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Review

Determination of aflatoxins in food products by chromatography

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ABSTRACT

Several chromatographic methods for the determination of aflatoxins in agricultural and food products are reviewed. During the past two decades, identification and determination of aflatoxins were done by thin-layer chromatography (TLC) because it was easy, fast and inexpensive. However, high-performance liquid chromatography (HPLC) using fluorescence detection is now the method of choice for determining aflatoxins and is also growing in popularity for their identification. The reasons for selecting HPLC over TLC can be summarized as the ability to analyze for a wide variety of compounds, including compounds that are easily degraded by heat, light or air, the ease of adaptation to confirmatory procedures, the potential for automation and the dramatic improvement in instrumentation, including the development of increasingly sensitive fluorescence and electrochemical detectors and short, high-resolution, reversed-phase columns.

CONTENTS

1. INTRODUCTION

Ever since the severe outbreak of Turkey "X" disease in the UK in the 1960s and the discovery of fluorescent compounds in a feed component, peanut meal, aflatoxins have been a major concern as a toxic contaminant of feeds and foods [1]. Aflatoxins are secondary metabolites produced by the fungi *Aspergillus flavus* and *Aspergillus parasiticus*. The word "aflatoxin" was derived from "a" from the genus *Aspergillus*, the "fla" from the species *flavus* and "toxin" meaning toxic [2]. Cole and Cox [3] listed sixteen compounds in the aflatoxin group, but only the aflatoxins B_1, B_2, G_1, G_2 and M_1 are routinely monitored in foods and feeds in commerce (Fig. 1). The International Agency for Research on Cancer has placed aflatoxin B_1 on their list of probable human carcinogens [4]. The fungi can produce aflatoxins on commodities in the field under stress conditions or in storage when conditions such as high moisture and warm temperature (25–30°C) are met [5].

Aflatoxin B_1 is the prevalent, acutely toxic and most carcinogenic of the aflatoxins and M_1 is excreted in the milk of animals ingesting aflatoxin B_1 [5]. Because of potential health hazards for humans, worldwide monitoring of aflatoxins in various commodities has been indicated an regulatory levels in different commodities have recently been documented [6]. Efforts to minimize aflatoxin contamination in susceptible commodities are the subject of research projects in many places.

Aflatoxin determination is no longer a particularly difficult task using current thin-layer chromatographic (TLC), high-performance liquid chromatographic (HPLC) and immunochemical techniques. Aflatoxins B_1, B_2, G_1, G_2 and M_1 can be readily separated and detected using either normal- or reversed-phase TLC or HPLC techniques, with HPLC becoming increasingly the method of choice.

The challenges inherent in aflatoxin analysis currently include sampling, subsampling and sample extraction methods and also the analytical variation associated with the chosen analytical method.

For this review, selected examples of techniques have been used, and therefore the literature cited is not exhaustive. For more information on specific techniques, the reviews by Rottinghaus [7], Beaver and Wilson [8], Wilson [9], Wilson *et al.* [10], Betina [11] and Beaver and Wilson [12] should be consulted.

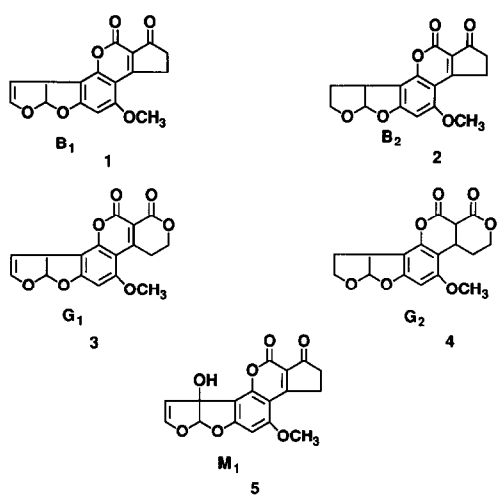

Fig. 1. Structures of the major aflatoxins. 1 = aflatoxin B_1; 2 = aflatoxin B_2; 3 = aflatoxin G_1; 4 = aflatoxin G_2; 5 = aflatoxin M_1.

2. SOLVENT EXTRACTION

Extraction of aflatoxins from various sample matrices has been accomplished using several organic solvents. Aqueous mixtures of methanol, acetonitrile, 2-propanol or acetone have been used for extraction of grains, oilseeds, cottonseed, nuts, meats and various other products. In most instances a two-phase extraction is carried out using chloroform–water or methanol–water.

