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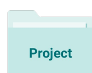

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REVIEW

Aflatoxin analysis at the beginning of the twenty-first century

Gordon S. Shephard

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Abstract Aflatoxin mycotoxins were first described in the early 1960s as important fungal toxins, which contaminate many different human foods and animal feeds. Accurate and sensitive determination of these carcinogenic compounds immediately became an important requirement to meet food safety concerns and new official legislated regulations. For these reasons, analytical methods for aflatoxins continued to develop over the decades, reflecting advances in analytical chemistry. Currently, a wide range of methods are available to analytical scientists, ranging from newly described multi-toxin liquid chromatography tandem mass spectrometry to rapid methods based on immunological principles. These latter methods can provide quantitative outputs or a simple rapid determination of contamination level above or below a pre-determined cutoff value. The newest official methods as validated by Association of Official Analytical Chemists International or Comité Européen de Normalisation rely on immunoaffinity column clean-up of conventional extracts, followed by high-performance liquid chromatography separation of the analogues with detection based on natural fluorescence or the fluorescence generated by various derivatisation methods. In selecting from this range of available methods, the analytical chemist must decide on the requirements of the analysis such that the method chosen is ‘fit for purpose’.

Keywords Aflatoxin · HPLC · Mass spectrometry · Fluorescence · Mycotoxin

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Introduction and background

The outbreak of the so-called Turkey X disease among turkeys in the UK in 1960, some 50 years ago, is the seminal event that led to the discovery of the aflatoxins and a proliferation in research on fungal toxins contaminating food and feed materials. The aflatoxins are mycotoxins produced mainly by the fungal species *Aspergillus flavus* and *Aspergillus parasiticus*, common saprophytes and opportunistic pathogens, which occur extensively in the tropics and semi-tropics on a wide range of agricultural commodities and food matrices. Of these two species, *A. flavus* produces aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2), whereas *A. parasiticus* produces these B aflatoxins as well as aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). Typical materials that are susceptible to aflatoxin contamination include maize and other cereals such as wheat and rice, groundnuts and other nuts such as pistachios and Brazil nuts, cottonseed, copra and spices. Ingestion of contaminated food or feed by female mammals leads to the excretion in milk of partially detoxified hydroxylated analogues, aflatoxin M_1 (AFM_1) and aflatoxin M_2 (AFM_2). In dairy cows, these contaminants can occur in commercial milk and can be processed into cheese, whereas in many developing countries, the presence of AFM_1 in human milk can lead to exposure to aflatoxins from infancy [1].

AFB_1 is a potent human carcinogen [2], which occurs in the low or sub micrograms per kilogram range and is regulated by legislation in the EU at 2 μg/kg in foods for direct human consumption [3]. AFM_1, although not as potent as AFB_1, is regulated in the EU at the level of 0.05 μg/kg, due to the high consumption of milk by infants and children. Analytical determination of contaminants at these low levels presents the analytical chemist with a range of challenges in isolating the analyte of interest and

separating it from co-extracted interfering impurities. In the case of the aflatoxins, the natural or induced fluorescence of these molecules aids their detection and the original thin-layer chromatographic (TLC) methods relied on analyst evaluation of fluorescent spots observed under a long wavelength UV light [4]. The designation of "B" and "G" analogues was based on their respective blue and green fluorescence colours. With the development of high-performance liquid chromatography (HPLC), fluorescence detection is still used as the method of choice in official analyses [5–9].

The need for analytical determination of aflatoxins has resulted in a plethora of methods to meet a range of analytical requirements by various analysts, from regulatory control in official laboratories to rapid test kits for factories and grain silos. These rapid methods generally involve the use of aflatoxin-specific antibodies, which can be specific for AFB_1, the most potent of the aflatoxins, and one which is frequently separately legislated or can give an analytical response for total aflatoxins based on cross reactivity of the antibody for all four B and G analogues. The purpose of this review is to examine the current status of methods and how recent advances are impacting on aflatoxin analysis.

