ANALYTICAL METHODS FOR DETECTION AND ESTIMATION OF AFLATOXINS*

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Aspergillus flavus and Aflatoxins

The aflatoxins are a group of highly toxic metabolites produced by the fungi Aspergillus flavus and Aspergillus parasiticus. Four of these metabolites designated aflatoxins B1, B2, G1, and G2 occur commonly in commodities infected with these fungi. The distinguishing letters refer to the colour of the fluorescence exhibited by the compounds on thin layer chromatograms (TLC) when viewed in ultra violet light (UV), and the suffixes refer to their respective positions on such chromatograms. The term aflatoxin is used to refer to any member of a group of these chemical compounds of related structure. Aflatoxins are extremely poisonous to farm and domestic animals and to humans and are known to have strong cancer-producing properties. Of these four naturally occurring aflatoxins, aflatoxin B1 is the most potent hepatocarcinogen.

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Aspergillus flavus and the closely related species A. parasiticus are the only aflatoxin-producing moulds that have so far been isolated from aflatoxin-contaminated commodities or commodities associated with an overt aflatoxicosis. A. flavus generally produces only aflatoxin B1 and occasionally B1 and B2, whereas A. parasiticus produces all four aflatoxins B1, B2, G1, and G2. Not all isolates of these two fungi produce aflatoxins, but there are indications that most are capable of toxin production. Aflatoxin B1 is normally present in highest concentration but the relative proportions of different aflatoxins vary according to the conditions of growth, substrate, and strains of A. flavus or A. parasiticus.

A. flavus and A. parasiticus can infect groundnuts and produce aflatoxins before harvest, during postharvest drying, and in storage. Aflatoxins can be found in groundnut kernels, in unrefined groundnut oil, in groundnut cake, and in peanut butter processed from aflatoxin-contaminated groundnuts.

Methods for Analysis of Aflatoxins

Although aflatoxins were found originally as contaminants of groundnuts they have since been isolated from a wide range of agricultural commodities in many parts of the world. In view of the extreme toxicity and carcinogenicity and the widespread distribution of the aflatoxins, much effort has been directed towards achieving

effective control of these mycotoxins in food and feedstuffs. This has necessitated the development of sensitive methods for the detection and quantification of aflatoxins in various agricultural commodities.

Two main types of assays have been developed for the detection of aflatoxins - biological and chemical assays. Biological assays are only qualitative or semiquantitative and are often non-specific. These assays are too time consuming for the routine analysis such as is required in a programme of quality control. Chemical assays are suitable for routine analyses, as required for quality control and survey work. Chemical assays are invariably quicker, cheaper, more specific, more reproducible, and more sensitive than biological assays. However, biological assays such as the duckling test may prove useful for confirmatory tests particularly under conditions of analytical difficulty that may arise in the analysis of certain food or feedstuffs.

Methods for the estimation of the aflatoxins in agricultural commodities are based usually on the characteristic fluorescence of these compounds in UV light, and most of the methods which have been developed depend on the quantitative or semiquantitative assessment of the fluorescence of suitable extracts of the material under test. Methods differ in the solvents used to extract the toxins from the sample and in the method of estimating the intensity of fluorescence. However, all analytical methods

for aflatoxins basically involve the same steps - sampling, extraction, cleanup, separation, and quantitation.

Sampling

The first problem encountered with aflatoxin analysis is to obtain a representative sample. This problem is most serious with whole grains. Within a given lot, aflatoxin contamination may be concentrated in a relatively small percentage of kernels. For example, individual groundnut kernels have been reported to contain aflatoxin B1 at levels as high as 1,100,000 ppb (1.1 mg/g) (Cucullu et al., 1966). The importance of adequate sample selection for aflatoxin analysis can not be overemphasized. Sampling plans for obtaining representative lot samples have been discussed by several workers (Whitaker and Wiser, 1969; Whitaker et al., 1970). In the case of seeds and nuts, analytical procedures involve reduction in particle size by grinding or milling for efficient extraction, along with good comminution and mixing of the entire sample to obtain a representative portion for analysis. Sample size is an important consideration in obtaining representative samples. The uneven distribution of aflatoxins in a commodity makes it desirable to test an entire batch or lot of suspect material, but this of course is impractical. Usually, lot samples are limited to sizes of 1 to 5 kg. From this sample the subsamples are taken. The size of the subsample also may vary, depending upon the method of analysis, and has

ranged from 20 to 100 g. A subsample size of 50 g is used in most methods and appears to be best to obtain both solvent economy and a representative sample.

