Confirmation of Identity of Aflatoxins

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ABSTRACT

Aflatoxins are detected and determined by analytical procedures based on physical and chemical properties, e.g., ultraviolet absorbance, fluorescence, solubility and chromatographic retention times during thin layer (TLC) or liquid chromatography. For acceptance of analytical results based on these properties, especially for regulatory purposes, proof of identity of the compound being measured is essential. Numerous tests have been devised for confirmation of identity. Included are tests based on toxicological effects observed in the duckling, zebrafish, chick embryo, *Bacillus megaterium* and many other species; chemical tests based on formation of derivatives such as the acetates and water adducts; and tests based on color changes of TLC spots after contact with spray reagents, e.g., sulfuric acid. All of the foregoing have inherent uncertainties in interpretation of identity. On the other hand, mass spectrometry (MS) is one of the most specific methods of identification available; however, it has been difficult to apply at the low concentrations at which aflatoxins are routinely detected. In this paper, the confirmation techniques for aflatoxins are placed in historical perspective and are reviewed and evaluated. A recently developed procedure for the application of negative ionization MS for the confirmation of identity of aflatoxins in foods or feeds at concentrations as low as 10 ng/g is described. This procedure consists of isolation of the aflatoxin by Association of Official Analytical Chemists' methods, purification by preparatory 2-dimensional TLC, in situ elution of the aflatoxin TLC spot and analysis of the sample by negative ion chemical ionization MS using a direct insertion probe.

INTRODUCTION

Identification of aflatoxins in 1961 as the agents responsible for severe economic losses by the British turkey industry, and the subsequent reporting of toxicological information, particularly that information identifying aflatoxin B_1 as a potent liver carcinogen in certain animal species, may be regarded as the fuse which led to an explosion in research efforts directed not only against aflatoxin, but against other mycotoxins, as well. These research efforts, continuing today, were directed primarily toward (a) development of analytical methods for the determination of low levels of aflatoxins in foods and feeds; (b) numerous surveys of foods and feeds for the presence of aflatoxins; (c) extensive studies of the toxicological properties of the aflatoxins and their metabolites; (d) the development of risk-benefit analyses and regulations by many countries in attempts to control human exposure to the aflatoxins; (e) the development of procedures for preventing contamination of foods and feeds by aflatoxin; and (f) development of ways of decontaminating aflatoxin-contaminated commodities. An important aspect of these activities has been the necessity for confirming the identity of the most toxic and most predominant aflatoxin, aflatoxin B_1. So germane was this issue to taking any sort of "regulatory action" that, in 1967, the Association of Official Analytical Chemists (AOAC) established an Associate Refereeship for Confirmative Methods. This refereeship was later discontinued with the understanding that all methods developed for aflatoxins include a "confirmatory step."

Various investigators have set different criteria for what constitutes a satisfactory "confirmation" of identity, given the circumstances in which the analysis is performed. The purpose of this paper is to describe and review the efforts exerted to date in arriving at a satisfactory technique for the confirmation of the identity of suspect aflatoxin.

CONFIRMATION OF AFLATOXIN BASED ON BIOLOGICAL ACTIVITY

Shortly after the discovery of the aflatoxins and the realization that the aflatoxins were potent hepatocarcinogens in certain animal species, efforts were exerted to develop analytical methods for the determination of low levels of aflatoxins in human foods. It was immediately recognized that these methods, based on thin layer chromatography (TLC), provided some degree of specificity, but that there would be circumstances that required further confirmation. It was necessary to provide techniques for "confirmation" of *presumptive* aflatoxin.

The first widely used confirmatory technique was a duckling bioassay (1). In this assay, extracts containing a few μg of aflatoxin B_1 were intubated into day-old ducklings; a week later the birds were sacrificed, and the livers examined for bile duct proliferation. This test was not nearly sensitive enough for practical regulatory purposes and was soon replaced by a bioassay using the fertile chicken egg (2,3—sec. 26.073).