Three different solvents were developed for aflatoxin extraction from peanut, cottonseed and corn. The AOAC CB method [13] uses chloroform saturated with water as the extraction solvent and is the standard by which other methods are judged. The CB method works very well with corn and peanuts but many interfering substances are seen when this method is used for cottonseed or mixed feeds. The other disadvantages of chloroform extraction include the expense of the solvent, toxicity and problems with waste disposal. The AOAC BF method [14], developed for TLC analysis of peanuts and peanut products, uses a methanol–water (55:45) extraction with hexane added to defat the sample. Aflatoxins are partitioned into chloroform prior to the TLC determination. Pons developed the AOAC cottonseed products method [15] which uses an acetone–water (85:15) extraction followed by a lead acetate precipitation, filtration, partitioning into dichloromethane, clean-up on a silica gel column and determination by TLC or HPLC. Acetonitrile plus water in various ratios has also been used frequently as the extraction solvent for aflatoxin M_1 and for multi-mycotoxin methods.

Shotwell and Goulden [16] compared the extraction efficiencies of the BF method and the AOAC cottonseed method with that of the CB method in corn. The BF method uses methanol–water (55:45) and the cottonseed method uses acetone–water (85:15) as extraction solvents. Neither of these solvents extracted aflatoxins from corn as efficiently as the CB chloroform–water (250:15) extraction. Bradburn *et al.* [17] evaluated aflatoxin extraction from corn using differing concentrations of aqueous acetone, aqueous methanol and aqueous acetone–methanol (1:1) as extraction solvents. With each system the amount of aflatoxin extracted increased as the ratio of organic solvent to water increased from 50:50 to 80:20 and then decreased or remained constant at 90:10. Aqueous acetone (80%) was found to extract 27% more aflatoxin than the corresponding 80% methanol, with 80% methanol–acetone (1:1) being intermediate. Hurst *et al.* [18] used methanol–water (55:45) as an extraction solvent for aflatoxins in peanut butter. Leitao *et al.* [19] used chloroform–water (10:1) in the determination of aflatoxins in various strains as *Aspergillus* in foodstuffs. Groopman and Donahue [20] used methanol–water (60:40) for extraction prior to affinity column clean-up for the determination of aflatoxins in foods and biological samples. The AOAC accepted method of extraction using methanol–water (60:40) was utilized by Dorner and Cole [21] in the analysis of peanuts after mini-column clean-up and by Boyacioglu and Gonul [22] in the analysis of raisins.

Selection of a particular extraction solvent depends on the type of chromatographic detection. Many other factors, including commodity, stability, interfering substances, cost and waste disposal, must be taken into consideration before selecting a method. In general, chloroform or dichloromethane is acceptable with the exception of immunochemical method which are not sensitive to chlorinated hydrocarbons. The immunochemical methods generally use various methanol–water extraction solvents. Matrix and sensitivity considerations should be the most important factors influencing the selection of extraction solvents.

3. SCREENING METHODS

In many instances only qualitative identification of the aflatoxins present in various media is needed. Ideally, the screening method should allow a determination of whether aflatoxins are present above a specific level without any sample preparation. The food and feedstuffs most frequently contaminated with aflatoxins are peanuts, cottonseed, corn and tree nuts. The simplest aflatoxin screening method is the use of black or long-wavelength UV radiation (365 nm) to examine cracked or coarsely ground corn. However, the USDA is considering halting the use of this method because of difficulty in interpreting the observed bright greenish yellow fluorescence (BGYF).

More reliable screening methods such as enzyme-linked immunosorbent assay (ELISA) and TLC normally involve and initial solvent extraction step prior to analysis.

3.1. Enzyme-linked immunosorbent assay (ELISA)

The development of commercially available ELISA kits that recognize different mycotoxins has made immunoassay an important tool for aflatoxin testing. The typical ELISA format for aflatoxins contains three specific reagents: the mono- or polyclonal antibodies that recognize a specific mycotoxin such as the aflatoxins and bind with them, an aflatoxin–enzyme conjugate and an enzyme substrate. Binding of the aflatoxin–enzyme conjugate by immobilized antibodies is prohibited by the presence of free toxin in the sample. The bound enzyme catalyzes the oxidation of a substrate to form a colored complex. Development of color indicates that the test sample contains no aflatoxin or a concentration below the level of interest. Two ELISA methods for aflatoxins were collaboratively studied. One method used microtiter wells [23] and the other used a membrane attached to a plastic cup [24]. The microtiter well method had minor problems and is being modified. The microtiter well method can also be used for semi-quantitative determinations when an ELISA reader is utilized to record the absorbance of the colored complex.

