where,

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

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

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

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

## 6.0 Determination of Aflatoxin M 1 in Milk, Cheese and Khoya

Aflatoxin M1 is 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 250 ml
- (2) Centrifuge
- (3) Chromatographic column – 22mm i.d X 300 mm length with a tap.
- (4) TLC plates
- (5) Filter paper No 1
- (6) Micropipettes 5- 100  $\mu$ l, 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) Sod Chloride solution – Saturated solution, about 40 gm / 100 ml
- (3) silica gel for column chromatography- E Merck silica gel No 60 (70 -230 Mesh) or equivalent. Stir 1hr in methanol, filter, treat similarly with  $\text{CHCl}_3$  Activate by drying 1 hour at 105  $^{\circ}\text{C}$ . Add water 1 ml / 100 gm, shake until thoroughly mixed and store 15 hrs in air tight container
- (4) Sod 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 50 ml milk, 10 ml of saturated salt solution (40 gm Na Cl / 100 ml water), and 120 ml chloroform at 30  $^{\circ}\text{C}$  in a 250 ml separating funnel and allow to separate for 2 minutes For milk powder reconstitute 5 gm with 50 ml water, for

cheese and Khoya blend 15 gm sample with 1 ml saturated salt solution, 5 gm diatomaceous earth or celite and 100 ml chloroform for 60 seconds in a blender jar.

Drain lower  $\text{CHCl}_3$  layer into 125 ml erlenmeyer flask. Centrifuge if layers do not separate (15 minutes at 2000 rpm). Add 10 gm anhydrous. Sod sulphate to  $\text{CHCl}_3$  with stirring. Filter into 100 ml graduated cylinder. Save filtrate for column chromatography.

#### 6.4 Column chromatography.

Fill column half full with  $\text{CHCl}_3$ . Add 2 gm silica gel slurry made with  $\text{CHCl}_3$ . Add 2 gm Sod sulphate above silica gel. Drain off excess  $\text{CHCl}_3$  and rinse silica off column sides with  $\text{CHCl}_3$ . Add sample extract and drain entire solution through column by gravity if flow rate slows stir sod sulphate gently. Rinse graduated cylinder with  $\text{CHCl}_3$  and add rinsings to column. Wash column with 25 ml toluene – acetic acid (9 + 1) to remove coloured compounds. Wash with 25 ml of hexane – ether – acetonitrile (5 + 3 + 2) to remove fat. Elute Aflatoxin M1 with 40 ml  $\text{CHCl}_3$  – acetone (4 + 1).

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

#### 6.5 Thin Layer chromatography.

Dissolve sample residue in 100  $\mu\text{l}$  of benzene – acetonitrile (9 + 1), mix well and spot on TLC or HPTLC.

Spot 20  $\mu\text{l}$  of test solution and 2, 4, 6, 8, and 10  $\mu\text{l}$  M1 standard (0.25  $\mu\text{g}/\text{ml}$ ).

Develop plate in chloroform - acetone – isopropanol (87 + 10 + 3) and calculate

Aflatoxin M1 in  $\mu\text{g}/\text{kg}$  or ppb as follows:

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

Where,

S = volume ml standard which matches with sample

Y = Concentration of M1 standard in ug / ml

V = Volume final dilution of sample extract in ul

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:- A,O,A,C 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-MSMS**

### **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 Vol. Flask, Para film, 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 (MeOH) HPLC grade, Tween-20, Acetonitrile HPLC, 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 120ppb and the working standards solutions of 1.0, 2.5, 5.0, 7.5, 10, and 12.5ppb concentration are prepared as follows;
- Aflatoxin Standard dilution Procedure:
  - Stock Solution (1 ml) in Acetonitrile- 20mg/ml (20ppm)
  - Add 250 $\mu$ l of the stock diluted to 50ml (50:50 V/V, MeOH: H<sub>2</sub>O)  
Vol. flask – 100ppb (Afla-Mix)

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

- 10 $\mu$ l of 100ppb (Afla-mix) +990 $\mu$ l of MeOH: H<sub>2</sub>O (50:50) – 1.0ppb
- 25 $\mu$ l of 100ppb (Afla-mix) + 975 $\mu$ l of MeOH: H<sub>2</sub>O (50:50) - 2.5ppb
- 50 $\mu$ l of 100ppb (Afla-Mix) +950 $\mu$ l of MeOH: H<sub>2</sub>O (50:50) – 5.0ppb
- 75 $\mu$ l of 100ppb (Afla-Mix) +925 $\mu$ l of MeOH: H<sub>2</sub>O (50:50) - 7.5ppb
- 100 $\mu$ l of 100ppb (Afla-Mix) +900 $\mu$ l of MeOH: H<sub>2</sub>O (50:50) – 10ppb
- 125 $\mu$ l of 100ppb (Afla-Mix) +875 $\mu$ l of MeOH: H<sub>2</sub>O (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  $\mu$ L

Mobile phase Gradient :

Time	Mobile phase-A	Mobile phase-B
0.0	50	50
4.0	10	90
7.0	10	90
7.2	50	50
9.0	50	50

**Instrument:**

Triple quadrupole HPLC-MSMS and Analytical Column RP-18 end-capped, 150x4.6mm, 5 $\mu$ m particle size or its equivalent, Flow rate: 0.3-1.0 ml per min.

Depending on column ID & length

Run time: 9-12 min.

**MSMS 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 25g of sample into a 250ml Conical flask, to this add 5g of AR/GR grade Sodium Chloride and 100mL of Aflatoxin extraction solution (containing 70% methanol & 30% water mix in case of Rice and incase of coffee & oleoresin the extraction solution shall be 80% methanol & 20% water mix). Cap & seal the conical flask with Para film, Shake at about 140rpm on a horizontal shaker for 30 minutes. After shaking keep the flasks standing for 5minutes 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 50ml graduated measuring cylinder and to this add 30ml of H<sub>2</sub>O.

If the sample is nutmeg, Oregano or black pepper, from the 150ml beaker take 10ml of the filtrate into 50ml graduated measuring cylinder 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 4 ml vial under the tip of the column and add 1mL of methanol to the column collect the eluate and again add 1ml H<sub>2</sub>O 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 ( $\mu\text{g/g}$  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  $\geq 3$ . 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  $\pm 5\%$  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  $\pm 20\%$  for peaks greater than 50% of the base peak and to  $\pm 50\%$  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/  $\mu\text{g}/\text{kg}$ ,  $m$ =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, 4<sup>th</sup> 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

## **8.0 Determination of Aflatoxins B1, B2, and G1 in Corn, Cottonseed, Peanuts, and Peanut Butter by Enzyme-Linked Immunosorbent**

### **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/g; no color development indicates that test sample contains aflatoxins at  $\geq 20$  ng/g.

## 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

Calibrate aflatoxin B1 standard according to 970.44 (see 49.2.02) and 971.22A-C (see 49.2.03).

- (a) Negative control test sample – Follow procedure in enzyme immunoassay for corn, J(a).
- (b) Threshold-level standard – Used to define lower limit of determination. Dispense 100  $\mu$ L working standard into test tube. Add 350  $\mu$ L methanol-buffer, E(d), (30+70), and mix. Follow procedure in enzyme immunoassay for corn, J(a), steps (2), (4)-(7).
- (c) Positive control test sample – Use working standard solution; follow procedure for enzyme immunoassay for corn, J (a), steps (2), (4)-(7).

Negative control test sample should develop blue color; positive control test sample should have no color development. Threshold standard should show no color development.