In the chick embryo bioassay, about 10 μg of aflatoxin B_1, isolated by preparative TLC, was injected into the air sac of the eggs. The eggs were then incubated up to 21 days and observed for the effects on the chick embryo. Over the years, extracts from over 400 food samples have been examined by this technique with 100% correlation between the bioassay results and chemical derivative confirmatory techniques. This bioassay, when used in combination with an isolation step, has been by far the most reliable and most widely used confirmative technique for aflatoxin B_1. Bioassays developed subsequently are listed in Table I. None of the techniques listed in the table can be reported as giving the needed degree of confidence desired for most purposes, although some of these techniques are useful for screening purposes.

It is important to realize that none of these bioassays confirms identity; rather they confirm the presence of a toxin. The duckling and chick embryo bioassays have been the most widely used because they come closest to characterizing the analyte by describing the type of lesion expected and, consequently, are more specific than those based on lethality.

CONFIRMATION OF AFLATOXIN BASED ON PHYSICAL PROPERTIES

At one time, it was felt that an excellent approach to confirmation of identity would be to measure the migration characteristics of an analyte using a series of solvent systems of widely varying polarities (3—secs. 26.013, 26.027 [c], 26.A08; 11) and the changes in R_f noted. The most useful examples of this technique involve the use of 2-dimensional TLC (12).

An outgrowth of this concept is the use of 2 different detection devices. For example, one might use a TLC detection step and a high pressure liquid chromatographic (HPLC) confirmatory step. Alternatively, one might use HPLC with 2 different detectors, e.g., an ultraviolet (UV)

TABLE I

Aflatoxin Confirmatory Techniques—Bioassay

Organism	Amt of sample required	Time required	Observation	Ref.
Duckling	ca. 10 μg	7 days	Bile duct proliferation	1
Chicken egg	>0.05 μg	21 days	Mortality	2,3 sec. 26.073
Mollusk eggs	>0.05 μg	18 hr	Inhibition of cell cleavage	4
Brine shrimp	0.5 μg/0.5 mL	24 hr	Mortality	5
HeLa cells	5-7 μg/mL	30 hr	Growth inhibition	6
B. megaterium	1 μg	15 hr	Growth inhibition	7
Maize leaves	0.25 μg	19 hr	Growth inhibition	8
Zebra fish larvae	0.4 μg	24-45 hr	Mortality	9
B. subtilis	1 μmol	12 hr	Cell death	10

and fluorescence detector in series (13) or a UV and an electrochemical detector. This approach, although applied recently in a method for roquefortine (14), has not, to date, been applied to the aflatoxins. UV and fluorescence techniques for identification have been applied to aflatoxin isolated from peanuts and purified by TLC (15). Another approach was applied in a method for zearalenone (16) in which the fluorescence ratio was used. This technique has not been applied to the confirmation of aflatoxins.

Finally, it is worth noting that 2 excellent techniques have been developed based on physicochemical measurements. The first of these is an infrared (IR) spectroscopic technique in which a satisfactory spectrum of aflatoxin B_1 is obtained with as little as 8 μg of sample (17). The second technique involves the use of MS; this technique is detailed in a subsequent section. Both of these techniques come close to being the "absolute" confirmation desired; however, both techniques, and particularly the MS techniques, are costly in terms of equipment required.

CONFIRMATION OF AFLATOXIN BASED ON CHEMICAL PROPERTIES

An even more satisfactory approach to resolution of the problem of aflatoxin confirmation has been the use of chemical derivatization techniques. This approach has been used successfully for many years. Perhaps the most extensively used method involves the acid-catalyzed hydration of the vinyl ether system in aflatoxins B_1 and G_1. This technique had been used in combination with the chick embryo bioassay for many years by the Food and Drug Administration (FDA) in handling regulatory samples; however, because an absolute correlation between the tests for a large number of contaminated samples of specific commodities was found, the requirement for the chick embryo assay has been dropped for those commodities.

