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Analytical Methods for Aflatoxins in Corn and Peanuts[1]

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Abstract. Aflatoxin determinations can be approached many ways. Peanuts and corn are more often contaminated with aflatoxins B_1 and B_2 than with aflatoxins B_1, B_2, G_1, and G_2. Some countries are only interested in B_1 content and others are interested in the total aflatoxin content. It is essential to safely handle all experimental materials associated with aflatoxin analyses or the aflatoxigenic fungi. Visual screening of suspect peanut lots, based on the presence of conidial heads of the *Aspergillus flavus* group, and screening corn for the presence of bright greenish yellow fluorescence (BGYF) are not chemical tests and such screening techniques may allow aflatoxin contaminated lots into commerce. Minicolumn screening procedures should always be used in conjunction with a quantitative method. Several thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) methods are suitable for quantitation and are in general use. Immunochemical methods such as the ELISA or affinity column methods are being rapidly developed. The chemical and immunochemical methods can be reliable if care is taken, using suitable controls and personnel that are well-trained. All analytical laboratories should stress safety and include suitable analytical validation procedures.

Aflatoxin contamination of foods and feeds is important to human and animal health, because the aflatoxins are toxic and carcinogenic. The toxic and carcinogenic properties of the aflatoxins make experimental safety a very important issue in all work with aflatoxins or the fungi *Aspergillus flavus* Link and *A. parasiticus* Speare that produce aflatoxins.

Safety

Chemical Safety

Guidelines for mycotoxin safety precautions are given at the beginning of Chapter 26 in the Association of Official Analytical Chemists (AOAC) Official Methods of Analysis book (1984). The mycotoxin analysis publication from the International Agency for Research on Cancer (IARC) also has a good discussion of safety precautions (Stoloff *et al.* 1982). The safety guidelines discussed in these books are appropriate for both crude and pure aflatoxin preparations. The chemicals should only be handled with gloves and used only in properly ventilated hoods or glove boxes. Since dry aflatoxins can disperse in the laboratory, most analytical laboratories should, if possible, buy commercially available prepared standards. However, the purity of standards should always be checked before use.

Spills and laboratory surfaces should be decontaminated by treating with 1% sodium hypochlorite bleach for 10 minutes followed by 5% aqueous acetone. Ideally before washing, glassware should be rinsed in methanol, soaked in 1% NaOCl bleach for 10 minutes after which 5% acetone should be added for 30 minutes.

Biological Safety

Spores and other viable propagules of *A. flavus*, *A. parasiticus* and other fungi can cause three types of

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disease in humans: allergy, poisoning and infection (Hill *et al.* 1984). *A. flavus* infection in humans is uncommon but possible. Airborne spores and dust containing propagules of the *A. flavus* group may cause allergies in some people and the inhaled particles may contain aflatoxins (Shotwell *et al.* 1981). Two TLC methods have been developed to measure aflatoxins in corn and grain dust (Ehrlich and Lee 1984; Shotwell *et al.* 1981).

Hill and co-workers (1984) found between 10^4 and 10^9 viable fungal propagules per m^3 of air containing corn dust; air containing peanut dust is probably equivalent. The majority of the *A. flavus* propagules in air samples were deposited on the stages of the Andersen sampler corresponding to the trachea, primary bronchi, and secondary bronchi in the human respiratory system (Hill *et al.* 1984). *A. flavus* spores and propagules in dust associated with inoculation, shelling, grinding and extraction procedures are sufficiently hazardous to require safe handling procedures including gloves, masks, protective clothing and efficient dust collection mechanisms.

Sampling

Samples taken for aflatoxin analyses are subject to large sampling errors because of the non-homogenous nature of aflatoxin distribution. The first consideration in any experimental or regulatory protocol should be how to take the sample. Protocols have been published on sampling techniques (Dickens and Whitaker 1986), field sampling techniques (Davis *et al.* 1980), test plot inoculation and sampling techniques (Widstrom *et al.* 1981, 1982) and lot sampling techniques (Stoloff *et al.* 1982; Whitaker and Dickens 1983) for peanuts and corn. Another important consideration is how to prepare the sample for analysis.