Extraction

No single extraction procedure is adequate for all commodities. This is due to the diverse nature of the commodities that may contain aflatoxins. Commodities with high contents of lipids and pigments require a different treatment from products that are low in these components. These interfering materials and aflatoxins are often soluble in the same solvents. Selective extraction of toxins or extensive purification procedures are required to produce clean extracts in these situations. The organic solvents most commonly used for extraction of aflatoxins are acetone, methanol, and chloroform. These solvents are mixed at a given ratio with a more polar solvent such as water, dilute acid, or aqueous solutions of salts, to aid in breaking of weak electrostatic bonds which bind mycotoxins to other substrate molecules, e.g. proteins. Natural fats and lipids are rather insoluble in these slightly polar solvents, resulting in cleaner extracts. By adding fat solvents such as hexane to the extraction solvent, many of the fats and lipids can be partitioned into the hexane portion of the solvent which can then be discarded. Again, this results in cleaner extracts. The ground sample, or preferably an aqueous slurry, is shaken with the extraction

solvent for 30-45 min or blended at high speed for 2-3 min. An explosion-proof blender is recommended for use with inflammable solvents such as acetone and methanol.

Purification and Clean-up

Purification and clean-up of aflatoxin extracts can be accomplished by liquid-liquid partitioning, followed by precipitation of impurities and their removal using column chromatography or preparative TLC.

Partitioning between solvents can occur during extraction as is the case with the solvent mixture of chloroform and water. When other aqueous solvents such as methanol-water and acetone-water are used, the toxins are partitioned into the chloroform layer after extraction. In these situations some prior concentration of the aqueous phase may be needed. Also, a prior cleanup step involving precipitation of interfering materials using lead acetate may be necessary. Lead acetate precipitation removes plant pigments, lipids, fatty acids and other unknown materials which may cause streaking on TLC plates (Pons et al., 1966). When the solvents used in the partitioning clean-up are immiscible, the partitioning can be done in a separatory funnel. When aflatoxins are partitioned from aqueous solutions into chloroform, emulsions are formed. These emulsions can usually be broken by adding salt solutions, anhydrous odium sulphate, or celite, or by warming or centrifugations.

Column chromatography may also be used to effect partitioning of aflatoxins from one solvent to another and thereby purify the extract. Columns packed with silica gel, cellulose, acidic alumina, or Florisil may be used to clean and purify an extract. The sample extract is usually added to the column in chloroform or another appropriate solvent, and then washed with one or more solvents in which the toxins are insoluble or less soluble than the impurities. After removal of impurities the toxins are eluted from the column using a solvent in which the toxin is soluble. The toxin solution can then be collected, concentrated, and examined for quantity of toxin present. Some loss of toxin on the column due to incomplete elution can occur, and the analyst needs to be aware of this possibility. In certain situations, the clean-up and purification step may be omitted. This may occur if the extracts are very clean to start with or if only qualitative screening results are required.

QUANTITATIVE ANALYSIS FOR THE DETECTION AND ESTIMATION OF AFLATOXIN IN GROUNDNUTS AND GROUNDNUT PRODUCTS

CB METHOD

This AOAC official method has been developed for the analysis of aflatoxins in groundnuts and groundnut products (apart from groundnut oil).

Extraction

(a) Place 50 g of the sample into a stoppered 500 ml conical flask and add 25 ml of distilled water, 25 g of diatomaceous earth, and 250 ml of chloroform. Stopper the flask, secure the stopper with tape, and shake for 30 min on a wrist-action shaker.

(b) Filter the extract through a fluted Whatman No. 1 paper. Collect the first 50 ml of the filtrate.

Column Clean-up

(a) Place a plug of glass wool in the bottom of a 22 x 300 mm chromatography column with the stop cock closed.

(b) Add 5 g of anhydrous sodium sulphate to give a level base for the silica gel.

(c) Fill the column about half full with chloroform; then add 10 g of silica gel. Wash the sides of the column with chloroform and stir to eliminate air bubbles and disperse the silica gel. As the settling rate slows, in order to facilitate settling, drain off the chloroform, leaving a space of 7 cm above the upper level of the silica gel.

(d) Add 15 g of anhydrous sodium sulphate, making sure that the silica gel layer is not disturbed, and drain off the chloroform to the upper level of this sodium sulphate.