Chromatographic methods

Aflatoxins are low molecular mass polar compounds, which possess significant UV absorption and fluorescence properties. For this reason, liquid separation techniques have predominated in their analysis, initially TLC, but subsequently HPLC. Although some interest has been shown in separation of aflatoxins using capillary electrophoresis, particularly with micellar electrokinetic capillary chromatography with laser-induced fluorescence detection, the technique has remained a research topic rather than finding application in routine analysis [10]. However, the ultimate in low detection limit was achieved by the application of multiphoton excited fluorescence and capillary electrokinetic chromatography in a narrow-bore 2.1 μm i.d. capillary containing carboxymethyl-beta-cyclodextrin, which achieved a detection limit for AFB_2 of 4.4 zmol (about 2,700 molecules) [11]. Mycotoxins contained in food or feed commodities must be extracted with a suitable solvent or solvent mixture, cleaned-up and concentrated before separation by HPLC. Commonly, quantification is achieved mainly by fluorescence detection and rarely by UV measurement. With the advent of atmospheric pressure ionisation techniques, the coupling of HPLC to mass spectrometry became a viable technology, and significant advances have been made in the determination of single and multiple mycotoxins, including aflatoxins (see Sulyok et al. in this volume).

Extraction

Extraction of aflatoxins from various food matrices generally involves aqueous mixtures of polar organic solvents such as methanol, acetone or acetonitrile, whereas extraction with chloroform, used in a number of previous methods, has largely been superseded by these other solvents as part of the international drive to reduce the consumption of chlorinated solvents. Despite the routine nature of aflatoxin extraction, a number of issues must still be considered. The volume of extraction solvent (and hence the waste generated in a large routine laboratory) was addressed in peanut butter analysis by reducing the sample size from 25 to 5 g and using a two-stage extraction procedure, firstly with methanol–15% sodium chloride solution and then with pure methanol [12]. Although this approach may be useful in a processed product in which some homogenisation has occurred, it should be used with caution in raw materials considering the highly inhomogeneous nature of aflatoxin contamination and the need for the analytical subsample to adequately represent the batch. Other authors have investigated more closely the various organic mixtures that have been proposed and their possible interaction with different matrices. The extraction of dry sample material can lead to compositional changes in the extraction mixture due to water absorption by the dry matrix such as infant formulae, animal feeds and paprika [13]. Such effects vary greatly between aqueous mixtures of methanol, acetone and acetonitrile, with the last showing the greatest effect in which absorption of water by the dry matrix can lead to significant elevation in the measured aflatoxin level. This apparent increase in level is dependent on the sample-to-extractant ratio, with an increasing effect as the ratio is changed from 10 g sample per 100 mL extractant to 40 g per 100 mL. At this latter ratio, recoveries of spiked AFB_1 from dry paprika were in the range 137–170%, whereas in the former ratio, recoveries were 78–89%. A common procedure is the addition of sodium chloride to an aqueous methanol extraction; yet if this procedure is transferred to an aqueous acetone or acetonitrile extractant, layer separation can occur with the aflatoxin being differentially distributed between the two layers formed. A similar effect can occur with aqueous acetonitrile in samples containing sucrose. Given that methanol is a more favourable solvent for subsequent clean-up by immunoaffinity column (IAC), due to the fact that antibodies can tolerate higher amounts of this solvent, aqueous methanol would appear to offer a more robust choice as extraction solvent [13]. However, other authors, who have investigated the extraction of AFB_1 from a defatted peanut powder certified reference material (CRM) with aqueous mixtures of the above organic solvents, have indicated that methanol–water (80+20) achieves only 59% of the level

achieved by acetonitrile–water (60+40), which gave a value within the certified range of the CRM [14]. As both extraction mixtures achieved acceptable recoveries, the extent to which solvent mixtures can extract incurred residues, as opposed to added spikes, is a question not frequently addressed.

Supercritical fluid extraction (SFE) has been used for a number of years in food processing (such as decaffeination and extraction of flavours and fragrances) and has found application in food analysis as it provides an extraction system which minimises or eliminates organic solvents by using supercritical carbon dioxide [15]. However, the application of this technology, either with or without an added organic modifier such as acetonitrile or methanol or their mixtures, to the extraction of polar aflatoxins has presented a number of problems with either low recoveries or high levels of co-extracted impurities such as lipids, which interfere with subsequent clean-up and chromatography [16]. Nevertheless, SFE has been used as the extraction step prior to liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of aflatoxins in the Chinese traditional medicine Zizyphi fructus [17]. As mentioned above, SFE is used to extract the flavours from spices and is popular as it eliminates the need for large volumes of solvent and provides an extract closer in composition to the essential spice oil. Given this poor recovery of aflatoxin from matrices extracted with pure carbon dioxide, it has been shown that the application of SFE to extract aflatoxin-contaminated paprika powder results in a considerable reduction in AFB_1 contamination of about 60% relative to the original paprika [18].