Of course, there have been many reports of use of a wide variety of other derivatives for confirmatory purposes. These include a large number of spray reagents (see Table II). It is important to realize that none of these reactions may be considered to be specific for aflatoxin.

Recently, a combination of a physical detection step (HPLC and fluorescence detectors) with a chemical derivatization step has been developed which appears to be specific for the aflatoxins (35,36). Such a combination of techniques may ultimately result in a practical, economical method for analyzing foods and feeds for aflatoxins.

CONFIRMATION OF AFLATOXIN BASED ON MASS SPECTROMETRY

It must be stressed that none of these techniques alone may be considered an "absolute confirmation" test. In recent years the realization of the need for a single, more certain

TABLE II

Aflatoxin Confirmatory Techniques—Chemical

Aflatoxin	Reagent	Product	Method of detection	Detection limit	Ref.
B_1,G_1	$HCO_2H/SOCl_2$				
	$HOAc/SOCl_2$	"H_2O adduct"	TLC-FL	0.25 μg B_1	18
	CF_3CO_2H				
B_1,B_2,G_1,G_2	2,4-Dinitrophenyl-hydrazine (DNPH)	Phenylhydrazones	TLC	–	19
	NH_2OH HCl	Oximes	TLC		
B_1,G_1	H_2SO_4		TLC-FL	–	20,3 sec. 26.083
	$FeCl_3$				
B_1,G_1	$HOAc/SOCl_2$	Acetates	TLC-FL	–	21
B_1,G_1	HCl/H_2O	"H_2O adducts"	TLC-FL	20 ng B_1/G_1	22,3 sec. 26.076, 23
	HCl/Ac_2O	Acetates			
B_1,B_2,G_1,G_2	*p*-Anisaldehyde	Condensation products	TLC-FL	–	24
B_1,B_2,G_1,G_2	*Di-o*-anisidine-tetrazolium chloride	–	TLC-FL	0.03 μg B_1,B_2 0.09 μg G_1,G_2	25
B_1	I_2	–	TLC-FL		26
M_1,M_2	Ac_2O,py	Acetate	TLC	0.04 ppb	27
B_1,B_2,G_1,G_2	2,4-DNPH	Phenylhydrazones	TLC	–	28
M_1		Acetate/hemiacetal	TLC-FL	30 ng	29
B_1,G_1	CF_3CO_2H	"H_2O adduct"	TLC-FL	0.5 μg/kg	30,3 sec. 26.083
B_1,B_2	$NaBH_4$	Reduction products	TLC	–	31
M_1	CF_3CO_2H	–	TLC-FL	1 ng	32
B_1,M_1	CF_3CO_2H	–	2D-TLC	0.5 ng	33,34
B_1	I_2/H_2O	–	HPLC-FL	–	35
M_1	CF_3CO_2H	–	HPLC-FL	1 ng	36

TABLE III

Aflatoxin Confirmatory Techniques—Mass Spectrometric

Aflatoxin	Commodity	Level	Technique[a]	Ref.
B_1,B_2,G_1,G_2,M_1	Cottonseed meal	750 ppb B_1 20 ppb B_2	TLC-direct probe/EI	37
B_1			TLC-direct probe/EI	38
B_1,B_2,G_1,G_2	Corn	15 ppb B_1 2.2 ppb B_2	FD	39
M_1	Milk powder, cheese	–	EI	40
B_1,B_2,M_1	Milk, bile, urine	ca. 1 μg	EI/HRSIM	41
B_1,B_2,G_1,G_2,M_1,M_2	Milk powder	–	EI	42
M_1	Milk	–	EI	43
B_1,M_1	Milk, ginger, melon seeds, peanut butter, corn	20 ppb M_1 10 ppb B_1	NICIMS	44

[a]FD = field desorption; EI = electron impact; NICIMS = negative ion chemical ionization mass spectrometry; HRSIM = high resolution single ion monitoring.

confirmation of aflatoxin identity has led to an investigation of the use of MS techniques (see Table III). That such techniques are needed and, in fact, are essential for the determination and confirmation of the identity of the aflatoxins may be stressed by considering 2 recent reports in which the chromatographic properties of certain extract components led to false identifications of aflatoxin B_1 (aflatoxin-like factor in tapioca [26], interferences in peanut butter extracts [3—sec. 26.031e], interference by ethoxyquin [45] and misidentification of aflatoxin M_1 [40]).