3.2. Immuno-dot

The cup or immuno-dot method is a "yes–no" test. Performance is assessed by examining the ability of the test to classify samples correctly into two categories, positive and negative at a predetermined detection level (20 ng/g) for the aflatoxins. Ideally, above 20 ng/g all results should be positive and all below should be negative. The 95% confidence intervals for correct identification of cottonseed and peanut butter as positive for aflatoxin contamination at 20 ng/g using this method were 79–100%, and for raw peanuts and corn 78–100% and 73–99%, respectively [24].

3.3. Thin-layer chromatography (TLC)

The most effective screening method uses TLC, which is the simplest of all the widely used chromatographic methods to perform. A developing tank containing the mobile phase, a coated plate and long-wavelength UV radiation are all that are required for separations and qualitative analyses. TLC of the aflatoxins has received the most attention over the years; consequently, it is the most refined and generally serves as a model for other mycotoxins. Most analysts prefer commercially prepared silica gel plates because of durability and homogeneity of the adsorbent layer. After sample application the plate is put in a developing tank containing mobile phase. The most commonly used mobile phases for aflatoxin analyses are chloroform–acetone (9:1), diethyl ether–methanol–water (96:3:1) and anhydrous diethyl ether.

A portion of a sample extract is evaporated and the oily residue along with a standard solution of aflatoxins are spotted on a TLC plate and developed with anhydrous diethyl ether [25]. The lipids move to the solvent front, while the four aflatoxins are separated. The plate is then examined under long-wavelength UV light. This method can easily detect aflatoxins at a level of 20 ng/g. The rate of correctly detecting contamination is 80% at 20 ng/g.

4. SAMPLE CLEAN-UP

There are three steps in the analytical procedures: extraction, purification and determination or analysis. The most common solvent system for extraction is a mixture of a chlorohydrocarbon and water, but is gradually being replaced with methanol–water or acetonitrile–water systems. The most significant improvement in the purification step is the use of solid-phase extraction (SPE). Clean-up of sample extract prior to instrumental analysis (TLC or HPLC) is used to remove other materials also extracted that often interfere in the determination of target analytes. The traditional use of column chromatography (silica gel) and liquid–liquid partition for clean-up has been relaced by SPE. SPE is rapid, solvent efficient and economical. The most commonly used stationary phases in SPE columns are silica gel [26], C_{18} bonded-phase [27], Florisil [28], Mycosep multi-functional clean-up [29] and antibody affinity types [30].

4.1. Silica gel columns

Wei *et al.* [31] used Sep-Pak silica cartridges in conjunction wih preparative TLC using silica gel to clean-up CB extracts of soy sauce and fermented soybean paste for HPLC determination of aflatoxins. Cohen and Lapointe [32] determined aflatoxins in corn and dairy feed using HPLC and fluorescence detection after clean-up with Sep-Pak silica

cartridges. The corn and dairy feed samples were extracted initially with acetonitrile–water and the aflatoxins were partitioned in chloroform prior to Sep-Pak silica SPE.

Trucksess *et al.* [33] used a 0.5-g disposable silica gel column to clean-up methanol–water extracts of corn and peanut butter after the extract had been partitioned with chloroform. Aflatoxin analyses were performed by TLC with gas chromatographic–mass spectrometric (GC–MS) confirmation. Hurst *et al.* [34] analyzed raw peanuts for aflatoxins by HPLC after the acetone–water extracts had been partitioned in dichloromethane and cleaned up by passage through Sep-Pak silica cartridges.

Tyczkowska *et al.* [35] used liquid chromatography to analyze for aflatoxin M_1 in milk after clean-up with Sep-Pak silica cartridges. The milk was extracted with acetone, defatted with hexane and the aflatoxin M_1 was partitioned in chloroform prior to Sep-Pak silica clean-up.

Hutchins *et al.* [36] evaluated a rapid Sep-Pak silica clean-up for the determination of aflatoxins in corn using HPLC and fluorescence detection after precolumn derivatization with trifluoroacetic acid. The corn samples were extracted with chloroform–water and mixed with hexane prior to clean-up on Sep-Pak silica cartridges. The results indicated that recoveries for the aflatoxins were greater than 97% overall. The working range for this method was from <1 ng/g to >100 μg/g of aflatoxin B_1.

It should be mentioned, however, that utilizing silica gel for clean-up involves the use of potentially hazardous solvents such as chloroform.