The possibility of achieving simultaneous extraction and clean-up has been investigated by various groups using matrix solid-phase dispersion (MSPD) for analysis of aflatoxins in peanuts and high-pigment matrices such as chilli powder, green bean and black sesame [19, 20]. MSPD involves the grinding together of the sample and a suitable sorbent (usually octadecylsilica, silica gel or alumina) using a pestle and mortar. The solid mixture is transferred to a glass column or cartridge containing a lower layer of carbon black in the case of the high pigment samples. The column may then be washed with a series of organic solvents, and the aflatoxins are eluted and determined by HPLC. Recoveries from spiked samples were reported to be between 78% and 95% with relative standard deviations between 4% and 7%.

Purification

The extracts of most matrices are unsuitable for direct chromatographic analysis due to the large number of co-extracted impurities. The recent exception to this has been the use of LC-MS/MS, where ‘dilute and shoot’ methods have been developed. These rely on the sensitivity and specificity of the mass analyser to overcome the problem of co-eluting impurities, which interfere with detection using spectrophotometric detectors [21, 22]. Purification on open glass columns packed with silica, although still employed in some laboratories, has largely given way to solid-phase extraction (SPE) methods, multifunctional columns or immunoaffinity columns [5–9, 23–25]. Both the latter two clean-up techniques have been developed commercially into a direct fluorometric method in which the column eluate is derivatised and the resulting fluorescence directly measured. Such methods should be applied with care as untested matrices may provide interfering compounds [26]. In one newly developed method for determination of both AFB_1 and ochratoxin A (OTA) in Mediterranean virgin olive oil, the final extract is applied to a silica SPE cartridge [27]. The toxins are adsorbed on the cartridge and then separated by elution with solvents of different polarities and finally analysed separately by HPLC. Other workers have reported the use of a SPE cartridge containing a hydrophilic–lipophilic-balanced water-wettable copolymer for the determination of aflatoxins in peanuts by HPLC with fluorescence detection [28].

The advantages of IACs are the effective and specific extract purification provided, the economic use of organic solvents and the improved chromatographic performance achieved with cleaner samples. An added advantage of improved clean-up is the fact that larger aliquots (up to 1 mL in some cases) can be injected into the HPLC, provided the elution strength of the injection solvent matches or is less than the HPLC mobile phase [29]. Large injections of this nature clearly benefit the detection and quantification limits of the analysis. As a result of the above advantages, IACs have been increasingly utilised in mycotoxin analysis in general and aflatoxin analysis in particular. A number of methods developed specifically in EU laboratories to meet the requirements of the low EU maximum tolerated levels have relied on the use of these clean-up methods, in which the extracts of various matrices can be purified by essentially the same protocol. The method for AFB_1 and total aflatoxins in peanut butter, pistachio paste, fig paste and paprika powder, which is now an official Association of Official Analytical Chemists method following its successful collaborative trial, is an example of this approach [30]. More recently, similar methods have been collaboratively studied and found suitable for aflatoxins in hazelnut paste [31], infant baby food [5] and maize samples [8]. The relative purity of the final eluate from IACs allows them to be used in method development for contaminated and complex matrices other than cereals and foods. Thus, methods have been developed to allow the contamination of medicinal herbs and plants to be determined [32–35]. Extracts of these medicinal herbs

can contain a complex mixture of chemical constituents, and the pH values of the extract can fall below pH 3 [36]. Dilution of these acidic extracts with the conventional phosphate-buffered saline (PBS, pH 7.4) failed to neutralise the solution, resulting in greatly reduced recovery of the aflatoxins, especially AFG_2. This problem was overcome by using a 0.1 M phosphate buffer (pH 8.0) for the dilution stage as it has a greater buffering capacity than PBS. Other applications include the analysis of animal liver (pig, chicken, turkey, beef, calf), an organ that is consumed as food and which is known to accumulate aflatoxins if the animal is fed aflatoxin-contaminated feed [37, 38]. Similar, a method was developed for determination of possible contamination of tiger nut-based soft drinks (horchata) available in Spanish and Belgian markets [39].