(e) Add 50 ml of the sample extract obtained above and allow the solvent to drain to the top of the sodium sulphate layer.

(f) Wash the column at maximum flow-rate with 150 ml of

hexane followed by 150 ml of anhydrous diethyl ether and discard the eluate.

(g) Elute the aflatoxins with 150 ml of chloroform:methanol (97:3), collecting the fraction from the time of addition until the flow stops.

N.B.: Do not allow the column to go dry at any time during the above operations.

Concentration

(a) Add a few anti-bump granules to the eluate and evaporate it to near dryness in a water bath.

(b) Quantitatively transfer the residue to a vial with chloroform and evaporate to dryness, under nitrogen.

(c) Reserve the residue for TLC for detection and estimation of aflatoxins.

TLC Procedure

(a) Use precoated Kieselgel 'G' plates or plates prepared from silica-gel GHR (0.25 mm thick layer of silica gel).

(b) Scribe a line at 16 cm from the bottom edge of a TLC plate as a solvent stop. Scribe lines 0.5 cm in from each side or remove 0.5 cm gel from each side to prevent edge effects.

(c) (See Figure 1). Spot successively 3.5, 5.0 and 6.5 µl portions of the sample extract. All spots should be of approximately the same size and < 0.5 cm in diameter. On the same plate, spot 3.5, 5.0 and 6.5 µl aflatoxin B1

standard solution (concentration 0.5 µg/ml). Spot 5.0 µl of the standard solution on top of the two 6.5 µl sample origin spots as internal standard (aflatoxins B1, B2, G1 and G2 standard mixture) to show whether adequate resolution is obtained.

(d) Place a sufficient amount of developing solvent chloroform:acetone (9:1) to obtain a solvent level of 1 cm height, in an unlined developing tank. Insert the TLC plate into the tank, seal the tank and develop the plate until the solvent reaches the solvent limit line. It may take about 45 min. Remove the plate from the tank and evaporate the solvent at room temperature. Place the developed TLC plate flat, with the coated side up under longwave UV light. Four clearly identifiable spots should be visible in the resolution reference standard. In order of decreasing Rf they are B1, B2, G1 and G2. Note colour differences between B (bluish fluorescence) and G (slightly green fluorescence) toxins. Examine the pattern from the sample spot containing the internal B1 standard. The Rf value of B1 used as an internal standard should be the same as of the B1 standard spots. Examine the pattern from the sample spot without internal standard. If B1 is present, its Rf value should be the same as that of the B1 standard spot. In such a case, the B1 spot from the sample containing the internal standard should be more intense than either sample or standards alone.

(f) Compare the fluorescence intensities of the B1 spot of

the sample with those of the standard spots and estimate its concentration visually or densitometrically. In visual estimation, the toxin spots of the sample(s) is(are) compared with those of the standards and it is determined which of the standard spots maches the sample spot.

The calculation of the concentration of aflatoxin in the sample is made using the following formula :

$$\mu g/kg = \frac{S \times Y \times V}{X \times W}$$

Where S = µl of aflatoxin standard equal to unknown

Y = concentration of aflatoxin standard in µg/ml.

V = µl of final dilution of sample extract.

X = µl sample extract giving a spot intensity equal to S.

W = mass of the sample, represented by the final extract in g.

BF METHOD

This method has been officially adopted by the AOAC for the analysis of aflatoxins in groundnuts and groundnut products.

Extraction

a) Place 50 g of a sample into a 1 litre blender jar. Add 250 ml of methanol:water (55:45), 100 ml of hexane and 4 g of sodium chloride. Blend at high speed for 1 min.

(b) Transfer the slurry to 200 m centrifuge bottles and

centrifuge at 2000 rev/min for 5 min. If no centrifuge is available let the mixture stand undisturbed when separation should occur within about 30 min.

Work-Up

(a) Transfer 25 ml of the aqueous methanol layer into a 125 ml separating funnel and extract with 25 ml of chloroform. Let the layers separate out. Run off the lower chloroform layer into a 100 ml beaker.

(b) Add a few boiling chips, evaporate the extract, under a stream of nitrogen, to near dryness in a water bath. Quantitatively transfer the extract to a small vial and evaporate to dryness under nitrogen. Dissolve the extract in 200 µl of chloroform in readiness for TLC.

