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# Aflatoxins: Their Measure and Analysis

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## 1. Introduction

Aflatoxins are natural secondary metabolites produced by some moulds (mainly *Aspergillus flavus* and *Aspergillus parasiticus*) and are contaminants of agricultural commodities in the field particularly in critical temperature and humidity conditions before or during harvest or because of inappropriate storage (Rustom, 1997; Sweeney & Dobson, 1998). Aflatoxins B1 (AFB1) and B2 (AFB2), produced by *A. flavus*, and aflatoxins G1 (AFG1) and G2 (AFG2), produced by *A. flavus* as well as *A. parasiticus*, can contaminate maize and other cereals such as wheat and rice, but also groundnuts, pistachios, cottonseed, copra and spices. Following the ingestion of contaminated feedstuffs by lactating dairy cows, AFB1 is biotransformed by hepatic microsomal cytochrome P450 into aflatoxin M1 (AFM1), which is then excreted into the milk (Frobish et al., 1986). Because of the binding of AFM1 to the milk protein fraction, in particular with casein (Brackett & Marth, 1982), it can be present also in dairy products manufactured with contaminated milk.

The WHO-International Agency for Research on Cancer (IARC) has classified AFB1, AFB2, AFG1, AFG2 and since 2002 also AFM1 as carcinogenic agents to humans (group 1) (IARC, 2002).

Considering their natural occurrence, it is impossible to fully eliminate their presence; so, coordinated inspection programmes aimed to check the presence and concentration of aflatoxins in feedingstuffs are recommended by the Commission of the European Communities.

National and international institutions and organizations such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) have recognized the potential health risks to animals and humans posed by consuming aflatoxin-contaminated food and feed.

To protect consumers and farm animals regulatory limits have been adopted. The current maximum residue levels (MRL) for aflatoxins set by the EC (Commission European Communities, 2006a) are 2 µg/kg for AFB1 and 4 µg/kg for total aflatoxins in groundnuts, nuts, dried fruits and cereals for direct human consumption. These have been extended to cover some species of spices with limits of 5 µg/kg and 10 µg/kg for AFB1 and total aflatoxins, respectively. These levels are about five times lower than those adopted in the USA. Limits of 0.1 µg/kg are established by the EC for AFB1 in baby foods and dietary foods. The current regulatory limit for AFM1 in raw milk is 0.05 µg/kg, while in baby foods and dietary foods has been set at 0.025 µg/kg. Taking into account the developments in Codex Alimentarius, recently EC has introduced the maximum accepted levels for aflatoxins in other foodstuffs, like oilseeds (2 µg/kg for AFB1 and 4 µg/kg for total aflatoxins),

almonds, pistachios and apricot kernels (5 µg/kg for AFB<sub>1</sub> and 10 µg/kg for total aflatoxins), hazelnuts and Brazil nuts (5 µg/kg for AFB<sub>1</sub> and 10 µg/kg for total aflatoxins) (Commission European Communities, 2010).

About animal feeds, only AFB<sub>1</sub> is regulated: EC has set a limit of 0.02 mg/kg in all feed materials and in most complete and complementary feedstuffs for cattle, sheep, goats, pigs and poultry, while it is 0.005 mg/kg in complete feedingstuffs for dairy animals and 0.01 mg/kg for complete feedingstuffs for calves and lambs (Commission European Communities, 2003).

Because of the toxicity of these molecules and considering the MRL set in food and in feedstuffs, analytical identification and quantification of such contaminants at these low levels has to be carried out with reliable methods: they must be able to provide accurate and reproducible results to allow an effective control of the possible contamination of food and feed commodities. For this reason, the EC has set also the performance criteria for the methods of analysis to be used for the official control of mycotoxins in general and aflatoxins in particular (Commission European Communities, 2006b).

Nowadays, many sensitive, specific, but also simple and rapid methods are available: in literature there is considerable attention to aflatoxin detection. As new analytical technologies have developed, they have been rapidly incorporated into mycotoxin testing strategies. Sometimes many works reflect advances in analytical science (the availability of mass spectrometry detectors is an example), but often modifications of existing methods are published to improve the analytical process. Several methods have been also validated for the determination of aflatoxins in various matrices, but the validation does not always comply with the more recent EC guidelines (Commission European Communities, 2006b; Commission European Communities, 2002; Commission European Communities, 2004).