4.2. Florisil columns

Kamimura *et al.* [37] purified chloroform–water extracts from corn, buckwheat, peanuts and cheese with a Fluorisil column and determined the aflatoxins utilizing high-performance TLC (HPTLC). Van Egmond *et al.* [27] compared six different methods for determining aflatoxin B_1 in feeding stuffs containing citrus pulp, and the preferred method involved purifying the chloroform extracts with Sep-Pak Florisil cartridges and Sep-Pak C_{18} cartridges before HPLC analyses. This method was recommended to the European Community (EC) for adoption following the results of a collaborative study in 1991 [38].

4.3. Mycosep multi-functional clean-up columns

Multi-functional clean-up (MFC) columns provide a rapid one-step extract purification. These columns work in just the opposite way to other clean-up columns. The MFC columns are designed to allow compounds of interest to pass through, while retaining compounds that could create interferences in most analytical methods. Wilson and Romer [29] purified acetonitrile–water extracts from several agricultural food products using MFC columns prior to HPLC analysis. Aflatoxin recoveries reported were above 95% with a sensitivity of <1 ng/g.

Romer [39] is planning to market a method for determining aflatoxins that is completely automated. The method involves on-line clean-up of sample extracts with MFC columns and HPLC analysis using fluorescence detection after postcolumn derivatization with bromine.

4.4. Monoclonal antibody affinity columns

Groopman and Donahue [20] used monoclonal antibody affinity columns to isolate aflatoxins rapidly from food and grain samples and aflatoxin M_1 from milk. Portions of methanol–water extracts were diluted and passed through the affinity columns. The aflatoxins were eluted from the column with methanol and subquently analyzed using reversed-phase LC. Holcomb and Thompson [40] used affinity columns to isolate aflatoxins from rodent feed prior to analysis by HPLC using fluorescence detection after postcolumn derivatization with iodine.

Farjam *et al.* [41] used an immuno precolumn packed with monoclonal or polyclonal antibodies for on-line analysis of aflatoxin M_1 in defatted milk. Mortimer *et al.* [42] used immunoaffinity column clean-up for aflatoxin M_1 in milk and analysis by HPLC.

As affinity columns isolate the aflatoxins from virtually all interfering compounds, sensitivities of <1 ng/g can easily be obtained.

4.5. Gel permeation chromatography

The use of gel permeation chromatography (GPC) for clean-up purposes has not been extensively utilized. However, GPC has the potential and has been used to purify sample extracts for aflatoxin analysis. Hetmanski and Scudamore [43] used GPC with a column packed with Bio-Beads S-X3 to clean

up extracts of cereals and animal feedstuffs prior to analysis by HPLC. Quantitative results were obtained down to 1 ng/g.

5. THIN-LAYER CHROMATOGRAPHIC QUANTIFICATION

Quantitative tests, with high precision and accuracy, have become a reality because of the improvements in instrumentation and the availability of a wide variety of adsorbents for use as stationary phases on TLC plates. Stationary phases have made great advances in recent years. Media of small particle size with a narrow size distribution have become available. HPTLC plates are made of such media. Various instrumentation for sample application, plate development and densitometry has recently been evaluated [44]. Optimum sensitivity, accuracy and precision were obtained by using a fully automated TLC sampler, an unsaturated conventional TLC glass chamber and a monochromatic fluorodensitometer. Recently a microcomputer was interfaced to a fluorodensitometer to simplify the data-handling procedure [45]. The system computes and records the amount of aflatoxin in the sample extract spots and the concentration of aflatoxin in the original extracted sample. TLC has maintained its analytical status because of the constant improvements in instrumentation and stationary phases.

5.1. One-dimensional

One-dimensional TLC includes one solvent, two solvents and bidirectional development systems. The one-solvent system is self-explanatory. In two-solvent development [46], first the plate is developed with a solvent that removes the interferences and then the plate is dried and developed with another solvent in the same direction for the separation of the toxins. In bidirectional development [47], sample extracts are spotted in the middle of the plate. After the first development with a non-polar solvent to remove the non-polar compounds, the top of the plate below the solvent front is cut off. The plate is then turned upside down (180°C) and developed with a more polar solvent to separate the toxins.

5.2. Two-dimensional

Two-dimensional TLC (2D-TLC) is the most powerful technique and offers greater resolution than other chromatographic techniques. It uses two solvents of different selectivity for the two developments. The usefulness of 2D-TLC for aflatoxin analysis has been demonstrated in a number of recent publications [26,27,48–50].

6. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC QUANTIFICATION

HPLC using fluorescence detection has already become the most accepted method for the determination of aflatoxins. HPLC is also fast becoming the method of choice for qualitative analyses of aflatoxins because of their native fluorescence of λ_{ex} = 360 nm for all four aflatoxins and λ_{em} = 440 nm for aflatoxins B_1 and B_2 and 470 nm for aflatoxins G_1 and G_2 [51].

HPLC methodology has several advantages over other methods but the most important seems to be the potential for automation. For a thorough review of HPLC data for aflatoxins, the surrey by Shephard [52] should be consulted.

Both normal- and reversed-phase HPLC can be utilized. Normal-phase methods have been developed using detection by UV monitoring at 254 and 365 nm, native fluorescence and fluorescence with a silica packed cell. Reversed-phase methods using both UV and fluorescence detection have been developed. Recently, reversed-phase HPLC methods for aflatoxins have received the most attention. A review of these methods is given.

6.1. Normal-phase

The early work of Pons [53] with applications chemists at DuPont resulted in the development of a normal-phase separation using a water-saturated chloroform–cyclohexane–acetonitrile–ethanol mobile phase. The initial separation was accomplished and aflatoxins B_1, B_2, G_1 and G_2 could be detected at either 254 or 365 nm using a UV detector. Kmieciak [54] reported that UV detection between 350 and 360 nm was more selective than at 254 nm. Garner [55] found that a silica gel column packed with material of 6-μm mean particle size was more efficient in separating aflatoxins than a column packed with particles of size ranging from 5 to 10 μm.

Pons and Franz [56,57] developed methods for

cottonseed and peanut products using normal-phase chromatography with UV detection at 365 nm. Pons and Franz [57] also determined that the use of UV detection at 365 nm was to be preferred for detection of aflatoxins B_1 and B_2 whereas fluorescence detection (excitation at 365 nm and emission above 450 nm) was preferred for aflatoxins G_1 and G_2.

The use of fluorescence detections has been hampered because the emission of the fluorescence of aflatoxins B_1 and B_2 is quenched by mobile phases containing chlorinated solvents. Two alternative methods were developed to overcome this limitation. First, the use of alternate mobile phases was investigated and second, a silica gel-packed flow cell was developed to enhance the fluorescence with normal-phase solvents.

Chang-Yen *et al.* [58] reported that the fluorescence of aflatoxins B_1, B_2, G_1 and G_2 was influenced by the solvent composition with various chloroform–methanol combinations. This dependence on solvent composition has limited the use of chloroform-based mobile phases without some type of fluorescence enhancement.

Manabe *et al.* [59] reported that a mobile phase consisting of toluene–ethyl acetate–formic acid–methanol (89:7.5:2.0:1.5) did not quench the fluorescence of aflatoxins B_1 and B_2 whereas mobile phases with chloroform, dichloromethane or methanol as the major components did quench the fluorescence. They reported that the relationship between peak area and concentration was linear up to 120 ng and that the method was sensitive to 10–20 ppb of total aflatoxins. Goto *et al.* [60] used toluene–ethyl acetate–formic acid–methanol (90:5.0:2.5:2.5) as the mobile phase to separate aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2 and developed a method with high recovery for the analysis of milk and milk products. Leitao *et al.* [19] also used a toluene–ethyl acetate–formic acid–methanol (90:6:2:2) mobile phase for aflatoxin determination in *A. flavus* group culture extracts. Howell and Taylor [61] used water-saturated toluene–ethyl acetate–formic acid (85:25:5) as the mobile phase to determine aflatoxins in mixed feeds using fluorescence detection with a limit of 1 ng/g. They changed the excitation wavelength from 360 to 330 nm with a consistent emission at 425 nm for confirmation. The 365/330 nm peak-height ratio from positive samples agreed within 10% of standard values for aflatoxins B_1, B_2, G_1 and G_2 [61]. Perhaps other solvent systems could be identified that improve the detectability of aflatoxins in normal-phase HPLC without adding a step to enhance the fluorescence.