#### A. Reagents

Items (a)-(h) are available as Immuno Dot Screen (IDS) Cup, (International Diagnostic Systems Corp., PO Box 799, St. Joseph, MI 49085, USA). Reagents from other 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.
- (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 0.23g  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ , 1.95 g  $\text{K}_2\text{HPO}_4 \cdot 3 \text{H}_2\text{O}$ , 8.70 g NaCl, 0.125 mL Tween 20 (polyoxyethylene[20]sorbitan monolaurate), and 10 mg thimerosal (ehtymercurithiosalicylic acid, sodium salt), in 900 mL  $\text{H}_2\text{O}$  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 g/L  $\text{H}_2\text{O}$ ), pH 8.3.



- (f) Substrate solution B – Hydrogen peroxide (0.02% H<sub>2</sub>O<sub>2</sub> in 0.13% aqueous citric acid solution), pH 3.0 (Kirkegaard and Perry Laboratories, Inc., 2 Cessna Ct, Gaithersburg, MD 20879, USA).
- (g) Methanol, hexane, and chloroform – Reagent grade.
- (h) Standard aflatoxin B1 – Approximately 28 µg as dry film.

### 8.3 Apparatus

Equipment specified is not restrictive; other suitable equipment can be substituted.

High- speed blender – With 500 mL jar

- (a) Micropipette and tips- recommended range 100-1000 µL; use with disposable polypropylene tips.
- (b) Glass culture (test) tubes- 10\*75 mm; 3mL.
- (c) Filters- Whatman No. 4 or equivalent.
- (d) Timer- Graduated in 1s intervals.
- (e) Carborundum boiling chips.

### 8.4 General Instructions

Store all kit components at 4-8 °C. Do not freeze. Before use, allow 1h for cups 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) of test samples. Negative control must be functioning properly (must develop blue colour in center of cup) 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). 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 6h at room temperature. To ensure shelf life of kit components promptly return reagents to refrigerator after use.

Because of difficulty in monitoring 1min timings 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 50 g test portion into blender jar. Add 100ml CH<sub>3</sub>OH-H<sub>2</sub>O (8+2). Blend 3 min at high speed. filter mixture and recover filtrate. alternatively, let mixture stand 10-15 min and recover supernatant liquid. Dilute extract in ratio 1:1 with extraction solvent.
- (b) Peanut butter- Weigh 50 g test portion into blender jar. Add 100 mL hexane and 250 mL CH<sub>3</sub>OH-H<sub>2</sub>O (55+45). Blend 3 min at high speed. Filter mixture and transfer filtrate to separatory funnel. Let layers separate for 10 min. Place 20 mL lower layer in 150 mL beaker. Add minimum of 15 boiling chips and heat in steam bath or on hot plate. Boil 3 min and let cool.

### 8.6 Preparation of Aflatoxin B<sub>1</sub> Standard Solutions

- (a) Stock solution- Add 3 mL CHCl<sub>3</sub> to vial containing 28 µL aflatoxin B<sub>1</sub> standard (ca 9ng/ 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  $\mu$ L buffer, E(d). prepare  $\leq 2$ h before use. Proceed as for diluted test extract.

### 8.7 Enzyme Immunoassay

(a) Corn, raw peanuts and whole cottonseed-

- (1) Allow 1 h for all reagents to reach room temperature (23-29° C).
- (2) Prepare fresh substrate in small culture (test) tube by mixing 500  $\mu$ L (10 drops) substrate solution A with 500  $\mu$ L (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.

[**Note:** Run 1 negative control cup and 1 positive standard cup each day to ensure that all reagents are functional. Threshold-level standard should be run with each set of new reagents. Negative control cup should be run by applying 100  $\mu$ L (2 drops) buffer to center of cup. For positive standard cup, apply working standard to cup as in (4). For both cups proceed the steps (5)-(7).]

- (3) Add 200  $\mu$ L test extract to 400  $\mu$ L buffer, E (d), (600  $\mu$ L total).
- (4) Thoroughly mix diluted test extract and apply one 150  $\mu$ L aliquot to center of cup. Using timer, wait 1 min and then add second 150  $\mu$ L aliquot of diluted test extract. Using timer, wait additional 1 min before proceeding to next step.
- (5) Apply 100  $\mu$ L (2 drops) enzyme solution to center of cup. Using time, wait 1 min.

- (6) Wash with 1.5 mL (30 drops) wash solution added drop wise. If more than 1 cup is being used, wash successively with 500  $\mu$ L (10 drops) per cup 3 times.
- (7) Add entire contents of substrate solution 1.0 mL (20 drops mixture) from each test tube to each cup. (Start time as soon as substrate mixture is added to cup.) Wait 1 min 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 h for all reagents to reach room temperature (23-29° C).
- (2) Prepare fresh substrate solution in small culture (test) tube by mixing 500  $\mu$ L (10 drops) substrate solution A with 500  $\mu$ L (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  $\mu$ L test extract to 500  $\mu$ L buffer, E(d), (1000  $\mu$ L total).
- (4) Thoroughly mix diluted test extract and apply one 200  $\mu$ L aliquot to center of cup. Using timer, wait 1 min and add second 200  $\mu$ L aliquot of diluted test extract. Using timer, wait additional 1 min and then add third 200  $\mu$ L aliquot of diluted test extract. Using timer, wait additional 1 min and then add third 200  $\mu$ L 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 disk (center of cup) for blue colour or no colour development at exactly 1min after adding substrate A and B mixture.

**Negative-** If disk (center of cup) turns light blue or darker, test sample contains total aflatoxin B<sub>1</sub>, B<sub>2</sub> and G<sub>1</sub> 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<sub>1</sub>, B<sub>2</sub> and G<sub>1</sub> 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

### 9.0 Determination of Total Aflatoxins (B<sub>1</sub>, B<sub>2</sub>, and G<sub>1</sub>) in Corn By Enzyme-Linked Immunosorbent Assay Method

(Applicable to detection of  $\geq 20$  ng total aflatoxins/g corn.)

Caution: Aflatoxins are carcinogenic in some animals.

The 95% confidence interval for correct identification of corn test samples positive for aflatoxin contamination at 20 ng/g level in the collaborative study was 83-100%. See Table 993.16 for the results of the interlaboratory study supporting the acceptance of the method.

## 9.1 Principle

See 990.34A (see 49.2.07).

## 9.2 Antibody Specificity

Cross-reactivities of polyclonal antibody used in the collaborative study of this method were 100, 70, 75, and <10% for aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>, respectively.

## 9.3 Sensitivity of ELISA Reagent

See **990.34D** (see 49.2.07).

## 9.4 Reagents

Antibody-coated solid support –Aflatoxin antibody-coated porous polyethly membrane, with 0.71 cm diameter opening, over layer of adsorbent material; support device consisting of high-density polyethylene housing (3.4 cm diameter \_\_\_ 2.65 cm height) with centered cone-shaped ring insert (3.09 cm diameter \_\_\_ 0.9 cm height) (Immunodot Screen Cup, International Diagnostic System Corp. [IDS], PO Box 799, S. Joseph, MI 49085, USA).

See **990.34E(b)-(h)** (see 49.2.07).

## 9.5 Apparatus

See 990.34F (see 49.2.07).

## 9.6 General Instructions

See 990.34G (see 49.2.07)

## 9.7 Extraction of Test Portion

See 990.34H (see 49.2.07).

## 9.8 Preparation of Aflatoxin B<sub>1</sub> Standard Solutions

- (a) *Stock solution.* – Dissolve 25 µg dry film aflatoxin B<sub>1</sub> in 2.5 mL methanol (10 ng/µL). Store below -20°C. In absence of acid or base, solution is stable for ca 6 months in clean container.
- (b) *Working standard.*– Dispense 250 µL stock solution into clean vial. Add 2250 µL methanol and mix. May be stored 1 month at ca 5 °C (1ng/µL).
- (c) *Buffer solution of standard.*–Prepare <2 h before use. Dispense 5 µL working standard, (b), into test tube. Add 300 µL methanol and 700 µL buffer, 990.34 E(d) (see 49.2.07), and agitate vigorously. Proceed as for diluted test extract, 990.34J (see 49.2.07).