The success of IACs as a purification medium for a single mycotoxin class has resulted in the development and commercialisation of multi-mycotoxin IACs, which contain antibodies specific to more than one mycotoxin. These are useful for analysis of commodities which can contain a number of different mycotoxins as a consequence of co-infection with different toxigenic fungal strains or species. The development of such methods necessitates the use of extractants that can achieve good extraction of the various mycotoxin moieties involved. Methods have been developed to allow simultaneous clean-up of aflatoxins and OTA from extracts of ginseng and ginger [40, 41], Spanish bee pollen [42] and a range of foods including peanut butter, maize, polenta, maize snacks and corn flour [43]. Further developments have added more antibody types to the IAC, and a method was developed for aflatoxins, OTA and zearalenone in rye, rice and pig feed [44]. The ultimate column of this type contains antibodies against aflatoxins, OTA, fumonisins, deoxynivalenol, zearalenone and T-2 toxin [45]. This method, which involves a double extraction of the mycotoxins from maize and clean-up on a single multi-mycotoxin IAC, was developed for LC-MS/MS quantification.

Apart from the above improvements in IAC design, other clean-up methods have also been developed. Anti-AFB_1 antibodies have been incorporated in an immunoaffinity disc for on-line extraction [46]. Other researchers have investigated the possible use of composite adsorbents formulated from calcium montmorillonite clay as a clean-up medium [47]. These materials were synthesised by self assembly of suspended clay aggregates onto the anionic surface of quartz particles using a cationic polyelectrolyte in aqueous solution. Subsequent analyses of rice and maize confirmed the concept, although spiked samples achieved low recoveries between 34% and 63% in initial experiments. Other clean-up concepts that have been applied to other mycotoxins and may show future promise for clean-up of aflatoxin extracts are molecularly imprinted polymers (based on the chemical generation of a simulated binding site for the mycotoxin in a polymeric lattice) [48] and DNA aptamers (based on the synthesis of single-stranded oligonucleotides) [49].

Separation and detection

As mentioned above, the chromatographic separation of aflatoxin analogues was originally performed by TLC. Semi-quantification was achieved by comparison of sample and authentic standards using visual estimation of fluorescence of the separated spots under long wavelength UV light. More recent advances have involved the development of methods based on overpressured-layer chromatography for determination of aflatoxins in red paprika, fish, maize and wheat [50, 51]. These methods were coupled with fluorescence densitometry as a quantification step and could achieve limits of detection as low as 0.5 μg/kg.

Separation of the aflatoxins has been performed for many years by HPLC [52]. Although both normal and reversed-phase columns have been used, the vast majority of separations are performed on reversed-phase systems with mobile phases composed of water, methanol and acetonitrile mixtures. It has been reported that binary systems of water and methanol resulted in broad HPLC peaks and long chromatographic run times, whereas binary mixtures of water and acetonitrile failed to yield baseline resolution [29]. As a result, the optimum mobile phase was found to be a ternary mixture of the three solvents, tailored to the individual characteristics of the HPLC column. Chromatographic performance has improved with column technology, particularly with reduced size of the column packing material. The introduction of packing materials with particle size 1.7 μm in Ultra-Performance Liquid Chromatography® has brought total run times down to about 3 min using a mobile phase of methanol–water with 0.1% formic acid and mass spectrometric detection [53].

The HPLC detection of aflatoxin analogues is most often achieved by application of fluorescence detection. Although the aflatoxins are naturally strongly fluorescent compounds, making them ideal subjects for fluorescence detection, various analogues exhibit solvent-dependent quenching in HPLC solvent systems. In the aqueous mixtures used for reversed-phase chromatography, the fluorescence of AFB_1 and AFG_1 are significantly quenched [54]. This is generally overcome by derivatisation of these two analogues at the reactive 8,9-double bond of the dihydrofuran moiety. Initially, precolumn derivatisation using trifluoroacetic acid (TFA), which causes hydration of the 8,9-bond, was used to produce the hemiacetals AFB_{2a} and AFG_{2a}, which possess similar fluorescence properties to AFB_2 and AFG_2. However, the relative instabilities of these derivatives and the advantages of automation offered by post-column derivati-