Among these, Commission Decision 2002/657/EC has set the performance and the procedures for the validation of screening and confirmatory methods.

Numerous methods have been developed to meet analytical requirements from rapid tests for factories and grain silos to regulatory control in official laboratories. This review will focus upon different analytical methods used for aflatoxin determination. They include thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) in combination with fluorescence detection with or without derivatisation, liquid chromatography tandem mass spectrometry (LC/MS) and immunochemicals methods, such as enzyme linked immunosorbent assay (ELISA), immunosensors, dipsticks, strip-test.

## 2. Chromatographic methods

Aflatoxins possess significant UV absorption and fluorescence properties, so techniques based on chromatographic methods with UV or fluorescence detection have always predominated.

Originally the chromatographic separation was performed by TLC: since when aflatoxins were first identified as chemical agents, it has been the most widely used separation technique in aflatoxin analysis in various matrices, like corn, raw peanuts (Park et al, 2002), cotton seed (Pons et al, 1980), eggs (Trucksess et al, 1977), milk (Van Egmond, 1978) and it has been considered the AOAC official method for a long period. This technique is simple and rapid and the identification of aflatoxins is based on the evaluation of fluorescence spots observed under a UV light. AFB<sub>1</sub> and AFB<sub>2</sub> show a blue fluorescence colour, while it is green for AFG<sub>1</sub> and AFG<sub>2</sub>. TLC allows qualitative and semi-quantitative determinations by

comparison of sample and standard analysed in the same conditions. Many TLC methods for aflatoxins were validated more than 20 years ago and also when there has been a more recent validation, the performance of the methods has often been established at contamination levels too high to be of relevance to current regulatory limits.

The combination of TLC methods with much-improved modern clean-up stage offers the possibility to be a simple, robust and relatively inexpensive technique (Vargas et al, 2001), that after validation can be used as viable screening method. Moreover, given the significant advantages of the low cost of operation, the potential to test many samples simultaneously and the advances in instrumentation that allow quantification by image analysis or densitometry, TLC can be used also in laboratories of developing countries in alternative to other chromatographic methods that are more expensive and require skilled and experienced staff to operate. Improvements in TLC techniques have led to the development of high-performance thin-layer chromatography (HPTLC), successfully applied to aflatoxin analysis (Nawaz et al, 1992).

Overpressured-layer chromatographic technique (OPLC), developed in the seventies, has been used for quantitative evaluation of aflatoxins in foods (Otta et al, 1998) and also in fish, corn, wheat samples that can occur in different feedstuffs (Otta et al, 2000).

Because of its higher separation power, higher sensitivity and accuracy, the possibility of automating the instrumental analysis, HPLC now is the most commonly used technique in analytical laboratories. HPLC using fluorescence detection has already become the most accepted chromatographic method for the determination of aflatoxins. For its specificity in the case of molecules that exhibit fluorescence, Commission Decision 2002/657/EC, concerning the performance of analytical methods, considers the HPLC technique coupled with fluorescence detector suitable confirmatory method for aflatoxins identification.

However, HPTLC and HPLC techniques complement each other: the HPTLC for preliminary work to optimize LC separation conditions during the development of a method or its use as screening for the analysis of a large number of samples to limit the HPLC analysis only to positive samples are not unusual.

Liquid chromatographic methods for aflatoxins determination include both normal and reverse-phase separations, although current methods for aflatoxin analysis typically rely upon reverse-phase HPLC, with mixtures of methanol, water and acetonitrile for mobile phases.

Aflatoxins are naturally strongly fluorescent compounds, so the HPLC identification of these molecules is most often achieved by fluorescence detection. Reverse-phase eluents quench the fluorescence of AFB1 and AFG1 (Kok, 1994); for this reason, to enhance the response of these two analytes, chemical derivatisation is commonly required, using pre- or post-column derivatisation with suitable fluorophore, improving detectability.