## 9.9 Enzyme Immunoassay

(Note: Allow 1 h for reagents to reach room temperature [20-23°C].)

Prepare fresh substrate in small tube by mixing 500 µL (10 drops) substrate solution A, 990.34E (e) (see 49.2.07), with 500 µL (10 drops) substrate solution B, 990.34E(f) (see 49.2.07), for each reaction site used.

Add 100 µL test extract, H, to 200 µL buffer (total volume, 300 µL) and thoroughly mix. Apply 100 µL diluted test extract to center of membrane, 990.34E (a) (see 49.2.07). Using timer, wait 1 min.

Apply 100  $\mu\text{L}$  (2 drops) enzyme solution, 990.34E(b) (see 49.2.07), to center of membrane. Using timer, wait 1 min. Wash 1  $\times$  with 1500  $\mu\text{L}$  (30 drops) wash solution, 990.34E(C) (SEE 49.2.07), added dropwise. If > 1 reaction site is used, wash successively with 10 drops per site, 3 $\times$ .

Add entire substrate solution, 1000  $\mu\text{L}$  (20 drops), from each tube to each reaction site. Using timer, wait 1 min from addition of substrate mixture to reaction site and immediately observe site for blue color development (negative) or no color development (positive).

### 9.10 Interpretation of Results

See 990.34K (see 49.2.07), except (1) method now detects aflatoxins in corn at  $\geq 20$  ng/g and (2) add that positive test samples should be confirmed by quantitative method, such as 99.31 (see 49.2.18).

### References

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

## 10.0 Determination of Aflatoxin in corn and peanuts by Thin Layer Chromatographic Method

(applicable to determination of 5-50 ng  $B_1/g$  corn, 3-15 ng  $B_2/g$  corn, 10-50 ng  $G_1/g$  corn, 3-15 ng  $G_2/g$  corn, 5-25 ng  $B_1/g$  raw peanuts and 1.5-7.5 ng  $B_2/g$  raw peanuts by densitometry; 10-50 ng  $B_1/g$  corn, 10-25 ng  $B_1/g$  peanuts, 7.5 ng  $B_2/g$  raw peanuts, and 10-25 ng  $G_1/g$  raw peanuts by visual comparison.



## 10.1 PRINCIPLE

Aflatoxin are extracted from samples with methanol-water. Filtrate is diluted with NaCl solution and defatted with hexane. Aflatoxins are partitioned into chloroform which is then removed by evaporation. Aflatoxins are purified by chromatography on 0.5 g silica gel column, and quantitated by thin layer chromatography (TLC) on silica gel 60 plate with densitometry or visual estimation.

## 10.2 Apparatus

- (a) Wrist-action shaker.- Capable of holding four to eight 250 mL flasks.
- (b) Silica gel column.- 6 mL disposable column, packed with 40 $\mu$ m (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 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 longwave ultraviolet (UV) lamp.
- (g) Fluorodensitometer (TLC scanner).- Capable of scanning in reflectance mode by fluorescence, equipped with high-pressure Hg lamp, monochromator for adjustment to excitation 366 nm, and emission cutoff filter 420 nm.

## 10.2 Reagents

- (a) Solvents- Methanol, hexane, chloroform, anhydrous ethyl ether (100%), dichloromethane, acetone and isopropanol.
- (b) Aflatoxin standard solution.- Prepare in benzene-acetonitrile (98+2) to contain 0.5  $\mu$ g/mL each B<sub>1</sub> and G<sub>1</sub> and 0.15  $\mu$ g/mL each B<sub>2</sub> and G<sub>2</sub>.

### 10.3 Extraction and Partition

Weight 50 g (ground to pass No. 20 sieve) corn or peanuts into 500 mL glass-stoppered Erlenmeyer flask. Add 200 mL methanol-H<sub>2</sub>O(85+15) and secure stopper with masking tape. Shake vigorously by hand until samples show no clumps. Shake 30 min on wrist-action shaker, B(a), and filter mixture through medium fluted paper. Collect 40 mL filtrate in 50 mL graduated cylinder. Transfer filtrate to 125 mL separatory funnel. Add 40 mL 10% NaCl solutions, mix, and add 25 mL hexane. Shake 1 min. Let phase separate, drain lower (aqueous) phase into second 125 mL separately funnel, and discard upper phase.

Extracts aflatoxins from aqueous phase with two 25 mL portions CHCl<sub>3</sub>; shake 1min each time. Combine CHCl<sub>3</sub> fractions in 125 mL Erlenmeyer flask and evaporate to dryness on steam bath.

### 10.3 Silica Gel Column Chromatography

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

Check column suitability by adding aflatoxin B<sub>1</sub> standard (3 mL dichloromethane containing 100 ng aflatoxin B<sub>1</sub>) to 0.5 g 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 1 mL portions of dichloromethane and add rinses to column. Wash column with 3 mL hexane, 3 mL anhydrous ethyl ether, and then 3 mL 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, B(d), under each column (test tube rack can be used to hold vials).

Elute aflatoxins (without vacuum) with two to four 3 mL portions (according to results of column suitability test) of  $\text{CHCl}_3$ -acetone (9 + 1). Evaporate eluate to dryness on steam bath under stream of nitrogen.

#### 10.4 Thin-Layer Chromatography-Fluorodensitometry Determination

Dissolve residue from E in 250  $\mu\text{L}$   $\text{CHCl}_3$ . Spot plate, B(e), with 5  $\mu\text{L}$   $\text{CHCl}_3$  test solution in duplicate and 2, 5, 10, and 20  $\mu\text{L}$  aflatoxin standard solution, C(b). 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 spotting plan, either on plate or in notebook, prior to spotting.

Develop plate 1 h with  $\text{CHCl}_3$ -acetone (9 + 1). Evaporate solvent 5 min in fume hood followed by 2 min in 50°C forced draft oven. Examine plate under longwave 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 1<sup>st</sup> or 2<sup>nd</sup> lane. Scans of test spots should be within  $\pm 5\%$ ; if not, rescan entire plate.

#### 10.6 Calculation

Calculate concentration of aflatoxin  $\text{B}_1$  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.

### 10.7 Thin-Layer Chromatography-Visual Estimation

Dissolve sample extract from E in 250 μL  $\text{CHCl}_3$  and proceed as in 968.22F(a)-(d) (see 49.2.08).

### References

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

### 11.0 Determination of Aflatoxins $M_1$ and $M_2$ in Fluid Milk By Liquid Chromatographic Method

#### 11.1 Principle

Aflatoxins  $M_1$  and  $M_2$  are extracted from milk on  $\text{C}_{18}$  cartridge, eluted with ether onto silica column, eluted with  $\text{CH}_2\text{Cl}_2$ -alcohol, and  $M_1$  is derivatized with trifluoroacetic acid. Liquid chromatographic peaks are detected fluorometrically compared with standard-TFA derivatives.