sation methods led to the adoption of this latter technique [29]. Although the use of TFA was not viable as a post-column reagent due to corrosion problems, reaction with halogens at the 8,9-double bond was found to be a suitable alternative. Originally optimised in the 1980s, iodine by post-column addition of a saturated solution and subsequent heating at 60–75 °C in a reaction coil provided good performance [55, 56]. Detection limits for this method were of the order 20 pg/injection for AFB_1. An added advantage of automated post-column methods is that the derivatisation can be switched off and the decrease in peak heights of the peaks representing AFB_1 and AFG_1 observed as a confirmation of their presence. However, the iodination method also has several disadvantages, including the need for a separate pump and a heated reaction coil, which can cause peak broadening, and the possible crystallisation of iodine in incorrectly operated systems. Consequently, reaction systems utilising the more reactive halogen, bromine, were introduced with the added advantage of a greater analyte response than achieved with iodine [29, 57]. Post-column bromination can be cleanly achieved in either one of two ways, the simplest being electrochemical generation in a so-called Kobra cell. For this method, potassium bromide is dissolved in an acidified mobile phase. The alternative method requires a pulseless pump for postcolumn addition of pyridinium bromide perbromide and the use of a short reaction coil at ambient temperature. The bromination methods have found wide applicability and have been incorporated in a number of methods, which have undergone validation by collaborative study. These include the determination of aflatoxins in peanut butter, pistachio paste, fig paste and paprika powder [30], baby food [5], hazelnut paste [31] and maize [8].

Photochemical derivatisation is an alternative and more economic post-column derivatisation method. This is achieved by passing the HPLC column eluate through a reaction coil wound around a UV light at ambient temperature, which causes hydration of AFB_1 and AFG_1 to their respective hemiacetals. A recent comparison of this method with the Kobra cell and iodine methods for peanuts and maize showed that the methods were analytically equivalent for peanuts but gave a slightly high bias for maize [58].

Enhancement of the fluorescence of aflatoxins can also be achieved without chemical derivatisation by the incorporation of specific cyclodextrins (CDs) in the mobile phase [59, 60]. CDs are cyclic oligosaccharides composed of multiple subunits of glucose in an alpha(1-4) configuration. They can be classified according to the number of glucose units and the type and degree of substitution. The cyclic nature of the structure gives rise to an internal cavity that can act as a host site for smaller molecules by forming an inclusion complex. The inclusion of certain molecules can lead to the enhancement of their fluorescence properties, the exact mechanism of which is not clear [59]. The first application of these effects for aflatoxin analysis was for separation and detection of aflatoxins by capillary electrophoresis using carboxymethyl-beta-CD or sulphated-beta-CD in the electrophoretic buffer and laser-induced fluorescence [11]. A HPLC method has been developed in which beta-CD or succinyl-beta-CD was added to the water–methanol mobile phase [61]. The fluorescence of AFB_1 and AFG_1 was significantly enhanced in the HPLC chromatogram, whereas the fluorescence of AFB_2 and AFG_2 and the retention time of all aflatoxins were little changed.

Arguably the greatest advance in mycotoxin analysis during the previous decade has been the introduction of mass spectrometry as a viable detector system. The coupling of HPLC to mass spectrometry via atmospheric pressure ionisation techniques such as electrospray ionisation (ESI) or atmospheric pressure chemical ionisation (APCI) has resulted in a range of new methods for single mycotoxin, single mycotoxin groups (such as aflatoxins) or for true multitoxin analyses [62]. The advantages of LC-MS or LC-MS/MS lie in the improved detection limits, the confirmation provided by on-line mass spectral fragmentation patterns and the ability to filter out by mass any impurities that interfere in spectrophotometric detectors. For aflatoxin determination, a number of instrument types have been used, including single quadrupole [20, 63], triple quadrupole [22] and linear ion trap instruments [21, 45]. Most reports deal with ESI, although a few workers have used APCI [17, 64], with both interface types being operated in the positive ion mode for aflatoxin detection. Comparison of ESI with atmospheric pressure photoionisation concluded that the latter source gave lower chemical noise, less signal suppression and a lower quantification limit for AFM_1 by a factor of 2, although the ESI source was found to be more robust [65, 66]. The use of matrix-assisted laser desorption ionisation time of flight mass spectrometry has also been described for the high-throughput screening of aflatoxins [67]. Quantification of aflatoxins is generally achieved by external calibration with mycotoxin standard solutions, although $[^{13}CD_3]$-AFB_1 has been used as an internal standard [68]. More recently, deuterated AFB_2 and AFG_2 were synthesised by catalytic deuteration of AFB_1 and AFG_1, respectively [69]. These two isotopically labelled compounds were used to develop a staple isotope dilution assay for aflatoxins in food by LC-MS/MS. Most authors report that sample clean-up is still performed prior to LC-MS/MS analysis. In this regard, the clean-up techniques described above have all been applied prior to LC-MS analysis. In particular, multimycotoxin IACs for aflatoxins, OTA and Fusarium toxins have been used as the clean-up step in multiple toxin analyses [45]. This may be necessary in multiple analyses as the problem