TLC Procedure

Use Kieselgel 'G' plates for spotting sample extracts and aflatoxin standards. Develop the plates in an unlined tank in chloroform:acetone (9:1). Estimate the amount of aflatoxin in the extract using the "comparison of standards' technique" either visually or densitometrically.

METHOD 3

This method, which is an adaptation by TPI of the "BF" method, provides a rapid assay for aflatoxin in groundnut kernels.

1. Slurry Preparation

Form a slurry by blending a 1 Kg sample of groundnuts with 2 litres of water in a 4 litre blender for 3 min.

2. Extraction

(a) Weigh 150 g of the slurry into a 1 litre blender and add 137.5 ml of methnol, 12.5 ml of water, 100 ml of hexane, and 2 g of sodium chloride. Blend the mixture for 3 min.

(b) Filter through a fluted Whatman No. 1 paper. Collect 100 ml of filtrate and divide this into two 50 ml aliquots.

Qualitative Assay

3. Work-up

Transfer one 50 ml aliquot of filtrate to a 250 ml separating funnel and add 25 ml of chloroform and 150 ml of water. Stopper the funnel and shake for 1 min taking care to avoid emulsion formation. Allow the layers to separate and run off the lower chloroform layer through a bed of sodium sulphate (1g), to dry the extract.

Minicolumn Assay

Using a 1 ml syringe transfer 2 ml of extract into a minicolumn prepared as described in Appendix 1. Allow the extract to drain through the column (slight positive pressure may be applied using a rubber teat), then elute with 2 ml of chloroform:acetone (9:1) When the meniscus of the solvent just reaches the adsorbent the column is ready to read. Do not allow the column to go dry. Examine the column under a

365 nm UV lamp; a blue band on the florisil layer indicates the presence of aflatoxin.

Quantitative Assay

Work-up

If the minicolumn indicates the presence of aflatoxin, transfer the second 50 ml aliquot of extract to a 250 ml separating funnel and extract with 25 ml of chloroform. Allow the layers to separate and run off the lower chloroform layer through a bed of anhydrous sodium sulphate into a 50 ml conical flask and wash the sodium sulphate with a further 2 ml of chloroform. Evaporate the combined extract and washings to near dryness in a water bath, preferably under nitrogen. Quantitatively transfer the extract to a vial with chloroform and evaporate the extract to dryness, under nitrogen. Dilute the extract with chloroform, depending on the aflatoxin content estimated from the minicolumn.

Carry out unidimentional TLC on precoated Kieselgel 'G' plates using chloroform:acetone (9:1) as developing solvent. Estimate the aflatoxin content by the "comparison of standards' technique" either visually or densitometrically.

Confirmatory tests

Inspite of all cleanup techniques used, there are still substances which behave like aflatoxins on TLC plates. In order to minimize the possibility of false-positives, the

identity of the mycotoxins in positive samples has to be confirmed.

Confirmatory tests for aflatoxins are generally based on the formation of a derivative which has different properties, eg. colour of fluorescence and polarity than the presumptive mycotoxin. Both mycotoxin standard and suspected sample are subjected to the same derivatization reaction. Consequently, in positive samples a derivative from the mycotoxin should appear, identical to the derivative from the mycotoxin standard. Confirmatory tests may be carried out directly on a TLC plate. The procedures for the confirmation of aflatoxin B1 were originally developed by Przybylski (1975) and Verhulsdonk (1977), and they have been adopted as official methods by AOAC.

In both methods, aflatoxin B1 is derivatized under acid conditions on TLC plate into its hemiacetal aflatoxin B2a, which has a blue fluorescence at a lower Rf than B1.

In the method of Przybylski this is achieved by superimposing trifluoroacetic acid directly on the extract spot before development. After reaction the plate is developed and examined under UV light for the presence of blue fluorescent spot of B2a, which can be recognised with the help of B1 standard, spotted on the same plate, which underwent the same procedure. As an additional confirmation, sulphuric acid (50%) is sprayed on another part of the plate where unreacted aliquots of extract and B1 standard were developed. The

sulphuric acid spray changes the fluorescence of aflatoxin from blue to yellow. This test only confirms the absence of aflatoxin, i.e. spots which do not turn yellow are positively not aflatoxin, whereas many materials other than aflatoxin may give a yellow spot with sulphuric acid.