Aflatoxin contamination in peanuts generally is more variable in single fields, single test plots or single lots than in corn and some other crops. A 22 kg sample is needed for peanut whereas a 4.54 kg sample is usually sufficient for corn, especially when several analytical samples are averaged to approximate the true mean (Whitaker and Dickens 1983). In peanut lots composed of comingled loads from different sources a larger initial sample should be taken. In the United States three 22 kg samples are taken from each peanut lot (Dickens and Whitaker 1986). The total sample should be ground to pass a 0.85 mm sieve, thoroughly mixed or divided and properly subsampled before analytical samples are taken. Sampling protocols for test plots must be part of the experimental design and need to be appropriate for the experimental objectives.

Standards

Criteria for mycotoxin standards (Rodricks 1973) and procedures for checking the concentration and purity of aflatoxin standards can be found in Chapter 26 of the AOAC Methods Book (1984). The use of calibrated standards in all analytical laboratories is essential. Prepared standards are available from several commercial companies at reasonable prices and analytical laboratories should, if possible, routinely use these standards. However, the purity of all standards should be checked before use. Velasco (1981) found that cyclohexane, heptane, and toluene could be substituted for benzene in standards if the solutions were not exposed to light, but aflatoxins in these solvents degraded rapidly upon exposure to light. Because Chang-Yen, Stoute and Felmine (1984) found that solvent composition affects aflatoxin fluorescence, analysts should take solvent composition into consideration when standards are prepared for HPLC.

Presumptive and Screening Methods

Aflatoxin analysis can be approached from many different levels. Peanuts and corn are more often contaminated with aflatoxin B_1 and B_2 than B_1, B_2, G_1 and G_2 (Hill *et al.* 1985). Some applications require only presumptive or screening tests while others require the quantitation of just B_1 or several of the aflatoxins. Peanuts at the buying point are visually inspected in the United States for evidence of *A. flavus* conidial heads and if present the suspect lots are not allowed into commerce for human consumption (USDA Peanut Marketing Agreement No. 146 1986). This visual test is not a chemical test and may allow contaminated lots into commerce or reject uncontaminated lots. The use of a black light to detect the bright greenish yellow fluorescence (BGYF) (Fennel *et al.* 1973; Shotwell and Hesseltine 1980) indicative of potential aflatoxin contamination of corn should only be used to identify lots for further chemical analysis. BGYF should never be used to set corn prices in the market place. The other commonly used screening technique is the application of one of several minicolumns to detect aflatoxin contamination above a predetermined level (Holaday 1981; Romer *et al.* 1979). Shannon and Shotwell conducted a collaborative study of two minicolumn methods and found that a combi-

nation method using the Holaday extraction and the Velasco minicolumn was the most satisfactory overall (Shannon and Shotwell 1979). It is important to understand that a minicolumn technique should not be used for quantitative purposes. Madhyastha and Bhat (1984) recently developed a minicolumn confirmation method for aflatoxins. These workers confirmed the identity of aflatoxins on the developed minicolumn by applying 20% H_2SO_4, 20% HCl, or trifluoroacetic acid (TFA) in 20% HNO_3. All acids changed the fluorescence from blue to yellow with the TFA in 20% HNO_3 having the lowest detection limit.

Quantitative Methods

Many of the methods adopted by scientific groups and government agencies are based on TLC detection and quantitation procedures that have been evaluated in one or more collaborative studies. The only reason that HPLC methods are not more often recommended is not that they are unsuitable but that few collaborative studies on HPLC methods have as yet been conducted. The Official Methods of Analysis (1984) published by the AOAC is probably the most widely consulted methods book for aflatoxin methods. Other societies and agencies that recommend or publish methods include: American Industrial Hygiene Association, American Oil Chemists Society, International Union of Pure and Applied Chemistry, Tropical Products Institute, American Association of Cereal Chemists, and International Agency for Research on Cancer. Information on recommended methods can be obtained by contacting the group or agency of interest.

Schuller *et al.* (1976) published an excellent review of sampling plans and collaboratively studied methods for aflatoxin analysis. People concerned with aflatoxin analysis should obtain this valuable paper to help devise or review analytical protocols. Nesheim (1979) and Shotwell (1983) also have excellent reviews on aflatoxin analysis.