## 11.2 Regents

- (a) *Solvents.* - Distilled in glass CH<sub>3</sub> CN, CH<sub>2</sub>CL<sub>2</sub>, isopropyle alcohol; reagent grade alcohol, ether (0.01% ethyl alcohol preservative), hexane, methanol, trifluoroacetic acid, and H<sub>2</sub>O (deionized, filtered through 0.45- $\mu$ m filter).
- (b) *Water-acetonitrile wash solution.*—95+5.
- (c) *Methylene chloride*—alcohol elution solution.—95+5.
- (d) *Mobile phase.*—prepare H<sub>2</sub>O-isopropyl alcohol—CH<sub>3</sub>CN (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 M<sub>1</sub> and M<sub>2</sub> (Sigma Chemical Co., or other suitable source). Prepare stock solutions (ca 200  $\mu$ g M<sub>1</sub>/mL and 100  $\mu$ g M<sub>2</sub>/mL) in CH<sub>3</sub> CN and determine concentrations according to 970.44 (see 49.2.02) and 971.22 (see 49.2.03), using extinction coefficients of 19 850 and 21 400 for M<sub>1</sub> and M<sub>2</sub>, respectively, in CH<sub>3</sub>CN. Make working standard solution containing 0.50  $\mu$ g M<sub>1</sub> and 0.10  $\mu$ g M<sub>2</sub>/mL in CH<sub>3</sub>CN.—benzene (1+9) for use in preparing M<sub>1</sub>—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 x 4.0 cm polypropylene Econo-Column with Luer tip, 35  $\mu$ g 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 (E. Merck, No. 9385) in 105°C oven for 1 h. Cool and add 1% H<sub>2</sub>O by weight. Shake in sealed container and equilibrate overnight before use. Assemble polypropylene column and 25 mL vacuum flask fitted with 1-hole stopper. Fill column to ca 2 mL mark with silica gel (ca 1 g). Pull gentle vacuum to pack bed and add ca 1 g anhydrous Na<sub>2</sub>SO<sub>4</sub> to top of silica gel bed.
- (c) *Extraction cartridges.*—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 M<sub>1</sub>-TFA derivative (e.g., Spectroflow 980, Applied Biosystems, Inc., 170 Williams Dr. Ramsey, NJ 07446, USA).
- (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) *Vacuum 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 1½ dram (4 or 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.

### 11.3 Extraction

Attach intel (longer) stem of C18 cartridge to Luer tip of 30-50 mL syringe. Assemble syringe, cartridge, and vacuum flask. Adjust vacuum to pull solvents through cartridge in fast dropwise manner (ca 5 mm Hg). Prime cartridge by adding 5 mL methanol, then 5 mL H<sub>2</sub>O 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  $\geq$  10 times to evenly distribute cream. Transfer 20 mL milk to graduate 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 40 mL warm diluted milk into syringe and gently pull liquid through cartridge at flow rate ca 30 mL/min (very fast drops). (Caution: Too fast a flow will not allow sufficient time for aflatoxin to adsorb, resulting in low recoveries.) Add 10 mL H<sub>2</sub>O-CH<sub>3</sub>CN wash solution to syringe and pull through. Plug syringe barrel with stopper and pull hard vacuum on cartridge for ca 30 s 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  $\mu$ L CH<sub>3</sub>CN to inlet bed support disk and let solvent soak into packing for 30 s. Attach cartridge to dry glass or plastic 10 mL Luer tip syringe, retaining same stem as inlet.

Insert silica gel cleanup column into 250 mL vacuum flask fitted with 1-hole rubber stopper. Wash column with 5 mL ether. Add 7 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 x 125mm collection tube in flask to catch eluate from column. Add 7mL elution solvent ( $\text{CH}_2\text{Cl}_2$ -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  $\leq 35^\circ\text{C}$ .

Transfer residue to 1 dram vial with  $\text{CH}_2\text{Cl}_2$  and evaporate to dryness under nitrogen on steam bath or in heating block  $\leq 50^\circ\text{C}$ . (Do not overheat dry residue.) Save for derivative preparation.

#### **11.4 Liquid Chromatography**

Prepare derivative of residue from D by adding 200 $\mu\text{L}$  hexane and 200 $\mu\text{L}$  trifluoroacetic acid to dry residue in vial. Shake on vortex mixer ca 5-10 s. Let mixture sit 10 min at  $40^\circ\text{C}$ , in heating block or bath; then evaporate to dryness



under nitrogen on steam bath or heating block (<50°C). Add 2 mL H<sub>2</sub>O-CH<sub>3</sub>CN (75 + 25) to vial to dissolve residue and shake well in Vortex mixer for LC analysis.

For derivative of standard M<sub>1</sub>, add 200µL hexane and 50µL trifluoroacetic acid to silylated vial and mix. Add 50µL M<sub>1</sub>-M<sub>2</sub> working standard solution directly into hexane-TFA mixture and shake on Vortex mixer 5-10 s. Treat as described for test solution. Stabilize instrument and detector for suitable period at flow rate of 1.0 mL/min with H<sub>2</sub>O-isopropanol-CH<sub>3</sub>CN (80 + 12 + 8). Adjust detector attenuator so that 50-100µL injection of standard (0.625-1.25 ng M<sub>1</sub>, 0.125-0.25 ng M<sub>2</sub>) gives 50-70% full-scale recorder pen deflection for aflatoxin M<sub>1</sub>. 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 50-100 µL) with standard injections interspersed to ensure accurate quantitation. Retention times M<sub>1</sub> (as TFA derivative) and M<sub>2</sub> are ca 4-5 min and ca 7 min, respectively. Calculate aflatoxin concentration.

$$\text{ppb (M}_1 \text{ or M}_2\text{)} = \frac{H \times C' \times VI' \times V}{H' \times VI \times W}$$

Where, H and H' = peak height 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 20 mL). Separately calculate concentration for M<sub>1</sub> and M<sub>2</sub>.

## References

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

## 12.0 Determination of Aflatoxin $M_1$ in Liquid Milk By Immunoaffinity Column by Liquid Chromatography

(Applicable to determine of aflatoxin  $M_1$  in raw liquid milk at  $>0.02$  ng/mL).

**Caution:** This method requires the use of solutions of aflatoxin  $M_1$ . Aflatoxins are carcinogenic to humans. Aflatoxins are subject to light degradation. Protect analytical work from daylight and keep aflatoxin standard solutions protected from light by using amber vials or 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 with an aflatoxin  $M_1$  (antigen) contained in the extract, to give an antibody-antigen complex. Other components of matrix are washed off the column with water. Aflatoxin $M_1$  from the column is eluted with acetonitrile. After the eluate is concentrated, the amount of aflatoxin  $M_1$  is determined by LC with fluorometric detection.

### 12.2 Performance Standards for Immunoaffinity Columns

The immunoaffinity column shall contain antibodies against aflatoxin  $M_1$  with a capacity of not less than 100 ng aflatoxin  $M_1$  (which corresponds to 2 ng/ML when 50 mL test portion is applied). Recovery of not less than 80% must be

obtained for aflatoxin M<sub>1</sub> when a calibrant solution containing 4 ng toxin is applied (which corresponds to 80 ng/L for a load volume of 50 mL).

Any immunoaffinity 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 50 mL capacity).
- (b) Vacuum system.—For use with immunoassay columns.
- (c) Centrifuge.—To produce a radical acceleration of at least 2000 g.
- (d) Volumetric pipets
- (e) Microsyringes.—100, 250, and 500  $\mu$ L (Hamilton, or equivalent).
- (f) Glass beakers.
- (g) Volumetric flasks.—50 mL.
- (h) Water bath.— $37^{\circ}\pm 2^{\circ}\text{C}$ .
- (i) Filter paper.—Whatman No. 4, or equivalent.
- (j) Conical glass tubes— 5 and 10 mL, stoppered.
- (k) Spectrophotometer.—Wavelength 200-400nm, with quartz face 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 50-200 $\mu$ L capacity; fluorescent detection with 365 nm excitation and 435 nm emission; and recorder, integrator, or computer-based processing system.
- (m) Reversed-phase LC analytical column— The following columns have been used satisfactorily: Octadecylsilane (ODS, ODS-1, ODS-2, ODS Hypersil,

Nucleosil C18 [Machery-Nagel], Chromospher C18, Nova-Pak C18 [Waters Corp.], LiChrosorb RP [Merck KGaA, Darmstadt, Germany], Microsphere C18; dimensions (mm): 100 × 2.3, 4.6, 5; 125 × 4; 200 × 2.1, 3, 4; 250 × 4.6; with and without guard columns.