In the case of very "dirty" extracts it may be difficult to notice the hemi-acetal of B1 (B2a) due to heavy background fluorescence. Then the two-dimensional method of Verhulsdonk should be the method of choice, in which a so-called separation-reaction-separation technique is carried out. Sample and standard are spotted on a TLC plate in the usual way as for two-dimensional TLC. Separation is first made in one direction after which hydrochloric acid is sprayed. After reaction separation is carried out in the second direction, under extactly identical conditions. The reaction of hydrochloric acid with aflatoxin B1 leads to the formation of a hemi-acetal B2a, which has a specific Rf value, lower than that of B1. This is recognised after subsequent chromatography in second direction.

Note: Both methods work as well for confirmation of the identity of aflatoxin G1.

MINICOLUMN METHODS FOR DETECTING AFLATOXINS

For convenience in large scale screening for the detection of aflatoxins minicolumn methods have considerable advantages. Their main advantages over the TLC methods are that the minicolumn methods are more rapid, less expensive, and simpler to use. Minicolumn methods do not require highly trained personnel for performing the aflatoxin analyses. However, the individual aflatoxin components (B1, B2, G1, and G2) cannot be quantified using these methods.

Since the introduction of the minicolumn technique by Holaday (1968), there has been much interest in improving the technique, and several minicolumn methods have been devised for detecting aflatoxins in groundnuts, cottonseed, maize, and several other commodities (Velasco, 1972; Pons et al., 1973; Holaday and Barnes, 1973; Holaday and Lansden, 1975; McKinney, 1975; Shannon et al., 1975; Romer, 1975; Holaday, 1976).

The minicolumn methods described here are those of Romer (1975) and Holaday and Lansden (1975).

METHOD 1 (ROMER ALL PURPOSE METHOD)

This method has been officially adopted by the Association of Official Analytical Chemists (AOAC) for the detection of aflatoxin in maize, groundnuts, and groundnut products, and cottonseed meal (limit of detection 10µg/kg total

aflatoxins).

Aflatoxins are extracted with acetone - water (85:15, v/v), and interferences are removed by adding cupric carbonate and ferric chloride gel. The aflatoxins are then extracted from the aqueous phase with chloroform and the chloroform extract is then washed with a basic aqueous solution. A Velasco-type minicolumn is used to further purify the extract and capture the aflatoxins in a tight band. The presence of aflatoxins can then be detected by their fluorescence under UV light. The presence of aflatoxins in the positive samples, can be confirmed and the toxins can be quantitatively measured, by subjecting a sample of the same extract as that used for the minicolumn to a TLC test.

The following steps are involved in the minicolumn detection method for aflatoxins :

1. Extraction

(a) Blend 50g of sample with 250 ml of acetone:water (85:15) in a 1 litre blender jar for 3 min.

(b) Filter the extract through a 24 cm diameter Whatman No. 4 paper in a 160 mm funnel into a 250 ml conical flask.

(c) Transfer 150ml of the filtrate to a 400 ml beaker.

2. Purification

(a) Prepare a mixture of 170ml of 0.2 N sodium hydroxide

solution an '0ml of 0.41N ferric chloride solution in a 500ml conical flask, mix well.

(b) To the sample extract in the 400 ml beaker (1.c.) add 3g basic cupric carbonate, mix well, then add to the mixture (2.a.) in the conical flask. Add 50g of diatomaceous earth, mix well, and then filter through Whatman No. 4 paper using a 160 mm funnel or a 10cm Buchner funnel.

(c) Transfer 150ml of the filtrate to a 500ml separating funnel; add 150ml of 0.03% sulphuric acid and then add 10 ml of chloroform. Shake vigorously for 2 min then allow the layers to separate out.

(d) Transfer the lower chloroform layer to a 250ml separating funnel. Add 100ml of potassium hydroxide wash solution (0.02 N KOH with 1% KCl), swirl gently for 30 sec then allow the layers to separate out. (If the layers do not separate and an emulsion is formed, drain this into a conical flask. Add 1g of anhydrous sodium sulphate, swirl the flask, and then filter through a fluted, 9cm Whatman No. 1 filter paper. OR transfer emulsion to a 125 ml separating funnel and wash with 50 ml of 0.03% sulphuric acid. Collect 3ml of the chloroform layer in a 10 ml graduated vial for minicolumn assay.