At the Bureau of Foods, FDA, we have recently developed an MS technique based on negative ion chemical ionization for confirming the identity of aflatoxins B_1 and M_1 (44). The experimental procedure is: (a) the aflatoxins are extracted and purified using previously developed procedures, i.e., the AOAC CB method (3—sec. 26.026, revised) for peanut butter, corn and melon seeds; the method of Trucksess and Stoloff (46) for ginger root; and a method devised for aflatoxicol (47) applied to aflatoxin M_1 in milk. (b) An extract equivalent to 10 g of original sample, dissolved in 100 μL of chloroform, is applied as a single spot on a 20 × 20 × 0.25 cm Silica Gel 60 precoated TLC plate. The plate is developed in 2 dimensions using the following solvents: for peanut butter, acetone/chloroform (1:9) and anhydrous ether/methanol/water (96:3:1); for corn, the identical solvents but in reverse order; for ginger root, acetone/chloroform (1:9) in both directions; for M_1 in milk, anhydrous ether/methanol/water (95:4:1) and chloroform/acetone/2-propanol (85:10:5). (c) The TLC plate, after development, is heated to 55 C for 1 min in a forced draft oven to evaporate the developing solvent. The aflatoxins are then located using long-wave UV light, and eluted in situ using an Eluchrom automatic elution system (Camag) and 2 mL of acetone/chloroform (2:8) for aflatoxin B_1, or 2 mL of chloroform/acetone/2-propanol (85:15:5) for aflatoxin M_1. It is, of course, important that overexposure of the aflatoxin to fluorescent lighting be avoided to minimize photodecomposition. The elution technique used here appears to be satisfactory without the addition of water that was required in a previous study (48). (d) The residue remaining after evaporation of the eluting solvent is dissolved in acetone in the receiving vessel and the solution and 2 acetone

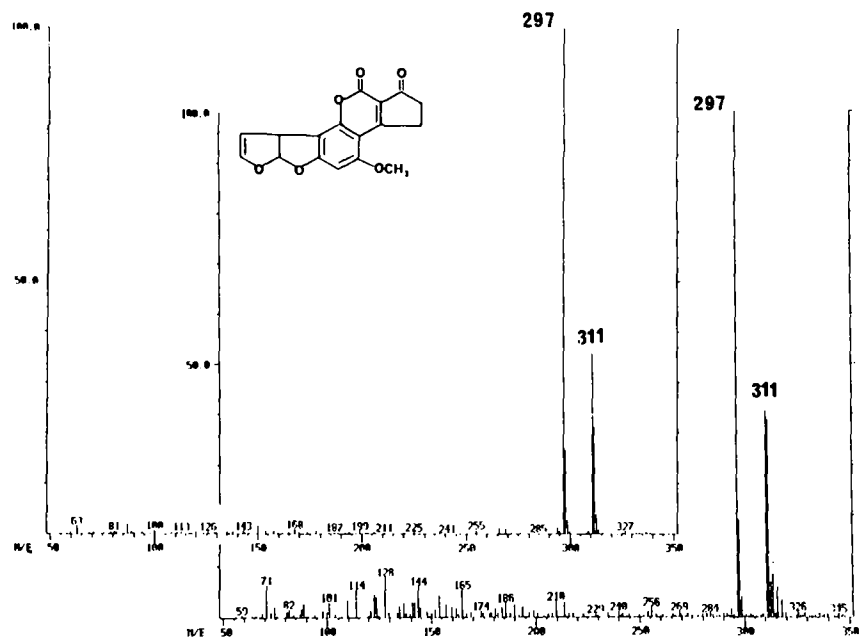

FIG. 1. Negative chemical ionization mass spectra of 43 ng reference aflatoxin B_1 (upper spectrum) and of aflatoxin B_1 isolated from peanut butter naturally contaminated at a measured level of 10 ng/g (lower spectrum). Full range of scan was 55-655 daltons; no ions with masses greater than 350 were detected.