The pre-column approach uses trifluoroacetic acid (TFA) with the formation of the corresponding hemiacetals (Stubblefield, 1987; Simonella et al, 1998; Akiyama et al, 2001) that are relatively unstable derivatives. The post-column derivatisation is based on the reaction of the 8,9-double bond with halogens. Initially, the post-column reaction used iodination (Shepherd & Gilbert, 1984), but it has several disadvantages, like peak broadening and the risk of crystallisation of iodine. An alternative method is represented by bromination by an electrochemical cell (Kobra Cell) with potassium bromide dissolved in an acidified mobile phase or by addition of bromide or pyridinium hydrobromide perbromide (PBPB) to mobile phase and using a short reaction coil at ambient temperature (Stroka et al, 2003; Manetta et al, 2005; Senyuva & Gilbert, 2005; Brera et al, 2007; Manetta et al, 2010). The

bromination methods offer the advantage to be rapid, simple and easy to automate, improving reproducibility and ruggedness and reducing analysis time.

A post-column derivatisation method that seems analytically equivalent to iodination and bromination is the photochemical one: it is based on the formation of hemiacetals of AFB1 and AFG1 as the effect of the irradiation of the HPLC column eluate by a UV light (Joshua, 1993; Waltking & Wilson, 2006).

A method based on the formation of an inclusion complex between aflatoxins and cyclodextrins (CDs) has been recently developed (Chiavaro et al, 2001): specific CDs are added to mobile phase (water-methanol) including aflatoxins in their cyclic structure, enhancing AFB1 and AFG1 fluorescence (Aghamohammadi & Alizadeh, 2007).

The introduction of mass spectrometry and the subsequent coupling of liquid chromatography to this very efficient system of detection have resulted in the development of many LC-MS or LC-MS/MS methods for aflatoxin analysis. Because of the advantages of specificity and selectivity, chromatographic methods coupled to mass spectrometry continue to be developed: they improve detection limits and are able to identify molecules by means mass spectral fragmentation patterns. Some of them comprise a single liquid extraction and direct instrumental determination without clean-up step (Cappiello et al, 1995; Kokkonen et al, 2005; Júnior et al, 2008). This assumption relies on the ability of the mass analyser to filter out by mass any co-eluting impurities. However, many Authors assert that further sample preparation prior to LC-MS analysis would benefit analysis (Chen et al, 2005; Cavaliere et al, 2006; Lattanzio et al, 2007) because ionisation suppression can occur by matrix effects. A number of instrument types have been used: single quadrupole (Blesa et al, 2003), triple quadrupole (Chen et al, 2005), linear ion trap (Cavaliere et al, 2006; Lattanzio et al, 2007). Atmospheric pressure chemical ionisation (APCI) is the ionisation source that provides lower chemical noise and, subsequently, lower quantification limit than electrospray ionisation (ESI) also if this one, on the other hand, is more robust. The use of mass spectrometric methods can be expected to increase, particularly as they become easier to use and the costs of instrumentation continue to fall.

Despite the enormous progress in analytical technologies, methods based on HPLC with fluorescence detection are the most used today for aflatoxins instrumental analysis, because of the large diffusion of this configuration in routine laboratories.

The recent availability of analytical columns with reduced size of the packing material has improved chromatographic performance. Today, numerous manufacturers commercialize columns packed with sub-2  $\mu\text{m}$  particles to use devices that are able to handle pressure higher than 400 bar, such as Ultra-Performance Liquid Chromatography® (UPLC). This strategy allows a significant decrease in analysis time: aflatoxins runs are completed in 3-4 min with a decrease of over 60% compared to traditional HPLC. In addition, solvent usage has been reduced by 85%, resulting in greater sample throughput and significant reduction of costs of analysis. UPLC system can be coupled to traditional detector or, using a mobile phase of water/methanol with 0.1% formic acid, to mass spectrometry detector.

For a short time capillary electrophoresis has been a technique of interest in aflatoxins separation, in particular its application as micellar electrokinetic capillary chromatography with laser-induced fluorescence detection (Maragos & Greer, 1997), but it has not found application in routine analysis.

In Table 1 some analytical methods for aflatoxin determination have been included with their performance characteristics.