Analytical laboratories can and should participate in one or more check sample programs. The American Oil Chemists Society conducts the Smalley Mycotoxin Check Sample Program (McKinney and Cavanagh 1977). Information on this program can be obtained from Sandra Burr, American Oil Chemists Society, P.O. Box 3489, Champaign, IL 61821, USA. The International Agency for Research on Cancer conducts the International Mycotoxin Check Sample Program (Friesen et al. 1980). Information on this program may be obtained from Dr. M. D. Friesen, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 2, France.

Thin Layer Chromatography (TLC)

The official AOAC CB method (1984) is the standard by which other methods are judged. A good discussion of this method can be found in the IARC mycotoxin book (1982). Shotwell and Goulden (1977) compared the AOAC BF peanut method and the AOAC cottonseed method with the CB method. The BF method uses a methanol-water (55:45) extraction solution while the cottonseed method uses an acetone-water (85:15) extraction solution. Neither of these solvents extracted aflatoxins from corn as efficiently as the chloroform-water (250:15) extraction of the CB method; however, the BF method is thought to be reliable in peanuts with aflatoxin contents below 50 ng/g. Lee and Catalano (1981) developed a scaled down cleanup column as a solvent saving modification of the CB method. Alexander and Baur (1977) described one of many two-dimensional TLC procedures which have been developed for use in samples with interfering substances. Laboratories which use fluorodensitometry for quantitative measurements need to be careful to avoid fading of aflatoxin spots on TLC plates. Nesheim (1971) found that fading could be delayed by covering the layer on the TLC plate with another glass plate.

The CB method is an excellent TLC method, but it has two major disadvantages: first, it is expensive because it uses large amounts of solvents which create a disposal problem and second, the major solvent is $CHCl_3$ which may be a hazard to workers. In many research applications, alternative methods may satisfy the experimental objectives in a less expensive and safer manner. One such screening method was developed by Dantzman and Stoloff (1972). They omitted the column chromatography step of the CB method and directly spotted the residual oil from corn extracted with $CHCl_3$-water. Spilman (1985) modified this screening method for corn by adding benzene-CH_3CN (98:2) to the residual oil and measuring the volume to obtain quantitative TLC results. Peanuts would have to be defatted with hexane before the $CHCl_3$-water extraction for these screening methods.

Many more aflatoxin analysis methods have been published but only a few can be considered in this paper. For example, Kamimura and co-workers (1985) recently published a simple rapid HPTLC method which compared favorably with the CB method. Davis *et al.* (1981) used a novel approach

by devising a method using the fluorescence of the iodine derivative of aflatoxin B_1 for quantitation and TLC confirmation. Shannon and Shotwell (1975) developed a method for determination of aflatoxin in roasted corn, and Bagley (1979) referred to a method especially adapted for corn detoxified by ammonia treatment. Josefsson and Moller (1977) developed a multimycotoxin screening method for the detection of aflatoxins, ochratoxin, patulin, sterigmatocystin, and zearalenone, while Seitz and Mohr (1976) and Thomas *et al.* (1975) developed methods for aflatoxin and zearalenone determination.

No matter which TLC method is used, the aflatoxin identity needs to be confirmed. A review of confirmation methods has been written by Nesheim and Brumley (1981). The AOAC aflatoxin confirmation method is based on the trifluoroacetic acid (TFA) reaction with B_1, G_1, or M_1 (Przybylski 1975). The TFA procedure or direct acetylation (Cauderay 1979) can be carried out on a TLC plate before development. Trucksess *et al.* (1984) recently published a rapid TLC method using a disposable silica gel column for cleanup and confirmation by gas chromatography/mass spectroscopy.

High Performance Liquid Chromatography (HPLC)

Aflatoxin analysis, using HPLC for separation and detection, is quite similar to TLC, because many of the same types of sampling and extraction procedures are used. The major advantages of HPLC over TLC are speed, automation, improved accuracy, and precision. Both normal phase and reversed phase HPLC separations have been developed for aflatoxin analyses. Early experimental work by Seitz (1975) and Garner (1975) on HPLC separations revealed that aflatoxins could be separated on normal phase columns and detected with either a UV detector or a fluorescence detector. Seitz (1975) noted that the fluorescence detector had limited usefulness for aflatoxin B_1 and B_2 with normal phase separations.