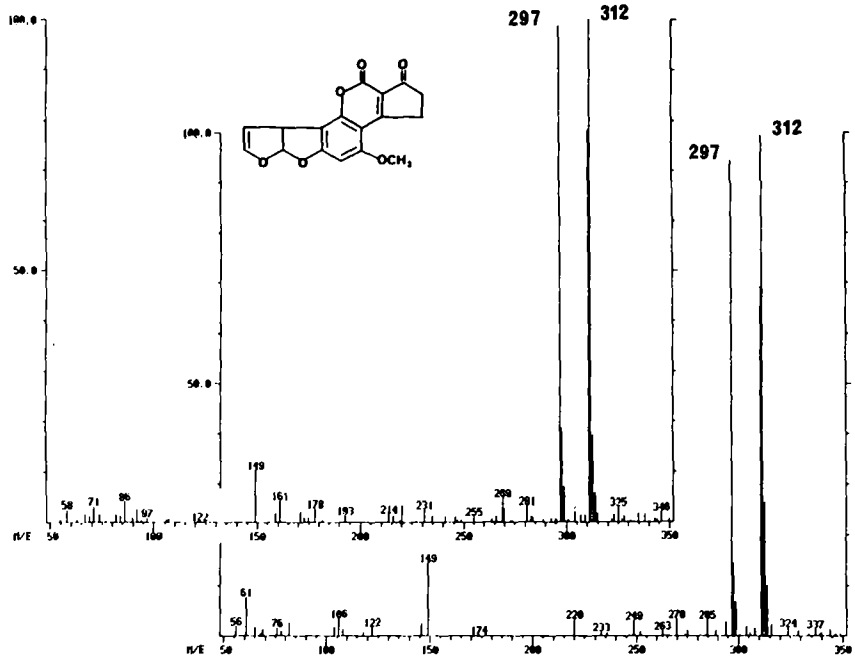

FIG. 2. Negative chemical ionization mass spectra of 12 ng reference aflatoxin B_1 (upper spectrum) and of aflatoxin B_1 isolated from corn naturally contaminated at a measured level of 13 ng/g. Full range of scan was 55-655 daltons; no ions with masses greater than 350 were detected.

washings are transferred to a concentrator tube for final evaporation, under a stream of nitrogen, in a warm water bath to about 10 μL. (e) An aliquot up to the full 10 μL of solution (depending on expected response), is transferred to a glass sample holder of the direct inlet probe of the mass spectrometer. Solvent is evaporated at low heat (ca. 60 C) in about 3-5 min. (f) The mass spectrum is obtained using methane as the reagent gas under negative chemical ionization conditions. The sample is ionized by resonance electron capture.

Figure 1 illustrates the spectrum of a standard of aflatoxin B_1 representing about 43 ng (top spectrum). The spectrum exhibits 3 major ions representing $M^{-\cdot}$, $(M\text{-}H^{\cdot})^-$ and $(M\text{-}CH_3{}^{\cdot})^-$ occurring at m/z 312, 311 and 297. Other masses in the scanned mass range (55-655 daltons) are of low relative abundance. The relative abundance of the 3 major ions varies with source temperature, and therefore, sample and standards need to be run under identical experimental conditions.

The lower spectrum on Figure 1 was obtained from aflatoxin B_1 isolated from a sample extract representing 10 g of peanut butter estimated by TLC to contain about 10 ng aflatoxin B_1/g. The spectrum exhibits the 3 major ions characteristic of aflatoxin B_1 and a relative absence of response for other ions in the scanned mass range.