Aflatoxin	Matrix	Method	Sample preparation	LOD (µg/kg)	LOQ (µg/kg)	R%	RSD <sub>R</sub> (%)	Reference
B1	Corn	HPLC/Fluor. Pre-column der. (TFA), Post-column (PBPB)	IAC	-	-	82-84	19-37	Brera et al, 2007
B1,B2, G1,G2	Corn, raw peanut, peanut butter	TLC/Densit.	SPE	-	-	95-139	26-84 (B1)	Park et al, 1994
B1,B2, G1,G2	Corn, raw peanut, peanut butter	HPLC/Fluor. post-column der. (iodine)	IAC	-		97-131	11-108	Trucksess et al, 1991
B1,B2 G1,G2 M1	Mould cheese	LC-MS/MS triple quadrupole (ESI source)	Only extraction	0.3(M1) 0.8(B-G)	0.6(M1) 1.6(B-G)	96-143	2-12	Kokkonen et al, 2005
B1,B2, G1,G2	Fish, corn, wheat	OPLC	Extraction and L-L partition	2	-	73-104	7-13 (RSDr)	Otta et al, 2000
B1	corn	Capillary electrophoresis / laser induced fluor.	SPE or IAC	0.5	-	85	-	Maragos & Greer, 1997
B1,B2 G1,G2	peanuts	HPLC/Fluor.	MSPD	-	0.125-2.5	78-86	4-7 (RSDr)	Blesa et al, 2003
M1	Milk	HPLC/Fluor. Pre column der. (TFA)	SPE or IAC	0.027-0.031	-	82-92	15-19 (RSDr)	Simonella et al, 1999
M1	Milk	colourimetric ELISA	none	0.006	-	100	11 (RSDr)	Simonella et al, 1999
M1	Milk, soft cheese	HPLC/Fluor. Post column der. (PBPB)	SPE	0.001-0.005	-	76-90	3-9 (RSDr)	Manetta et al, 2005
M1	Hard cheese	HPLC/Fluor. Post column der. (PBPB)	SPE	0.008	0.025	67	4-7 (RSDr)	Manetta et al, 2009
M1	Milk	HPLC/Fluor.	IAC	-	0.005	74	21-31	Dragacci et al, 2001
M1	Milk	HPLC/Fluor.	IAC	0.006	0.015	91	8-15	Muscarella et al, 2007
M1	Milk	Chemiluminescent ELISA	none	0.00025	0.001	96-122	2-8	Magliulo et al, 2005
M1	Milk	LC-MS/MS linear ion trap (ESI and APCI source)	carbograph-4 cartridge	-	0.006-0.012	92-96	3-8	Cavaliere et al, 2006

M1	Milk	Membrane-based flow through enzyme immunossay	IAC	0.05	-	97	-	Sibanda et al, 1999
M1	Milk	Electrochemical biosensor	none	0.01	-	-	-	Paniel et al, 2010
M1	Milk, milk powder	LC-MS/MS triple quadrupole (ESI source)	IAC	0.59-0.66	-	78-87	-	Chen et al, 2005
M1	Milk, milk powder	LC-MS/MS triple quadrupole (ESI source)	Multifunction column	9-14	-	7-16	-	Chen et al, 2005

Legend: Fluor.: fluorescence detection; Densit.: densitometry; der.: derivatisation.

Table 1. Performance characteristics of some analytical methods for aflatoxins

3. Sample preparation

Aflatoxins present in food and feed commodities must be extracted from the matrices by a suitable solvent or mixture of solvents and cleaned-up prior to analysis.

Sample preparation technology is one of the most relevant field of analytical science.

The pretreatment of sample (protein precipitation, defatting, extraction, filtration) is an important phase for removing many interferences and for having, in this way, extracts without impurities to allow accuracy and reproducibility in the subsequent instrumental step.

The first phase is the extraction of the toxins from the matrices: it generally involves chloroform, dichloromethane or aqueous mixtures of polar organic solvents as methanol, acetone, acetonitrile, the aqueous mixture being recently the most used ones because more compatible not only with environment but also with the antibodies involved in the subsequent step of clean-up with immunoaffinity columns that are increasingly utilised.

Supercritical fluid extraction (SFE) has some applications in food analysis because this system of extraction uses supercritical carbon dioxide and not organic solvents or involves them only in small amounts. However, in aflatoxins analysis this technique of extraction has not been successfully used because of the low recoveries of aflatoxins and the presence in the extracts of impurities such as lipids that are the main interferences with the purification step and with the chromatographic separation.