Panalaks and Scott (1977) developed a silica-gel packed flow cell for fluorometric detection of B_1, B_2, G_1, and G_2 with normal phase aflatoxin separations. A silica-gel packed cell was used by Pons (1979) and Thean *et al.* (1980) in two different HPLC methods for the determination of aflatoxins. The major disadvantage of the packed cell is the lack of stability. The cell needs to be repacked often and the detector signal weakens with time. The advantages of a packed cell method are that no derivative is necessary for detection and the mobile phase can be recycled.

Reversed phase HPLC separations of aflatoxins are more widely used than normal phase separations. However, the fluorescence intensities of B_1 and G_1 are diminished in reversed phase solvent mixtures, so the B_{2a} and G_{2a} derivatives are generally prepared before injection. Analysts should be aware that the B_{2a} and G_{2a} derivatives are not stable in methanol and methanol should be used with caution, especially in the injection solvent. Acetonitrile-water mixtures do not degrade B_{2a} and G_{2a} rapidly and are preferred to methanol-water mobile phases.

Several reversed phase methods have been published (Cohen and Lapointe 1981; Stubblefield and Shotwell 1977) including three with comparisons to the CB method (DeVries and Chang 1982; Hutchins and Hagler 1983; Tarter *et al.* 1984). Stubblefield and Shotwell (1977) found that M_1 and M_2 as well as B_1, B_2, G_1, and G_2 could be resolved and detected with a UV detector at 350 nm, using reversed phase chromatography. The methods developed by Hutchins and Hagler (1983), DeVries and Chang (1982) and Tarter *et al.* (1984) use TFA derivitization and apparently compare favorably with other methods. Diebold *et al.* (1979) used laser fluorometry to detect aflatoxin B_{2a} after reversed phase chromatography.

Davis and Diener (1980) found that the iodine derivative of B_1 could be used for confirmation and developed a reversed phase method with fluorescence detection for this derivative. This work led to the development of a post-column iodination method to enhance B_1 and G_1 fluorescence after reversed phase chromatography. Shepard and Gilbert (1984) investigated the conditions needed for the post-column iodination reaction to enhance fluorescence of aflatoxin B_1 and G_1. The detection limit for B_1 was about 20 pg at a signal to noise ratio of 3.

Immunochemical Methods

Aflatoxin B_1 in peanuts can be determined by solid phase radio-immunoassay (RIA), (Langone and Van Vunakis 1976; Sun and Chu 1978) monoclonal antibody affinity columns (Groopman *et al.* 1984) or enzyme-linked immunosorbent assay (ELISA) techniques (Chu and Ueno 1977; El-Nakib *et al.* 1981; Lawellin *et al.* 1977; Pestka *et al.* 1980). ELISA or monoclonal antibody affinity column techniques are more suited to field use than RIA

techniques and probably will be extensively developed and utilized. The major advantages for ELISA and monoclonal antibody affinity column methods include speed, ease of sample preparation, ease of use and a potentially low cost per analysis. The disadvantages include different antibody specificities for B_1 and cross reactivity with other aflatoxins. At the present time, ELISA procedures are qualitative or semi-quantitative at best and are temperature sensitive. The major application for ELISA procedures at present is screening for aflatoxin B_1 below a predetermined concentration. The color developed by the enzyme mediated reaction gives an indication of the amount of B_1 present. More development is needed before immunochemical techniques will be generally useful for applications where quantitation is critical. Methods also need to be developed that will distinguish between B_1, B_2, G_1, and G_2 individually or collectively.

Selection of Analytical Approach

Regulatory and experimental applications of methods for aflatoxin analysis do not always need to be the same. Regulatory applications need to be quantitative and legally acceptable, but acceptable methods may vary within a country or between countries. However, it is important to use validated methods for regulatory applications.

Aflatoxin analysis in experimental work must be tailored to the objectives and method selection should be a part of the experimental design. Inexpensive minicolumn data may be sufficient for some experimental purposes whereas quantitative data on B_1, B_2, G_1, and G_2 may be required for other purposes. Costs and data requirements can sometimes seemingly be at odds when quantitative data is necessary. TLC and immunochemical methods may not always be cheaper than HPLC in the long run, because HPLC requires a single large initial investment, and TLC and ELISA both use disposable plates. Large analytical laboratories are possibly more suited for HPLC while laboratories with only a few samples may be more suited for TLC. With further development, immunochemical methods will probably be versatile and suited for a wide variety of applications.

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