Figure 2 illustrates the spectra obtained from a 12-ng standard of aflatoxin B_1 (top spectrum) and aflatoxin B_1 isolated from an extract representing 10 g of yellow corn estimated by TLC to contain about 13 ppb of aflatoxin B_1 (lower spectrum). As in the case of the peanut butter sample, the negative ion spectrum exhibits the 3 ions characteristic of aflatoxin B_1 and a relative absence of respose for all other ions in the scanned mass range.

These are full scan mass spectra from m/z 55 to 655. Final extract components other than aflatoxin B_1 that ionize by resonance electron capture will also be detected. Thus, the ability of the extraction and cleanup procedures to remove all ionizable components is the major factor limiting the level at which the identity of aflatoxins can be confirmed.

In summary, the realization of the necessity for confirmation of presumptive aflatoxin has led to an immense research and development effort. At first, these efforts resulted in relatively nonspecific bioassay confirmative procedures which were uniformly expensive, inconvenient and inconclusive, in the absence of a large amount of historical data on aflatoxin contamination. The research, therefore, soon turned to the use of chemical derivatization techniques which were much more specific, but still not absolutely specific. More recently, MS techniques have been developed that give a high degree of specificity but at relatively high cost. The result of this extensive effort is that we now have a large variety of techniques and combinations of techniques which allow for the certainty of confirmation required for any particular situation.

Confirmation of identity has, as in the case of aflatoxin, a historical perspective. In a commodity having a history of high incidence of aflatoxin contamination and no interfering compounds, the use of a single chromatographic procedure (TLC, HPLC) can provide adequate confidence in identity. With decreased experience with new commodities, and increased penalty for error (e.g., samples involved in economic or legal dispute), there is increased need for certainty; this can only be attained by using a multiplicity of tests or increasing the resolving power and specificity.

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Pons Scaled-Down Clean-Up Column Adapted for Use in Solvent-Saving Modification of the CB Method for Aflatoxin

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ABSTRACT

The solvent-saving procedure devised by Pons using a small chromatographic tube (Bio-Rad Laboratories glass Econo-Column, 10 mm id × 300 mm long) has been adapted and extended for use in modifications of the Official AOAC procedure for quantitative determination of aflatoxins in corn, peanuts, soybeans, coconut and pistachios. Thirty mL of each of 3 solvents for column washes was used instead of the 150 mL specified by the Official CB Method. The analytical aliquot was also reduced 80%, but sample size and extracting solvent volume were not changed, so that there was no loss in sensitivity. Toxins ranging from 3 to over 1,000 μg/g of sample were quantitated after clean-up using both procedures with no statistically significant difference between results.

INTRODUCTION

Since recognition of the aflatoxin problem in 1960, methods recently summarized (1,2) have been developed to quantitate this toxin in contaminated products. Methods of analysis for various commodities are described in the Association of Official Analytical Chemists (AOAC) Official Methods of Analysis (3). The CB (Contaminants Branch, FDA) Method is one such official method of analysis for aflatoxin in peanuts and pistachios (3). The procedures for determining aflatoxins in corn, soybeans and coconuts all use the CB column clean-up procedure. The CB clean-up procedure uses a silica gel column of 22 mm id × 500 mm that requires three 150-mL aliquots of eluting solvents. In 1977, Pons and Franz proposed a high pressure liquid chromatographic (HPLC) method for the quantitation of aflatoxins in cottonseed products (4). Part of the procedure involved use of a small column for extract clean-up. We have substituted this small Bio-Rad Laboratories glass Econo-Column, 10 mm id × 300 mm, hereafter termed "Pons" column, for the official one used in the CB procedure. The silica gel required for packing and the 3 elution solvents are reduced 5-fold from those required by the official procedure. The aliquot taken from the initial extraction for analysis is also reduced 5-fold. The sample size and volume of original extracting solvent are not changed, so there is no loss in sensitivity. We applied the solvent-saving modification to tests of corn, peanuts, soy-