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