3. Minicolumn Assay

Add 2 ml of the chloroform sample solution (2.d.) to a minicolumn prepared as described in Appendix 1. Allow this to drain through; this should take 15-30 min. This process may be speeded up by applying a slight air pressure (rubber bulb attached to top of minicolumn) to force the solution down through the top layers of the column at a rate not exceeding 10 cm per min. After the solvent reaches the top of the adsorbent florisil layer, all further draining must be done by gravity alone. Then elute with 3ml of elution solvent chloroform:acetone (9:1, v/v) and allow the solvent to drain by gravity alone until the solvent reaches the top of the adsorbent. Do not allow the column to go dry. View the minicolumn under a 365 nm UV lamp; a blue fluorescent band at the top of the florisil layer indicates the presence of aflatoxin.

Preparation of Reference Minicolumns

To prepare reference columns containing 10ng each of aflatoxins B1 and G1, place 1ml of chloroform in a small vial and add 5ul of the standard solution. Transfer the entire solution to a packed minicolumn and allow it to drain until the solution reaches the top of the adsorbent layer. Add 3ml of elution solvent, chloroform:acetone (9:1) and allow to drain

Reference columns prepared by using chloroform extracts from an uncontaminated sample, spiked with a suitable ratio and level of aflatoxins, will be best for quantitative approximation. The reference column is not necessary for the screening tests; however, it aids in locating and becoming familiar with the aflatoxin band. Any quantitative estimation performed by using reference columns containing pure aflatoxins is at best an approximation.

N.B. : Packed sample and reference minicolumns are available from Myco-lab Co., P.O. Box 321, St. Louis, MO 63017, USA.

METHOD 2 - HOLADAY MINICOLUMN METHOD

This method has been officially adopted by the AOAC for the detection of aflatoxins in maize and groundnuts (limit of detection 10 µg/kg total aflatoxins).

The following steps are involved in the minicolumn detection method for aflatoxins :

1. Extraction

   (a) Blend 50 g of the sample with 100 ml of methanol:water (80:20) in a 1 litre blender jar for 1 min at high speed.

   (b) Filter the extract through a fluted, 24 cm diameter Whatman No. 1 filter paper into a 250 ml conical flask.

   (c) Transfer 15 ml of the filtrate to a graduated culture tube fitted with a stopper.

2. Purification

   (a) Add 15 ml of salt solution (sodium chloride/zinc acetate solution) to the filtrate (1.c) in the culture tube, stopper the tube and shake vigorously for 10 sec. A small separatory funnel may be substituted for the culture tube. The salt solution (sodium chloride/zinc acetate solution) is prepared by dissolving 300 g of sodium chloride, 300 g of zinc acetate and 7.5 ml of acetic acid in 2 litres of water.

(b) Filter 15 ml of the mixture through a fluted, 9 cm diameter Whatman No. 1 filter paper into a second culture tube. Collect 15 ml of the filtrate.

(c) Add 3 ml of benzene to 15 ml of filtrate in a culture tube (2.b.), stopper the tube, shake gently for 10 sec and then allow the layers to separate. Collect the upper benzene layer into a graduated tube.

3. Minicolumn Assay

(a) Pipette 1 ml of benzene extract (2.c.) into the top of a minicolumn prepared as described earlier. Allow the extract to drain to the top of the adsorbent layer.

(b) Add 3 ml of elution solvent chloroform:acetone (9:1) and allow it to drain through. When the meniscus of the solvent just reaches the top of the adsorbent the column is ready to read. Do not allow the column to go dry.

(c) Examine the column for the presence of aflatoxin as described for Method 1 above.

APPENDIX 1

MINICOLUMN PREPARATION

Minicolumn - glass column that transmits 365 nm UV light, 6mm (i.d.) x 190mm (l), tapered at one end to 2mm (i.d).

Column Packing Materials

Florisil - 100-200 mesh
Silica gel - 60-240 mesh
Alumina (neutral)- 80-100 mesh
Drierite (non-indicating)- 20-40 mesh
Glass wool.

N.B. : These packing materials should be dried at 110°C for 1-2 hr before use.

Minicolumn Preparation

(a) Block the lower, tapered, end of the column with a plug of glass wool of about 2-3mm long.
(b) Insert the tip of a small plastic funnel into the open, upper, end of the column.
(c) Pour drierite, non-indicating, 20-40 mesh (calcium sulphate, anhydrous) into the column to a depth of 8-10 mm.
(d) Add florisil (100-200 mesh), also to a depth of 8-10 mm.

(e) Add a layer of silica gel (60-240 mesh) to a depth of 16-20 mm.

(f) Add a layer of neutral alumina (80-100 mesh) to a depth of 8-10 mm.