- (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 M<sub>1</sub> standard solutions.—
- (1) Stock standard solution.—1μg/mL. Suspend a lyophilized film of reference standard aflatoxin M<sub>1</sub> in acetonitrile to obtain the required concentration. Determine the concentration of aflatoxin M<sub>1</sub> 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 noting an undistorted shape of the recorded peak. Calculate the mass concentration (C, μg/mL) from the equation:

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

Where, A is the measured absorbance at the maximum wavelength, M is the molecular mass of aflatoxin M<sub>1</sub> (328 g/mol), and ε is the absorption coefficient of aflatoxin M<sub>1</sub> in acetonitrile (1985m<sup>2</sup>/mol). Store this stock solution in a tightly stoppered amber vial below 4°C. 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, (c)(1), into an amber vial and evaporate to dryness under a steady stream of N. Dissolve the residue vigorously in 500 µL acetonitrile using 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 day of use. Bring working standard solution, (c)(2), to ambient temperature. Prepare a series of standard solutions in the mobile phase, **C(n)**, of concentrations that depend upon the volume of the injection loop in order to inject, e.g., 0.05-1.0 ng aflatoxin M<sub>1</sub>.

## 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 50 mL. Let immunoaffinity column reach room temperature. Attach syringe barrel to top of immunoaffinity cartridge. Transfer 50 mL (V<sub>s</sub>) of prepared test portion with volumetric flask or volumetric pipet into syringe barrel and let it pass through immunoaffinity 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 20 mL water at steady flow rate. After washing completely, blow column to dryness with nitrogen stream. Put another dry clean barrel on the cartridge. Slowly elute aflatoxin M<sub>1</sub> from column with 4 mL pure acetonitrile. Allow acetonitrile to be in contact with column at least 60 s. Keep steady slow flow rate. Collect eluate in conical tube. Evaporate eluate to dryness using gentle stream of nitrogen. Dilute to volume

$V_f$  of mobile phase, i.e., 200 $\mu$ L (for 50 $\mu$ L injections) to 1000  $\mu$ L (for 250 $\mu$ L injections).

## 12.6 LC Determination with Fluorescence Detection

Pump mobile phase at steady flow rate through LC column. 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  $M_1$  from other extract components. As a guideline for conventional columns (with a length of 250 mm and id of 4.6mm), a flow rate of ca 0.8mL/min gives optimal results. Check optimal conditions with aflatoxin  $M_1$  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  $M_1$  calibrant solution until stable peak areas or heights are obtained. Peak areas or heights corresponding to consecutive injections must be within  $\pm 5\%$ . Retention times of aflatoxin  $M_1$  can vary as a function of temperature and must be monitored by injecting a fixed amount of aflatoxin  $M_1$  calibrant solution at regular intervals.

- (1) Calibration curve of aflatoxin  $M_1$ .—Inject in sequence suitable volumes  $V_i$  depending on the injection loop, aflatoxin  $M_1$  standard solutions containing from 0.05 to 1 ng. Prepare a calibration graph by plotting the peak area or peak height against the mass of injected aflatoxin  $M_1$ .
- (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  $M_1$  calibrant with every 10 injections. Determine aflatoxin  $M_1$  peak area or height corresponding to the analyte, and calculate aflatoxin  $M_1$

amount  $W_a$  in test material for the calibration graph, in ng. If aflatoxin  $M_1$  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  $M_1$  mass concentration of the test sample, using the following equation:

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

Where  $W_m$  = the numerical value of aflatoxin  $M_1$  in the test sample in ng/mL (or  $\mu\text{g/L}$ );  $W_a$  = the numerical value of the amount of aflatoxin  $M_1$  corresponding to the area or height of the aflatoxin  $M_1$  peak of the test extract (ng);  $V_f$  = the numerical value of the final volume of redissolved eluate ( $\mu\text{L}$ );

$V_i$  = the numerical value of the volume of injected eluate ( $\mu\text{L}$ )

$V_s$  = 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 also known as 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 x 50 mm )
- (3) Filter flask – 125 ml fitted with a rubber stopper having a hole to hold chromatographic tube
- (4) TLC Plates – Precoated 20 x 20 cm silica gel plates. Dip plates in 15 %  $\text{AlCl}_3$  solution prepared by dissolving 1.5 gm  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  with 15 ml water and 85 ml alcohol.. Let stand in vertical position 5 mins to drain.  
Remove residual  $\text{AlCl}_3$  from back of plate with wet paper. Air dry 2 hrs and activate 1 hour at 105 °C. store in dust tight cabinet.
- (5) Viewing cabinet fitted with long wave 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 - 20 gm  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  in 100 ml alcohol
- (5) Deoxynivalenol Standard solution
  - (a) Stock Solution – 0.5 mg / ml. Weigh 5.0 mg DON into 10 ml glass stoppered volumetric flask, dilute to volume with ethyl acetate – methanol (19 + 1) and shake to dissolve.



(b) TLC working standard – 20 ug / ml – Pipette 1 ml of DON stock solution into 25 ml 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 20 mesh sieve

### 13.1.4 Extraction

Weigh 50 gm prepared sample into 500 ml glass stoppered conical flask.

Add 200 ml CH<sub>3</sub>CN- H<sub>2</sub>O (84 + 16), secure stopper with tape and vigorously shake 30 minutes on shaker. Filter and collect 29 ml filtrate in 250 ml graduated cylinder.

### 13.1.5 Column chromatography

Secure chromatographic tube on 125 ml filter flask. Place small ball of glass wool at bottom of tube and add about 0.1 gm celite. Weigh 0.7 gm charcoal, 0.5 gm alumina, and 0.3 gm celite. Place in 50 ml 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 20 ml 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 10 ml 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 Al 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 1.5 ml portions of ethyl acetate. Evaporate to dryness and retain dry residue for TLC. Final extract represents 5 gm sample

### 13.1.6 Thin Layer Chromatography

Dissolve residue in vial in 100ul chloroform – acetonitrile ( 4 + 1). Apply 5 and 10 µl aliquots alongside 1, 2 , 5 . 10 and 20 ul working standard solution (20 µg DON/ ml) Develop plate with CHCl<sub>3</sub> – 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 7 min in upright position at 120 °C. Place plate on cool surface in dark 1 minute. Observe DON as blue florescent spot under long wave UV light at R<sub>f</sub> 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:

$$\text{DON ng/ gm} = S \times (C / X) \times (V / W)$$

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 :- A,O,A,C 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 *Byssoschlamys* 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

#### 14.11 Principle

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

#### 14.12 Apparatus

- (1) Chromatographic tubes -22x 300 mm with Teflon stopcock, reservoir type (250ml) for 50 gm sample.
- (2) Hollow polyethylene stoppers – 13 mm top dia, 7 mm bottom dia.
- (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 250 ml and 25 ml.
- (8) Thin layer chromatographic apparatus and a spray bottle of 125 ml to produce a fine even spray. and sample streaker for preparative TLC

**14.13 Reagents**

- (1) solvents Benzene, ethyl acetate – redistilled
- (2) Sodium Sulphate anhydrous
- (3) Silica gel 60 (0.063 -0.2 mm) or eqvt.
- (4) Patulin standard solution –
  - a) Stock standard solution – weigh 0.5 mg pure crystalline patulin to nearest 0.001 mg in a 5 ml volumetric flask and dissolve in  $\text{CHCl}_3$  to prepare 100  $\mu\text{g}$  / ml stock solution
  - b) Working standard solution - Introduce 1 ml of stock solution into a 10 ml volumetric flask and dilute with  $\text{CHCl}_3$  to make 10  $\mu\text{g}$  / ml solution
- (5) 3 methyl – 2 benzothiazolinone hydrazone ( MBTH) hydrochloride solution – Dissolve 0.5 gm MBTH in 100 ml water.