Clean-up is another very critical step. It is necessary for removing many of the co-extracted impurities and obtaining cleaner extracts for the subsequent instrumental determination, to have the most accurate and reproducible results. The traditional techniques, such as liquid-liquid partition or purification on conventional glass columns packed with silica, are time and solvent consuming. Nowadays, new sample preparation technologies, based on extraction by adsorbent materials, are available.

Solid-phase extraction (SPE) and Immunoaffinity Chromatography (IAC) represent very efficient systems that combine in one step filtration, extraction, adsorption and clean-up, allowing to obtain extracts without interferences, to reduce the analytical time and the volumes of solvents used, to improve the reproducibility and the accuracy, to be easily

automatable. With these sample treatment techniques the analytes present in solutions can be concentrated, improving detection limit.

The SPE can be a powerful method for sample preparation: it represents a very significant improvement in the purification step. It is based on the separation mechanism of the modern chromatography: the sample extract is loaded on a cartridge packed with a selective adsorbent material, on which the analytes to be detected are adsorbed and then separated by elution with suitable solvent. In this process the molecules of interest that are in the sample are separated on the basis of its different partition between a liquid (solvent of extraction) and a solid (sorbent phase). The eluent and the adsorbent material compete in the affinity with the analytes: the components of the sample that have higher affinity for mobile phase are easily eluted, while the molecules with affinity for stationary phase are retained. In this technique one or more washing steps are necessary to remove the interferences co-adsorbed on a sorbent stationary phase.

Different types of adsorbent material are available, silica and octadecyl-bonded phase being the most used ones for aflatoxins B and G and for aflatoxin M, respectively.

Matrix solid-phase dispersion (MSPD) is the innovation of the SPE, although it has not yet found application in routine analysis. The MSPD has the advantage to combine extraction and clean-up in one step: the sample is homogenised in a specific sorbent phase in a mortar. Then, the mixture is transferred in a cartridge constricted between two frits and after the column has been washed with suitable solvents, the analytes are eluted for the subsequent instrumental detection. In literature some applications of MSPD to aflatoxins analysis are reported (Blesa et al, 2003; Hu et al, 2006) with high recoveries and satisfactory precision.

IAC is a very efficient technique of purification: it is based on the high specific interactions among biological molecules, so that such chromatography is able to complete the separation of complex mixture in one step. In a cartridge, like that used for SPE, the stationary phase is constituted by a ligand that is specific for the substance to be separated. The ligand is immobilized on a chromatographic bed material and it can be a polyclonal or monoclonal antibody *vs* the analyte to be separate. When the sample is loaded into a cartridge, only the analytes of interest are retained, bound to their antibody, while the other components are eluted. The analyte is then eluted with suitable solvent that is generally methanol. The advantages of IAC is the effective and specific purification provided that allows to achieve cleaner eluates also starting from complex matrices. As a result, performances improve, especially in terms of detection and quantification limits; an added advantage is the limited use of organic solvents. So, IAC has become a major tool for mycotoxin analysis and, in particular, for aflatoxins determination. Another important advantage of this purification method is the fact that the extract of different matrices can be purified by essentially the same protocol. As a consequence, many methods developed to meet the requirements of the low EU maximum tolerated levels have relied on this purification technique and, perhaps for the same reason, many methods involved in collaborative studies and in validation protocols are based on the IAC purification step (Trucksess et al, 1991; Stroka et al, 2001; Dragacci et al, 2001; Stroka et al, 2003; Senyuva et al, 2005; Brera et al, 2007; Muscarella et al, 2007). IACs were thought to be more robust in terms of applicability to different matrices without the need for major adjustments to the method. Immunoaffinity columns offer the opportunity to concentrate large volumes of sample extract to achieve high sensitivity, which is for example the requirement for aflatoxins in baby foods. Moreover, immunoaffinity columns are less demanding in terms of the skills and the experience required.