(g) Add a second layer of drierite to a depth of 8-10 mm.

(h) Finally, insert a small plug of glass wool on top of column.

N.B. : Tap column after addition of each material to ensure even packing.

IMMUNOASSAYS FOR ANALYSIS OF AFLATOXINS

In recent years, Enzyme-linked immunosorbent assay (ELISA) has emerged as the major immunoassay for analysis of aflatoxins. Several ELISA techniques have been described for analysis of aflatoxins in groundnuts and peanut butter (El-Nakib et al., 1981; Chu, 1984; Morgan et al., 1986). In large scale screening for the detection of aflatoxins ELISA techniques have considerable advantages. Their main advantages over conventional analytical procedures are that the ELISA procedures are more rapid, more sensitive and specific, and simpler to use.

PRODUCTION OF ANTIBODOY AGAINST AFLATOXINS

Aflatoxins are not antigenic and must be conjugated to a protein before immunization. Conjugation of the mycotoxins to a protein is complicated by the functional groups present in the molecules. Aflatoxins lack a reactive group. In such cases, a reactive carboxyl or other group must first be introduced to the toxin molecule. Methods for preparation of mycotoxin conjugates have been developed, and specific antibodies against aflatoxin B1 and B2a have been produced (Chu et al., 1977; Gaur et al., 1981; Morgan et al., 1986).

Once the conjugates is prepared, antibody is produced in rabbits by injecting a mixture of conjugate and complete Freund's adjuvant (Chu and Ueno, 1977) at multiple sites on

the back of rabbits. Antibodies having sufficient titer are generally obtained 5 to 7 weeks after the initial immunization. Subsequent booster injections are made through the thigh once every month using incomplete adjuvant as the dispersion agent (Chu, 1984).

The specificity of an antibody is determined primarily by the antigen used in antibody production. Before running any ELISA, the specificity of an antibody preparation must be tested thoroughly by a competitive binding assay. When rabbits immunized with aflatoxin conjugated through the cyclopentane portion of the molecule, such as the carboxymethyl oxime of AFB1 (Chu and Ueno, 1977), the antibodies generally recognize the dihydrofuran portion of the molecule. When conjugates were prepared through the dihydrofuran portion of the aflatoxin molecule, such as AFB2a (Gaur et al., 1981), the antibody had a specificity directed toward the cyclopentane ring.

Competitive ELISA Technique

Competitive ELISA is based on the competition between unlabelled toxin in the sample and labelled toxin for the specific binding site of an antibody molecule. In the competitive ELISA, mycotoxin is first conjugated to an enzyme which should remain active after conjugation. The mycotoxin-enzyme conjugate is then used as the labelled toxin. Experimentally, specific antibody is first coated to a solid phase such as a microplate. After washing with

buffer, the sample solution or standard toxin is incubated simultaneously with mycotoxin-enzyme conjugate. The plate is washed again, and the residual enzyme bound to the antibody determined by addition of a specific substrate solution containing hydrogen peroxide and appropriate oxidizable chromogens. The resulting colour is measured spectrophotometrically or by visual comparison with standards.

Protocol for Competitive ELISA for Analysis of Aflatoxin (Chu, 1981)

A. Coating of plate

1. Add 50 µl of BSA (0.02%) to each well of a polystyrene microtiter plate.
2. Air dry for 24-48 hr (label plates "BSA"). In humid weather, plates may be dried with warm air (25° - 37°C) from a hair dryer.
3. Add 50 µl of GA-PBS and let stand at room temperature for 30-60 min.
4. Aspirate the contents and wash 5 times with distilled water by shaking gently and air dry overnight (label plates "Glut").
5. Add 50 µl of diluted antiserum and dry under hair dryer.

B. Competitive ELISA for Aflatoxin

1. Wash precoated plates 3 times with Tween-PBS (filling the wells with solution and aspirating out well contents) and air dry.
2. Fill all wells with 0.2 ml of BSA-PBS solution, and incubate the plate at 37°C for 30-60 min.
3. Wash 2-3 times with Tween-PBS and aspirate dry.
4. Add 25 µl of aflatoxin standard or sample solution to wells.
5. Add 25 µl of aflatoxin-peroxidase conjugate to each well, cover the plate and incubate at 37°C for 1 hr.
6. Carefully aspirate out contents completely after incubation and fill wells with 50 µl Tween-PBS and aspirate.
7. Fill wells with 0.10 ml Tween-PBS and aspirate.
8. Wash 4 times again using 0.25 ml of Tween-PBS each time.
9. After wash, aspirate wells completely and add 0.1 ml of ABTS substrate.
10. Incubate at room temperature for 15-45 min.
11. Add 0.1 ml of HF-EDTA solution to stop the reaction. Plate can be read at this point.