Store in refrigerator and prepare fresh every 3 days.

If necessary determine concentration of patulin as follows:-

Using precision syringe withdraw 5 ml of working solution (10  $\mu\text{g}$  / ml ) into 4 dram vial and evaporate to dryness under nitrogen. Immediately add 5 ml 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 A at wavelength of maximum absorption (about 275 nm) using following equation  $\mu\text{g patulin / ml} = A \times \text{MW} \times 1000 \times \text{CF} / E$  Where CF is correction factor, MW = 154 and E = 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 Al foil from flask until contents have reached room temperature.

#### 14.14 Extraction

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

#### 14.15 Column chromatography

Place glass wool plug firmly in bottom of chromatography tube containing about 10 ml benzene and add slurry of 15 gm 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 and discard eluate. Eluate patulin with 200 ml benzene – ethyl acetate (75 + 25) at about 10 ml / min. Evaporate eluate to near dryness under N on steam bath. Quantitatively transfer residue to 4 dram vial with  $\text{CHCl}_3$  and evaporate to dryness under Nitrogen on steam bath. Immediately dissolve residue in 500  $\mu\text{L}$   $\text{CHCl}_3$  with the aid of vortex mixer. Seal with polyethylene stopper if TLC is not performed on the same day, store test solution in freezer to avoid evaporation of solvent.

#### 14.16 Thin layer Chromatography

- (a) Preparation of plates – weigh 30 gm silica gel into 300 ml glass stoppered flask, add water as recommended, shake vigorously and pour into applicator. Immediately coat five 20 x 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 Thin Layer Chromatography – 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 bottom edge of plate. Spot 5 µL of standard solution on top of the 5 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 hood. Spray plate with 5 % MBTH solution until layer appears wet, then heat about 15 minutes in 130 °C oven. Examine plate in transmitted and reflected long wave UV light. Patulin appears as 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 conc is more than 0.05 µg. Examine TLC patterns. Fluorescent spot from test solution thought to be patulin must have Rf value and colour identical to patulin standard spot when test solution spot and internal standard spot 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

**14.17 Calculation**

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

Where, S = uL standard equal to test solution

Y = Concentration of standard in  $\mu\text{g} / \text{ml}$

V = Volume of test solution

X =  $\mu\text{L}$  test solution spotted giving fluorescent intensity equal to S ( standard)  
 Sprayed TLC plate shall slowly turn to blue on standing in air for few hrs unless covered by second glass plate.

(Ref:- A.O.A.C 17th edn 2000 Official Method 974.18 Patulin in Apple Juice)

**Note:-** Patulin can also be determined by Liquid Chromatographic method.  
 A,O,A,C 17th edn 2000, Official Method 995.10 refers

**15.0 OCHRATOXIN**

There is no regulatory limit for Ochratoxin under FSS Act, 2006 so far. In case determination has to be carried out refer to A.O.A.C 17th edn , 2000 Official Method 973. 37 Ochratoxins in Barley.

**16.0 Determination of Beta-Lactam Antibiotics in Milk By Affinity Quantitative Determination**

(Applicable to levels  $\geq 0.01$  IU penicillin G/mL or  $\beta$ -lactam equivalent.)

## 16.1 Principle

Assay is based on specific, irreversible affinity of  $\beta$ -lactam antibiotics for certain enzyme sites on cell wall of microorganisms.  $^{14}\text{C}$ -labeled penicillin and *Bacillus stearothermophilus* are added to milk test portion. Antibiotic in test portion competes with  $^{14}\text{C}$ -penicillin for binding sites. Amount of bound carbon-14 is counted and compared with control to determine presence of  $\beta$ -lactam antibiotic.

## 16.2 Reagents

(Note: Standards and reagents are conveniently measured into tubes, stoppered, and frozen at  $-20^{\circ}\text{C}$  if held  $>1$  day. Keep reagents at  $<4^{\circ}\text{C}$  when used. Milk should be  $<15^{\circ}\text{C}$  when test starts.)

- (a) *Penicillin*. -free milk.- Reconstitute penicillin-free whole milk powder (Yankee Milk, Newington, CT 06111, USA) with  $\text{H}_2\text{O}$ .
- (b) *Penicillin-free skim milk powder*
- (c)  *$^{14}\text{C}$  Penicillin*.- Containing  $103\ \mu\text{Ci}/\mu\text{mole}$  (Charm Sciences, Inc., 36 Franklin St, Malden, MA 02148-4120, USA). Use  $0.0027\ \mu\text{Ci}$  in each assay.
- (d) *B. stearothermophilus*.- Use vegetative cells, ca 47 mg wet weight in each test.
- (e) *Penicillin G standards*.- (1) Zero standard.- Use penicillin-free milk, (a). (2)  $0.01\ \text{IU/mL}$  standard.- Add  $1.00\ \text{IU}$  sodium or potassium penicillin G USP reference standard to small amount of reconstituted skim milk powder in  $125\ \text{mL}$  bottle and freeze-dry. Add  $100\ \text{mL}$  penicillin-free milk, (a), and mix well.



### 16.3 Apparatus

- (a) *Heater*.- Constant temperature, dry well tube heater for 13x100 mm glass tubes (Constantemp, Roeco Manufacturing Co.).
- (b) *Cold plate*.- Accommodates 13x100 mm tubes (Charm Sciences, Inc., or equivalent).
- (c) *Radiation counter*.- Models 150 or 160 mm (Charm Sciences, Inc., or equivalent).
- (d) *Pipets*.- Semi-automatic (Absoluter, Tri-Continent Scientific, 12541 Loma Rica Dr. Graff Valley, CA 95945, USA, or equivalent).

### 16.4 Procedure

Pipet 5 mL test portion into 13 x 100 mm glass tube. Pipet in 200  $\mu\text{L}$   $^{14}\text{C}$ -penicillin and mix. Pipet in 200  $\mu\text{L}$  *B. stearothermophilus* suspension and mix. Incubate 3 min in 90°C dry well heater. Centrifuge 4 min at 1200 x g.

Decant milk and swab out fat ring, using 2 cotton swabs. Rinse tube twice with  $\text{H}_2\text{O}$  from wash bottle; do not disturb precipitate at bottom of tube. Add ca 300  $\mu\text{L}$   $\text{H}_2\text{O}$  and re-suspend precipitate, using tube miser.

Place Al planchet on 400°C hot plate. Pour suspension into planchet, touching mouth of tube to planchet to remove last drop. Rinse tube twice with 300  $\mu\text{L}$   $\text{H}_2\text{O}$  and add washing to planchet. Let planchet dry. Place dry planchet in penicillin analyzer and measure radiation from  $^{14}\text{C}$  for 8 min. Compare count with predetermined control point to determine whether test sample is positive or negative.

### 16.5 Control Point Determination

Analyze 10 zero standards and average. If any zero standard falls  $\pm 20\%$  from average with new standard and determine new average. Control point = 0.80 x

average count. Test samples fall below control point if they contain  $\beta$ -lactam antibiotic or if there is a test failure.

To identify test failure, makes second determination of positive test samples, zero standard, and 0.01 IU/mL standard at same time, Zero standard count should be greater than control point to confirm positive determination.

## References

AOAC Official Methods of Analysis (2000), Ch.33.2.55 Method, 982.15

### 17.0 Determination of Beta-Lactam Antibiotics in Milk By *Bacillus stearothermophilus* Qualitative Disc Method II

(Applicable to level  $\geq 0.008$  IU penicillin G/mL.)