of matrix effects such as ionisation suppression could occur in such analytical systems. However, other groups have used the power and sensitivity of the LC-MS/MS to achieve an extensive multimycotoxin method [21, 22]. Spanjer and co-workers [22] report the analysis of 33 mycotoxins extracted from peanut, pistachio, wheat, maize, cornflakes, raisins and figs using an acetonitrile–water mixture, dilution of the extract and analysis on triple quadrupole LC-MS/MS with positive ESI and multiple reaction monitoring. All analytes were monitored by two transitions (parent and two-fragment ions), one for quantification and the other for confirmation. Matrix effects were overcome by matrix-matched standards. Another group has reported determination of 87 analytes, which required two chromatographic runs on a triple quadrupole linear ion trap instrument operating with positive and negative ESI in the separate runs [21, 70]. Validation was performed in wheat and maize samples, with ion suppression being experienced on some toxins in the maize sample. This could largely be overcome by matrix-matched standards, although some toxins such as fumonisins and nivalenol were not fully extracted in the extraction solvent optimised for the majority of the analytes.

Given that aflatoxin analysis is readily achieved by standard HPLC with fluorescence detection, it may be considered that few routine laboratories will invest in LC-MS/MS as their primary methodology for aflatoxins, but that the LC-MS/MS instrumentation will form part of a broader range of their analytical requirements or commitments. In this scenario, the instrument used for aflatoxin and its configuration will most probably reflect the bulk of the requirements of the analytical work of the individual laboratory.

Immunology-based methods

The application of aflatoxin-specific antibodies has produced a range of immunoassay analytical methods which rely on the recognition of an aflatoxin epitope by a specific antibody. Using the ingenuity of researchers, these immunoassays have been developed into a wide range of formats to meet the specific demands of analysts. The IAC clean-up columns described above have been commercialised into systems in which the IAC eluate is used directly for aflatoxin quantification based on derivatisation and subsequent reading of fluorescence in a commercial fluorometer. A number of commercial enzyme-linked immunosorbent assays (ELISAs) are well established and available. The essential principle of these assays is the immobilisation on a suitable surface of antibody or antigen and the establishment of a competitive process involving this resource and components of the analytical solution [71]. In this manner, commercial companies have developed and marketed ELISAs which are well defined in their applicability, analytical range and validation criteria and merely require the laboratory to ensure their ability to perform the assay satisfactorily and within the constraints of matrix and analytical range specified by the manufacturer. ELISA technology for aflatoxin analysis has thus become a mature technique. Nevertheless, the development of new ELISA methods is still reported, especially to meet the challenge for cost-effective analysis in a range of commodities in developing countries [72]. An ELISA developed by the International Crops Research Institute for the Semi-Arid Tropics has been used to study aflatoxin contamination in peanuts grown in western Kenya [73].

A number of other immunoassay techniques have been developed and, in some cases, commercialised. One of the simplest and fastest technologies is the lateral flow device, usually in the format of a strip or dipstick [74–77]. Aflatoxin if present in the sample extract interacts with colloidal gold conjugated anti-aflatoxin antibodies at the base of the stick. Both bound and unbound antibodies move along the stick membrane, passing a test line composed of immobilised mycotoxin which will bind free antibody to form a visible line indicating a level of aflatoxin contamination below the test cutoff value. Typically, commercial kits contain a control line further along the stick composed of anti-antibodies as control for complete extract migration along the strip. Issues related to this technology, beside the matrices for which the test is valid, include the cutoff limit set by the producer and the degree of false negatives during testing. Since this is a screening technology, false positives are less serious, as such samples would normally be further tested by a fully quantitative method. In order to introduce an element of quantification into this technology, photometric strip readers have been used and even commercialised [74, 77]. A related qualitative test technology, namely a membrane-based flow-through device in which the test and control lines are generated by an enzyme–substrate colour reaction, has also been commercialised but carries similar concerns as to substrate applicability and the proportion of false positive and negative results [78].