The primary fluorescence enhancement technique for normal-phase HPLC has been to use a detector flow cell packed with silica gel. The silica gel-packed flow cell was developed by Panalaks and Scott [62] and was reported to be linear up to about 2 ng injected. A normal-phase HPLC method was developed by Pons [63] for corn using a methanol–10% aqueous sodium chloride extraction, precipitation with zinc acetate and preliminary clean-up using a small silica gel column and detection using a packed flow-cell. Pons [63] compared the methanol–10% aqueous sodium chloride extraction with the CB extraction in samples containing only aflatoxins B_1 and B_2 and reported that the results were essentially identical, while methanol–10% aqueous sodium chloride extracted more aflatoxins when the samples contained aflatoxins B_1, B_2, G_1 and G_2. The average recovery was above 90% for all aflatoxins tested. Awe and Shranz [64] and Francis *et al.* [65] developed methods for spices and peanut butter using a silica gel packed flow cell. The AOAC method [15] for cottonseed products uses normal-phase HPLC with detection either with UV monitoring at 365 nm or fluorescence detection with a packed cell.

6.2. Reversed-phase

Development of reversed-phase HPLC methods for aflatoxin determination was similar to normal-phase HPLC development. The use of reversed-phase methods is now more common than that of normal-phase methods. Seitz [66] investigated the use of dry packed octadecyl and phenyl reversed-phase columns and found that they were incapable of resolving the aflatoxins. Hurst and Toomey [67] used a 5-μm reversed-phase C_{18} column to develop a method for the determination of aflatoxins in peanut products. The eluate from the column was passed to a UV detector operated at 365 nm to determine the aflatoxins and then into a fluorescence detector to improve the accuracy aflatoxin of B_2 and G_2 determination. Hurst *et al.* [68] also developed a reversed-phase method for cocoa beans using UV detection at 365 nm for aflatoxins B_1 and G_1 and fluorescence detection of aflatoxins B_2 and

G_2. A silica gel-packed flow cell was used by Knutti *et al.* [69] for the determination of aflatoxins in peanut kernels. The packed cell enhanced the fluorescence signals of aflatoxins B_1 and G_1, eliminating the need for dual detectors.

A high-affinity monoclonal antibody specific for aflatoxins was used in an immunoaffinity column by Groopman *et al.* [70] and Groopman and Donahue [20] in methods developed for determination of aflatoxin metabolites in urine and aflatoxins in foods. They used a 5-μm ODS reversed-phase column and a UV detector with isocratic elution with 18% ethanol for 20 min followed by an 18–25% ethanol gradient over 25 min. The mobile phase was buffered at pH 3.0. The common aflatoxins and several aflatoxin metabolites were separated and determined using this technque.

UV detection has the disadvantage of not being as sensitive or selective as fluorescence detection and interfering peaks are more common. Therefore, methods development has focused on the more sensitive and selective fluorescence detection with excitation at about 365 nm and emission at about 440 nm.

A kinetic study of the acid-catalyzed conversion of aflatoxins B_1 and G_1 and B_{2A} and G_{2A} was published by Pons *et al.* [71] in 1972. The rate of conversion of aflatoxins B_1 and G_1 to the corresponding saturated hydroxy derivatives, B_{2A} and G_{2A}, was found to be first order and strongly pH dependent. This work formed the basis for the derivatization of aflatoxins B_1 and G_1 to B_{2A} and G_{2A} with trifluoroacetic acid on TLC plates or in test-tubes prior to HPLC separation. In reversed-phase HPLC mobile phases aflatoxins B_1 and G_1 are not highly fluorescent whereas B_{2A} and G_{2A} are easily detected. The conversion of aflatoxins B_1 and G_1 to B_{2A} and G_{2A} was accomplished by Takahashi [72,73].

Takahashi [72] developed an HPLC method for the determination of aflatoxins in wines and other liquid, using a 10-μm ODS column with water–acetonitrile–methanol (15:3:2) as the mobile phase. Aflatoxins B_1 and G_1 were converted into B_{2A} and G_{2A} before injection, whereas B_2 and G_2 were not affected by the treatment with trifluoroacetic acid. The recovery of aflatoxins B_1, B_2, G_1 and G_2 from various liquid products spiked at 1 ng/ml was 80–116% with the detection limit being about 0.02 ng/ml. Reversed-phase methods using precolumn derivatization were soon developed for green coffee and peanut butter [74], corn and dairy feeds [32], pistachio nuts [75] and animal feeds [50]. De Vries and Change [76] reported a correlation of 0.991 between reversed-phase HPLC using precolumn derivatization and the CB method with corn and peanut butter. Tarter *et al.* [77] published an improved method using precolumn derivatization and reversed-phase HPLC which was suitable for use with peanut, various tree nuts and pumpkin seed. This method was used in a successful AOAC collaborative study on corn and peanut butter. This new AOAC method [78] for corn and peanut butter uses precolumn trifluoroacetic acid derivatization with separation on an RP-C_{18} column accomplished using water–acetonitrile–methanol (700:170:170) as the mobile phase and fluorescence detection (excitation at 360 nm, emission at 440 nm).