#### 17.1 Culture Media

- (a) *Agar medium B.*- See 979.14 (see 5.3.01).
- (b) *Agar medium P.*- Dissolve 3.0 g beef extract, 5.0 g peptone, 1.7 g tryptone, 0.3 g soytone, 5.25 g dextrose, 0.5 g NaCl, 0.25 g  $K_2HPO_4$ , 1.0 g polysorbate 80, 0.06 g bromcresol purple, and 15.0 agar in  $H_2O$  and dilute to 1L. Adjust if necessary asi that after sterilization pH is  $7.8 \pm 0.02$ . (Difco PM Indicator Agar has been found satisfactory.)
- (c) *Agar medium M.*- See 979.14A (b) (see 33.2.57).
- (d) *Broth medium D.*- Dissolve 17.0g pancreatic digest of casein, 3.0 g papaic digest of soybean, 5.0 g NaCl, and 2.5 g  $K_2HPO_4$  in  $H_2O$  and dilute to 1 L. Adjust is necessary so that after sterilization pH is  $7.3 \pm 0.2$ . (BBL Trypticase Soy Broth without dextrose has been found satisfactory.)

## 17.2 Reagent and Apparatus

- a. *Penicillin stock solution*. - See 979.14B(a) (see 33.2.57). (Difco PM Positive Controls, Penicillin G, and Charm Sciences, Inc. penicillin standards have been found satisfactory.)
- b. *Penicillinase (beta-lactamase)*. - See 979.14B(a) (see 33.2.57). (Commercial Penase Discs [12.7 mm] have been found satisfactory.)
- c. *Filter paper disc, blank*. - Use S&S 740 E, 12.7 mm disc or discs of equivalent absorption performance qualities and purity.
- d. *Control discs*. - Prepare fresh daily from positive control milks containing 0.008 IU penicillin/mL.
- e. *Petri dishes (plates)*. - See 957.23C(b) (see 5.3.01).

## 17.3 Stock Culture of Test Organisms

Maintain *B. stearothermophilus* ATCC 10149 on agar medium M (c), transferring to fresh slant weekly. Inoculate slant of agar medium M test organism and incubate overnight at  $55 \pm 2^\circ\text{C}$ . Inoculate three 300 mL Erlenmeyers, each containing 150 mL broth medium D, A (d), with 1 loopful of test organism. Incubate at  $55 \pm 2^\circ\text{C}$  and periodically make spore stains to determine extent of sporulation. When ca 80% sporulation has occurred (usually in 72 h), centrifuge cell suspension 15 min at 5000 rpm. Decant supernate, resuspend cells in saline solution, 957.23 (r) (see 5.301), and recentrifuge. Repeat saline washing. Resuspend washed cells in 30 mL saline and store at  $4^\circ\text{C}$ . Spore suspension will remain viable 6-8 months. Check viability periodically by preparation of trial test plates. (Commercially prepared spore suspension has been found satisfactory).

## 17.4 Preparation of Plates

Inoculate aliquot of liquefied agar medium B, A (a), or P, A(b), cooled to  $55$  or  $64^\circ\text{C}$  with previously spore suspensions. Adjust inoculum level to provide

clear, readable zones of inhibition from penicillin-positive control discs, **B(d)** after 3-4 h incubation at  $55\pm 2^{\circ}\text{C}$  or 2-3 h incubation at  $64\pm 2^{\circ}\text{C}$ . Pour 6 mL inoculated agar medium B or P into each plate, and let harden on flat, level surface. Use within 5 days.

#### 17.4 Assay

**Screening.**- With clean dry forceps, touch paper disc to surface of well-mixed milk and let milk be absorbed by capillary action. Drain excess milk by touching disc to inside surface of test portion vessel. Immediately place disc on agar surface, pressing gently to ensure good contact. Identify each disc or section on which it is placed. Place control disc containing 0.008 IU penicillin/mL on plate. Invert plate and incubate at  $55\pm 2^{\circ}\text{C}$  or  $64\pm 2^{\circ}\text{C}$  until well-defined zones of inhibition (17-20 mm) are obtained with the 0.008 IU/mL control. Examine plate for clear zone of inhibition surrounding discs. Clear zone of  $>14$  mm indicates presence of inhibitory substances. Zones of  $\leq 14$  mm are read as negative. Confirm presence of inhibitor.

**Confirming.**- Heat test samples to  $82^{\circ}\text{C}$   $>2$  min. Cool promptly to room temperature. With clean, dry forceps, touch paper disc to surface of well-mixed milk and let milk be absorbed by capillary action. Also fill penicillinase-impregnated disc or add 0.05 mL penicillinase to 5 mL test portion vessel. Immediately place each disc on agar surface, pressing gently to ensure good contact. Place control disc containing 0.008 IU/mL on plate. Invert plate and incubate at  $55\pm 2^{\circ}\text{C}$  or  $64\pm 2^{\circ}\text{C}$  until well-defined zones of inhibition (17-20mm) are obtained with 0.008 IU/mL control. Examine plate for clear zone of inhibition ( $>14$  mm) surrounding disc, indicating presence of inhibitory substance.

## 17.5 Interpretation

Assay of test milk in screening and confirmatory test may produce following results:

**17.5.A** No zone around disc containing untreated milk in screening test is negative test for inhibitory substances.

**17.5.A** Zone around disc containing untreated milk but no zone around disc containing penicillinase-treated milk in confirmatory test is positive test for  $\beta$ -lactam residue.

**17.5.B** Clear zone of equal size around both discs in confirmatory test indicates presence of inhibitors other than  $\beta$ -lactam residues.

**17.5.C** Clear zone around penicillinase-treated milk substantially smaller than around untreated milk disc in confirmatory test indicates presence of  $\beta$ -lactam residue as well as another inhibitor(s).

Penicillin-positive control solution at 0.008 IU/mL should produce clear, well-defined zones of inhibition (17-20mm). If no zone of inhibition is produced by penicillin-positive control, test sensitivity is not adequate and test should be repeated.

## References

1. AOAC Official Methods of Analysis (2000), Ch.33.2.58 Method, 982.17

## 18.0 Determination of Beta-Lactam Antibiotics in Fluid Milk Products by Qualitative Color Reaction Tests

(Applicable to levels  $\geq 0.005$  IU/mL penicillin G. Multi test system not applicable to chocolate products).

### 18.1 Principle

Test is based on rapid growth rate and acid production of *Bacillus stearothermophilus* var. *calidolactis*. Acid changes color of bromocresol purple to yellow in absence of  $\beta$ -lactam inhibitors. In presence of inhibitors, purple colour remains. Applicable for detecting and confirming levels of  $\beta$ -lactam residues  $\geq 0.005$  UI/mL processed fluid milk product and raw milk. Test may be performed by using ampule or multi-test kits.

### 18.2 Reagents and Apparatus

- (a) *Delvotest*<sup>®</sup> –*P-Ampule test kit*.- (Gist-Brocades USA, PO Box 241068, Charlotte, NC 28224-1068, USA). Containing 100 test ampules, seeded with *B. stearothermophilus* var. *calidolactis* in plain solid agar medium (store at 4-15°C ); 100 nutrient tablets containing tryptone (0.5 mg), glucose (5.0 mg), nonfat dry milk (2.0 mg), and bromocresol purple (0.025 mg) (store at 4-15°C until opened and then store at room temperature); plastic forceps to transfer nutrient tables; and plastic syringe with 100 disposable tips for sampling and dispensing 0.1 mL portions of milk.
- (b) *Delvotest*<sup>®</sup> –*P-multi test kit*.- (Gist-Brocades USA). Containing hermetically sealed Al bag containing 3 plates with 96 cups each of *B. Stearothermophilus* var. *calidolactis* in solid medium with bromocresol purple indicator. Each plate

can be divided into 6 blocks of 16 cups each. Similar Al bag contains 3 similar plates with 1 nutrient tablet per cup, and sealing tapes to cover all block individually. Store at 4-15°C until opened; then store at room temperature.