More recently, the desire for multiple analyses has resulted in the development of array biosensors, which can be used for simultaneous analysis of multiple samples or simultaneous analyses of multiple target analytes [79, 80]. The multiple targets for this technology included large pathogenic bacteria (*Campylobacter* sp.), as well as mycotoxins AFB_1, ochratoxin A, deoxynivalenol and fumonisin B_1. Silanised microscope slides were patterned with suitable capture species for the sandwich immunoassay used for the bacterial assay and the competitive immunoassay for the mycotoxin assay. The glass slides acted as a waveguide for the detection system,

which involved incident laser light launched into the end of the waveguide and charge-coupled device camera recording of the fluorescence of surface-bound species resulting from excitation by the evanescent wave. The possibilities of multiple analyte screening by immunoassay have recently been reviewed [81]. The problem of aflatoxin and ochratoxin A contamination in spices has also been addressed by the development of a specific clean-up tandem immunoassay column with two detection layers [82] and also the development of a flow-through device based on ELISA [83]. The novel tandem column incorporates a clean-up sorbent at the base of a syringe tube, with the detection layers placed separately above. Diluted sample extract was pulled through the column from the base so as to pass through the sorbent and interact with the capture species in the detection layers in the upper half of the tube packing. Subsequent steps involved reversal of flow to remove the extract, followed by a wash step and colour development in the capture layers, the intensity of which is inversely proportional to the mycotoxin levels in the sample. The device was suitable for highly coloured chilli extracts with an AFB_1 cut of 5 μg/kg [82].

The immunoassays described above rely on a visual or optical endpoint. However, electrochemical detection systems have also been described. In their simplest format, these are analogous to conventional competitive ELISAs, but the immobilised antibody is bound to the surface of a screen-printed electrode, and the final enzymatic stage develops a reaction product that can be measured by its electrical properties. Examples are the oxidation of 3,3,5′,5′-tetramethylbenzidine by horseradish peroxidase/hydrogen peroxide [84] or the dephosphorylation with alkaline phosphatase of 1-naphthyl phosphate and differential pulse voltametry of the resultant 1-naphthol [85]. This latter assay was also run in microtitre plates with spectrophotometric detection of 4-nitrophenol generated by the same alkaline phosphatase conjugate. The detection limits for the two assays were 20 and 30 pg/mL AFB_1 for the spectrophotometric and electrochemical immunosensor, respectively. This system was subsequently developed into a 96-well method using screen-printed electrodes and a microplate reader operating with intermittent pulse amperometry [86]. A similar read-out system was used for an enzyme-linked immuno-magnetic electrochemical array, in which the aflatoxin–bovine serum albumin conjugate for the competitive ELISA format was immobilised on magnetic beads held on the surface of a screen-printed electrode by a neodymium magnet [87]. A number of other formats have been developed, including the use of a piezoelectric quartz crystal immunosensor with gold nanoparticles [88] or the application of linear sweep voltametry to measure metallic silver deposited on an electrode after silver ion reduction by ascorbic acid generated by the dephosphorylation of ascorbic acid 2-phosphate by alkaline phosphate conjugated on the secondary antibody [89]. An alternative concept has been the development of a micro-comb electrode, on to which a layer of nanogold is affixed [90]. Onto this surface is immobilised first the AFB_1 antibody and then horseradish peroxidase to complete the immunobiosensor. Incubation in AFB_1 standard or sample extract allows interaction of the antigen with bound antibody. The subsequent detection step requires oxidation of iodide in an acid solution and measurement of conductance differences. The final assay gave acceptable accuracy as compared with ELISA and a detection limit of 0.1 ng/mL.

Other optical techniques have been adapted for mycotoxin determination. An immunoassay based on surface plasmon resonance has been developed for AFB_1 and has a linear range of response of 3–98 ng/mL [91]. Application of surface plasmon-enhanced fluorescence spectroscopy allows AFM_1 in milk to be determined at levels as low as 0.6 pg/mL [92].

The assays described above all involve antigen–antibody interactions at the surface of various supports. However, similar interactions within the analytical solution form the basis for fluorescence polarisation methods [93]. Briefly, these methods rely on measurement of the rate of rotation of fluorescent molecules in which smaller molecules (such as a fluorescent-labelled mycotoxin substrate) rotate faster than larger molecules (such as the same fluorescent-labelled mycotoxin which has competed with unlabelled analyte for binding on the relevant antibody). As aflatoxins are most effectively extracted by aqueous polar organic solvent mixtures, these assays require antibodies that can tolerate these solvents. A method for determination of total aflatoxin has been investigated for maize, sorghum, peanut butter and peanut paste [94]. The results of fluorescence polarisation measurements correlated well with HPLC determination but were lower in value, possibly due to a low cross reactivity with aflatoxin analogues other than AFB_1.