The major problem in the chromatography of aflatoxins B_{2A} and G_{2A} is the relative instability of these compounds. Both are very unstable in methanol and precautions must be taken to protect their stability in injection solvents. Usually this is accomplished with an acetonitrile–water injection solvent. The low stability of aflatoxins B_{2A} and G_{2A} can present major problems when samples are placed in an autosampler hours before injection. Therefore, precautions must always be taken when using methods for aflatoxins B_{2A} and G_{2A}.

The use of precolum derivatization in HPLC separation also introduced an additional step in the analysis. Postcolumn enhancement of fluorescence is desirable because it offers and easily controlled in-line step. Davis and Diener [79] reported that the iodine derivative of aflatoxin B_1 was 25 times more fluorescent than the parent aflatoxin. The structures of the iodine derivatives of aflatoxins B_1 and G_1 were not determined because the derivatives appeared to be unstable. However, their observations led to the development of a postcolumn iodine derivatization method by Thorpe *et al.* [80]. Tunistra and Haasnoot [51] improved this method and Shepherd and Gilbert [81] investigated conditions for postcolumn iodination for the enhancement of aflatoxin B_1 fluorescence. Shepherd and Gilbert [81] reported that optimum postcolumn iodination with iodine saturated water solutions consisted of a 5000 × 0.3 mm I.D. reactor coil operated at 75°C with a

reagent flow-rate of 0.5 ml/min. The HPLC used a 5-μm Spherisorb ODS column maintained at 35°C and eluted with water–acetonitrile–methanol (60:30:10) at 0.75 ml/min with fluorescence detection (excitation at 365 nm, emission at 440 nm).

Theil *et al.* [82] developed a postcolumn iodine reversed-phase method for determining aflatoxins in corn, peanut butter, sorghum malt and duckling mash. The separation and conditions were similar to those developed by Shepherd and Gilbert [81]. The iodine solution was prepared daily and confirmation of aflatoxins B_1 and G_1 was accomplished by stopping the iodine flow and observing the disappearance of the B_1 and G_1 peaks. Methods using postcolumn iodination were developed by Hurst *et al.* [34] for peanut products, by Paulsch *et al.* [83] for feedstuffs containing citrus pulp and by Chamkasem *et al.* [84] for multi-mycotoxin screens in grains, oilseeds, and animal feeds. Beaver *et al.* [85] compared postcolumn iodine derivatization with the CB TLC method and found a correlation coefficient of 0.99 for aflatoxin B_1.

An AOAC–IUPAC collaborative study was conducted by Trucksess *et al.* [86] to evaluate the Aflatest immunoaffinity column for the determination of aflatoxins in corn, peanuts and peanut butter. The method used reversed-phase HPLC with postcolumn iodine derivatization. The samples were extracted with methanol–water (7:3) and diluted to $<30\%$ methanol before application to the affinity column. The column was washed with water before elution with 1 ml of methanol. The methanol solution was diluted to 2 ml with water and 50 μl were injected into the HPLC system. The chromatographic conditions were similar to those of Shepherd and Gilbert [81] except that a 5-μm C_{18} column was generally used with a mobile phase consisting of water–acetonitrile–methanol (3:1:1). Recoveries were 81, 81 and 83% for samples spiked at 10, 20 and 30 ng/g, respectively. The collaborative study results were acceptable for within-laboratory and between-laboratories precision and the method was adopted as an AOAC–IUPAC method [87]. The major disadvantages of the postcolumn iodine method are the need for daily preparation of the iodine solution and the necessity for two pumps.

The development of a method using postcolumn derivatization with electrochemically generated bromine by Kok and co-workers [88,89] overcomes the need for a second pump but adds an electrochemical KOBRA cell for the generation of bromine. Kok *et al.* [88] described the optimum operating characteristics of the KOBRA cell and applied this technique in a method for determining aflatoxins in cattle feed [89]. An RP-C_{18} column was used to accomplish the chromatographic separation with a mobile phase of water–methanol–acetonitrile (13:7:4) containing 1 m*M* potassium bromide and 1 m*M* nitric acid. The reaction coils provided reaction times of 4, 8 and 24 s at a flow-rate of 0.5 ml/min. The aflatoxins were detected using a fluorescence detector (excitation at 360 nm, emission at >420 nm). The detection limits were reported to be 0.04 ng for aflatoxins B_1 and G_1 and 0.02 ng for B_2 and G_2. Trucksess *et al.* [90] evaluated the KOBRA cell reversed-phase method using corn with three different clean-up procedures. TLC results were similar to HPLC results and the HPLC method could be automated.