- (c) *Heaters*.-Block heater and H<sub>2</sub>O bath, both thermostatically controlled at 65±2°C. Check daily.
- (d) *Dispensing pipet*.- Disposable, as in ampule test , or micro-pipettor (SMI Micro pettor, Baxter Dade Div., SMI Liquid Handling Products, 97950 NW 25<sup>th</sup> St, Miami, FL33172-2209, USA).
- (e) *Phosphate buffer*.- 1%, pH 6.0. Dissolve 8.0 g anhydrous KH<sub>2</sub>PO<sub>4</sub> and 2.0g anhydrous K<sub>2</sub>HPO<sub>4</sub> in H<sub>2</sub>O and dilute to 1L with H<sub>2</sub>O.
- (f) *Penicillinase (beta-lactamase)*.- Concentrate, store at 0-4.4°C (Difco, BBL, or Charm Sciences, Inc., 36 Franklin St, Malden, MA 02148-4120, USA, material is satisfactory).
- (g) *Penicillin Stock solution*.- Accurately weigh ca 30 mg USP potassium penicillin G reference standard and dissolve in pH 6.0 butter to give known concentration of 100-1000 IU/mL. Store at 0-4.4°C not>2 days. Stock solution may be diluted inhibitor-free milk to final desired concentration, distributed in small quantities in tubes, sealed, and frozen ≤6 months.
- (h) *Inhibitor-free milk*.- Any fluid milk product (butterfat content 0.00-3.50%, total solid <13%) may be used after being tested with this method to verify it is inhibitor-free. User for diluting standards and as negative control.

### 18.3 Check run Procedure

Perform on each new lot number of ampule or test kits and on new batches of prepared reagents.

Prepare 10 mL volumes of penicillin standards in inhibitor-free milk, containing concentrates 0.002, 0.004, 0.006, 0.008, and 0.010 IU/mL, 10 mL H<sub>2</sub>O, and 10 mL inhibitor-free milk in test tubes. Transfer 5 mL aliquot from each tube into a corresponding tube. Heat both sets of tubes to 82°C for 3 min H<sub>2</sub>O bath. Remove; cool rapidly to room temperature. Add 0.2 mL penicillinase to 1 series of tubes. Shake well; let stand 15 min at room temperature.

For ampule test, remove and identify 1 ampule for each test tube from test kit. Break off neck of ampule and place ampule in lid of ampule box, or other suitable rack. With clean, dry forceps, place 1 nutrient tablet in each ampule. For multi test, remove 1 plate from each of the 2 foil bags. Score foil covering at cutline on end block of each plat and break off 1 block of agar cups and 1 block of cups containing nutrient tablets. Open both blocks by carefully tearing back foil. Place block containing agar upside down, exactly on top of those with nutrient tablets. Holding both blocks together, invert them and tablets will fall into each corresponding cup. Light tapping of tablet cup may be needed to get all of tablets out. Arrange test tubes of controls according to cup locations in the block. Attach dry sampling pipet to plastic syringe. Completely depress plunger, place end of pipet into tube, ca 1 cm below top of test portion level. Allow plunger to return slowly under pressure of spring. Level of test portion should reach wide part of pipet. I air bubble appears, slowly expel test portion back into tube and let plunger slowly return again. Do not contaminate syringe. If drop of milk clings to outside of pipet tip, gently touch it off on edge of tube. This colume is ca 0.1 mL. Empty pipet into appropriate ampule or cup. Remove pipet tip and replace with new pipet for each test portion, control, or standard. If using micropipettor, wipe outside with tissue and rinse 3 times in test portion before removing test aliquot. Continue this pipetting procedure until all tubes have been sampled.



For ampule test, place ampules into appropriate holes in heater block. Incubate at  $65 \pm 2^\circ\text{C}$  exactly 2.5 h. Remove ampules immediately. Read penicillinase-treated ampule and its corresponding untreated ampule side by side, looking through agar against white reflective background; compare and record colors as yellow, purple, or yellow-purple. Disregard intense color surrounding nutrient tablet.

For multi test, carefully seal block of cups with strip of adhesive tape enclosed in kit. Very carefully float sealed block in  $65^\circ\text{C}$   $\text{H}_2\text{O}$  bath. Incubate exactly 2 h and 45 min at  $65 \pm 2^\circ\text{C}$ . Remove block from  $\text{H}_2\text{O}$  bath, reach, and record colors developed. Reach from bottom side of block. Compare and record colors as yellow, purple, or yellow-purple.

Following colors are satisfactory in check run: yellow for  $\text{H}_2\text{O}$  inhibitor-free milk, and 0.002 IU/mL standard; yellow or yellow-purple for 0.004 IU/MI; purple for 0.006, 0.008, and 0.010 IU/MI standards.

[**Note:** Occasionally kits of a particular Lot No. may require a longer incubation time for color to fully develop. If  $\text{H}_2\text{O}$  and inhibitor-free milk test portions are not truly yellow and/or 0.006 and 0.008 IU/mL standards are not completely purple at end of 2.5 h incubation (2 h 45 min for multi test) continue incubating until proper colors are developed. Check color development at 10min intervals and record optimum incubation time required for each Lot No.]

## 18.4 Screening Procedure

Use 1 negative and 1 positive control (0.008 or 0.010 IU/MI). Test portions may be heat-treated, as in check run, if desired.

Prepare test ampules or blocks of cups as in check run and arrange test portions in same order as ampules or cups. Shake test portions 25 times through arc of 1 ft in 7 s. Using new pipet tip for each test portion, control, and standard, proceed with sampling and incubation as in check run. Remove ampules or blocks of cups and record colors.

All-yellow test portions are negative and need not be confirmed. Purple or yellow-purple test portions must be confirmed before reporting.

## 18.5 Confirmation Procedure

Heat-treat two 5mL portions of each test portion to be confirmed and the 0.002, 0.004, and 0.006IU/ml standards. Do not treat negative control or 0.008 IU/mL standard. Add 0.2 mL penicillinase to 1 portion of each heat-treated test portion and the 3 low concentrates of standards. Prepare ampules or blocks of cups, test portion, and incubate as in check run. Remove ampules or blocks and read treated and untreated test portions side by side and record color results.

## 18.6 Interpretation

All results are reported as positive or negative for  $\beta$ -lactam residue.

Negative: Solid yellow in screening test.

Negative: Heated test portion yellow, penicillinase-treated test portion yellow.

Negative: Heated test portion purple, penicillinase-treated test portion purple.

Negative: Heated test portion yellow-purple, penicillinase treated test portion yellow purple.

Positive: Heated test portion yellow-purple, penicillinase-treated test portion yellow.

Positive: Heated test portion purple, penicillinase-treated test portion yellow.

Test sample containing heat-stable natural inhibitor give true negative test for  $\beta$ -lactam residues. Test samples containing heat-stable natural inhibitor plus penicillin may result in false negative test for  $\beta$ -lactam residues. Test samples containing other inhibitory substances (e.g., tetracycline) will give true negative test for  $\beta$ -lactam residues. Test samples of chocolate flavoured products are difficult to read in the multi test kit because of light-distorting colours from adjacent cubes.

## References

AOAC Official Methods of Analysis (2000), Ch.33.2.60 Method, 962.14.

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Annexure-1**Maximum residue limits of Aflatoxin M<sub>1</sub> in different countries**

	<b>Eu</b> µg/kg	<b>Codex</b> µg/kg	<b>Canada</b> ppm	<b>Japan</b> ppm	<b>PFA</b> ppm
<b>Aflatoxin (M<sub>1</sub>)</b>	<b>0.05</b>	<b>0.5</b>	-	-	-

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