Aflatoxins have also been determined in flow injection analysis systems containing immunosensor detection. One example of this technique is the low ppt determination of AFM_1 in milk (limit of detection 11 ng/kg), in which diluted milk is incubated with the antibody and a tracer of AFM_1-bound horseradish peroxidase [95]. After equilibration, the mixture is injected into a flow injection system containing a column of immobilised protein G, which binds the antibody. Free tracer flows through the column is mixed with 3,3′,5,5′-tetramethylbenzidine and hydrogen peroxide and passes through a reaction coil prior to amperometric detection on a glassy carbon electrode. A good correlation was found with HPLC. A more direct measurement has been obtained by incorporating an optical waveguide lightmode spectroscopy (OWLS) technique into a flow

injection system [96]. The OWLS sensors were prepared with AFB_1 antibody or with immobilised AFB_1. The former format proved less sensitive, probably due to the small relative change in refractive index caused by the binding of a low-molecular mass mycotoxin to a large protein antibody. The latter format gave a sensitive detection range between 0.5 and 10 ng/mL and compared well with an ELISA determination in cereals.

Immunology-based methods represent a diverse group of rapid methods of varying quantitative capability. For most routine mycotoxin analytical laboratories, only the commercialisation of these technologies makes them viable choices provided they are affordable and meet requirements in terms of applicable matrices and analytical ranges or cutoff points. For example, flow-through devices designed to provide cutoff points that meet EU regulatory levels are seldom applicable in other jurisdictions. Nevertheless, this could potentially be overcome by changes in antibody and/or conjugate dilutions or changes in sample extract concentrations, in consultation with the manufacturer.

Biomarkers

The application of analytical chemistry to aflatoxin analysis extends beyond the determination of levels in foods and feeds. The study of the impact of aflatoxins on human and animal health is best achieved by using individual biomarkers of aflatoxin exposure. These biomarkers involve the analysis of various aflatoxin metabolites in blood, urine or liver samples. As a xenobiotic chemical, AFB_1 is metabolised by various cytochrome P450 isozymes to form a number of products, including the hydroxylated compounds AFM_1, aflatoxin P_1 and aflatoxin Q_1 [97]. Of these, AFM_1 is the most important, being excreted in milk, and despite its reduced toxicity is further consumed by humans, particularly infants. The hydroxylated products can be excreted in urine (together with unmetabolised aflatoxin precursor) and determined by analytical methods analogous to AFB_1 in which the samples are cleaned-up on IACs or SPE cartridges and subjected to reversed-phase HPLC with post-column bromination [98, 99]. A more important metabolic pathway is the epoxidation of AFB_1 by the cytochromes CYP1A2, 3A4 or 3A5 to the 8,9-epoxide and the ultimate formation of DNA and protein adducts. As this is the pathway of its toxicological effects, the two most extensively studied biomarkers are the urinary aflatoxin *N*-7-guanine adduct, which results from AFB_1 reaction with DNA, and aflatoxin-lysine adduct, which is obtained by digestion of the aflatoxin–albumin adduct occurring in serum. Both these adducts can now be determined by LC-MS/MS techniques [100, 101]. The latter adduct, which due to the long half-life of albumin in the human body is considered to reflect more the longer term intake of aflatoxin B_1, was originally determined by ELISA [102]. Correlation of the LC-MS/MS and ELISA methods showed good correlation, except that ELISA produced proportionately higher levels [103]. This result suggests that the ELISA antibodies may be sensitive to other aflatoxin metabolites co-extracted with the lysine adduct. These biomarkers can be found to varying extents in many animal species due to the widespread occurrence of cytochrome P450 enzymes. Measurement of AFB_1–albumin adducts and AFB_1–DNA adducts in a cross-species study of rodents with differing tumour susceptibility (rats, mice, hamsters and guinea pigs) was used to indicate that the albumin adduct reflects hepatic DNA damage [104].

Conclusions

Analysis of aflatoxins has advanced considerably since their discovery. In some cases, these advances have mirrored the improvements in general analytical methodology, including the introduction of solid-phase extraction and the advances in instrumental design that have seen HPLC move to the fore as a separation technique and MS move from the realm of physics to an almost universal HPLC analytical detector. In other cases, developments in aflatoxin analysis have shown the way forward for rapid screening methodologies required in many practical situations. Given that aflatoxin contamination of foods and feeds will be an issue into the foreseeable future, methods for its analysis will continue to be developed and improved.

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