7. GAS CHROMATOGRAPHIC–MASS SPECTROMETRIC CONFIRMATION

Confirmation of the identity of aflatoxins by means of MS analysis in the past required additional clean-up such as TLC isolation or SPE [91], as the presence of impurities in the sample extracts caused problems. New approaches involve interfacing gas chromatography with mass spectrometry (GC–MS), which uses GC to separate the impurities in the extracts and MS to confirm the identities of the aflatoxins. Trucksess *et al.* [92] were the first to use GC–MS to analyze for aflatoxin B_1. The extract samples were injected directly on to the GC column at 40°C. Immediately after sample injection, the column temperature was raised to 250°C in 4 min. A 6 m × 0.2 mm I.D. methylsilicone-coated fused-silica column was used and the effluent was analyzed by negative-ion chemical ionization (NICI) MS.

Goto *et al.* [93] used GC with flame ionization detection to analyze a mixture of four aflatoxins. The initial and final temperatures were set at 50 and 300°C, respectively, and the rate of heating was set at 15 or 20°C/min. A 5% phenylmethylsilicone capillary column was used to separate aflatoxins B_1, B_2, G_1 and G_2 with 2, 2, 4 and 4 ng being injected, respectively. This technique coupled with MS could be used for the determination and confirmation of the aflatoxins.

Holcomb *et al.* [94] used thermospray mass spectrometry (TSP-MS) to characterize the reaction products of aflatoxins B_1 and G_1 with iodine in methanol–water. About 2 μg of the derivatives were injected into the HPLC–TSP-MS system. The mass spectra showed ions at *m/z* 471 and 488, corresponding to the $[M+H]^+$ ion of the derivatized aflatoxins B_1 and G_1, respectively. These results indicated that the reaction products were adducts with one iodine atom and a methoxy group on the furan ring. Hurst *et al.* [95] used an HPLC–TSP-MS method for the confirmation of aflatoxins in peanuts. A 5-μm C_{18} column (25 cm × 4.6 mm I.D.) was used with a mobile phase of 0.1 *M* ammonium acetate–methanol–acetonitrile (56:22:22) at a flow-rate of 1.0 ml/min. The detection limits for B_1, B_2, G_1 and G_2 were 60, 40, 100 and 100 pg, respectively.

8. FUTURE PROSPECTS AND CONCLUSION

Methods for the determination of aflatoxins by TLC and HPLC have been well developed. The separation and determination of aflatoxins B_1, B_2, G_1, G_2 and M_1 in various matrices are no longer difficult. The individual aflatoxins can be routinely determined in almost any well equipped laboratory. The fact that the aflatoxins are fluorescent and can be selectively analyzed has helped in methods development. The advent of immunochemical technology has made aflatoxin detection possible in many different environmental conditions.

The primary difficulty with aflatoxin analysis lies with sampling and subsampling and not with the analytical method. Whitaker *et al.* [96] calculated the sampling, subsampling and analytical variances associated with testing corn. At 20 ng/g, the relative standard deviations associated with a 4.5-kg sample a 1-kg coarse-ground subsample, a 50-g fine-ground analytical sample and one CB TLC analysis were 21, 8, 11 and 26%, respectively. The use of HPLC or other more precise methods could reduce the variation of the analytical step to below 5% [97].

The greatest need is to improve the methods for sampling, subsampling and analytical sample preparation in order to reduce overall variations and make determinations of aflatoxin content in a given lot more accurate. Only limited improvements may be possible because of the heterogeneous distribution of aflatoxins in contaminated food and feed. At present, these sources of error far outweigh the variance contributed by the analytical method.

Analytical methods can still be strengthened by improving both extraction efficiencies and chromatographic detection. Further development of monoclonal antibodies in preliminary clean-up will help in HPLC methods for many commodities that contain many interfering substances. Advances in mobile phases, columns and detectors will probably allow routine aflatoxin determinations at the picogram level. New extraction techniques such as supercritical fluid extraction and detection methods need to be developed to eliminate the need for fluorescence enhancement and produce lower costs, increased efficiency, improved safety and meeting waste disposal requirements. Future methods for aflatoxin determination will certainly be more sensitive and improvements made in sampling will make aflatoxin control easier.

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