Buffers and General reagents

1. Phosphate buffered saline solution (PBS): 0.1 M sodium phosphate buffer containing 0.15 M NaCl, pH 7.5.
2. Tween-PBS: Add 0.5 ml of Tween 20 to 1 liter of PBS

(0.05% Tween in PBS).

3. BSA (0.02%).
4. Glutaraldehyde solution (GA-PBS): 0.2% glutaraldehyde in PBS.
5. BSA-PBS solution : 1% BSA in PBS.
6. BSA-PBS-Tween solution : 5% BSA and 0.5% Tween 20 in PBS.
7. Enzyme substrate solution (ABTS) : 1 ml of 40 mM ABTS stock solution (0.22 g of 2,2' -azino-di- 3-ethyl-benzthiazoline-6 sulfonate per ml, stable at 4° C) and 0.03 ml of 30% H_2O_2 in 100 ml of pH 4.0 CB.
8. Citrate buffer (CB) : Add 9.6 g citric acid to 500 ml of distilled water, adjust to pH 4.0 with 1.0 M NaOH and make up to 1 liter with distilled water.
9. HF-EDTA solution : Add 100 µl of EDTA solution (0.4 g of ethylenediamine tetra-acetic acid-tetra Na salt per ml of distilled water) to 100 ml of hydrofloric acid solution. (3.47 ml of 48% HF and 6 ml of 1N NaOH per liter HF).

Special reagents:

1. Antiserum : Antiserum against aflatoxin B1. Appropriate dilution in PBS should be used.
2. Aflatoxin B1 - peroxidase conjugate : Appropriate dilutions in BSA-PBS-Tween should be used. The undiluted antisera and toxin-peroxidase solution should be kept in the freezer and the diluted working solutions should be kept in the refrigerator for no more than 2 days.

Standard Aflatoxin Solutions

1. **Stock solution A : Aflatoxin B1 in benzene (1 µg/ml).**
2. **Stock solution B: Take 0.2 ml of stock solution A, dry under nitrogen to remove trace amount of benzene and redissolve in 1 ml of methanol (200 ng/ml).**
3. **Working solutions: Withdraw 0.5 ml of stock solution B and dilute to 1 ml with PBS containing 0.25% Tween 20; then make the following series of dilutions with PBS-containing 50% methanol - to give a final concentration of 0, 0.1, 0.25, 0.5, 1, 2.5, 5 and 10 ng of aflatoxin B1 per ml.**

Sample preparation :

An extration step is necessary for analysis of aflatoxin B1 in groundnut kernels, groundnut meal and peanut butter. Aflatoxin B1 can be extracted from these commodities using any one of the extraction methods described above. After extration, withdraw appropriate volume of chloroform extract (equivalent to 10 g of sample) and bring to dryness. The sample will be redissolved in 5 ml of methanol followed by an additional 5 ml of PBS - containing 0.5% Tween 20. Further dilutions should be made with 50% methanol - PBS - 0.25% Tween 20 solution.

Double Antibody ELISA for aflatoxin B1

Morgan et al (1986) have described a double antibody ELISA procedure that can detect 0.1 pg of aflatoxin B1 per well.

In this procedure, aflatoxin-protein conjugate (keyhole limpet haemocyanin (KLH) - aflatoxin B1 conjugate) is coated on to the microtitration plate (Nunc Immunoplate 1). Sample or standard aflatoxin B1 is added to the wells followed by an aliquot of anti-aflatoxin antibody. The amount of antibody bound to the solid phase is detected by the addition of goat anti-rabbit IgG-alkaline phosphatase conjugate which is subsequently reacted with para-nitrophenyl phosphate to give a colored product. The aflatoxin B1 content of the sample is determined by reference to a standard curve.

The improvement and extent of use of ELISA procedures for detecting aflatoxins in food and feedstuffs depend on the availability of specific antibodies. It is important to develop efficient methods for producing specific antibodies against aflatoxin B1 and G2, with high titers. It is also important to use simple extraction procedures with ELISA techniques. There is great potential for wide application of ELISA techniques for routine detection of aflatoxin.

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