Recently, IAC has been improved by the introduction of cartridges containing antibodies that are specific to more than one analyte, allowing the simultaneous clean-up of different classes of mycotoxins, like aflatoxins and ochratoxin A and zearalenone (Gobel & Lusky, 2004), and also aflatoxins, ochratoxin A, zearalenone, fumonisins, deoxynivalenol and T-2 toxin (Lattanzio et al, 2007).

In addition to the high cost of immunoaffinity columns, a critical factor in the IAC clean-up procedure is the fact that antibodies are sensitive to organic solvents; this is a problem because sample extracts generally contain high concentrations of acetonitrile, methanol or acetone, obligating to dilute them before application to the column. Acetonitrile, in particular, although it is a good extraction solvent used for SPE clean-up, is rarely used as an organic solvent for IAC because of the production of insoluble substances that can affect aflatoxins recovery (Patey et al, 1991). Very recently, some Authors have proposed a novel immunoaffinity column for aflatoxin analysis in roasted peanuts and some kinds of spices that shows satisfactory organic solvent tolerance, allowing acetonitrile extraction (Uchigashima et al, 2009).

In both SPE and IAC the final eluate can be concentrated evaporating the solvent, improving detection and quantification limits.

#### 4. Immunological methods

High performance liquid chromatographic methods with fluorescent detection are mainly used in routine aflatoxins analysis. They are often laborious and time-consuming and require knowledge and experience of chromatographic techniques to solve separation and interference problems. The big demand in analytical chemistry to have sensitive, specific, but also simple and fast methods for an effective monitoring of aflatoxins in food and feed commodities, has produced analytical methods that combine simplicity with high detectability and analytical throughput. This can be realized by means of immunological methods in conjunction with a highly sensitive detection of the label.

As IAC methods, these assays involve antigen-antibody specific interactions at the surface of various supports. Previously conventional enzyme immunoassay for aflatoxin analysis use antibodies immobilized on well polystyrene microtiter plates: they are based on a competitive process involving antigen and antigen labelled with an enzyme (horseradish peroxidase, generally) and on colorimetric detection with chromogenic substrates (Thirumala-Devi et al, 2002). Enzyme-linked immunosorbent assay is the best established and the most available immunoassay in aflatoxin rapid detection, using the 96 well plate microtiter format. Many commercial companies have developed and commercialised ELISAs which applicability, analytical range and validation criteria are well defined. Despite the increasing use of LC-MS techniques, antibody-based methods for aflatoxins analysis continue to be investigated. The development of these immunochemical methods and their evolution from single to multiple analyte screening, including topics on ELISA, immunosensors, fluorescence polarization and rapid visual tests (lateral-flow, flow-through and dipstick) have been developed. In literature there are many applications to aflatoxins analysis by ELISA: AFB1 determination in deep-red pepper (Ardic et al, 2008), which requires a clean-up by IAC prior ELISA test; many commercial AFB1 screening test in feedstuffs often without purification; AFM1 in milk (Freymy & Chu, 1984; Thirumala-Devi et al, 2002), that needs only defatting step prior to analysis, resulting in a useful screening test for routine quality control of milk of different farms before mixing the different milk bulks,

especially when the absence of AFM1 above the regulatory limit needs to be documented. Enzyme labels can be detected also by chemiluminescent substrates, such as the luminol/peroxide/enhancer system for horseradish peroxidase (HRP) or dioxetane-based substrates for alkaline phosphatase, resulting in a very sensitive detection system in immunoassay. Chemiluminescent detection allows the use of 384 well plates with an assay volume of 20  $\mu$ l, which is at least five times lower than that used in the conventional 96 well microtiter format (Roda et al, 2000). A 5-fold reduction in antibody, labelled probe and chemiluminescent mixture volume reduce the costs of the assay, maintaining the same analytical performance. Thanks to the combination of the chemiluminescent detection of enzymatic activity with the use of a 384 well microtiter format, a highly sensitive, accurate, reproducible, simple and robust chemiluminescent enzyme immunoassay has been developed for AFM1 in milk samples (Magliulo et al, 2005), with a reduction of costs and increased detectability compared with other immunological methods and commercial available kits for AFM1 in milk.

In the case of immunosensors for aflatoxins, antibodies are immobilized on the surface of a screen-printed electrode, magnetic beads held on the surface of a screen-printed electrode (Piermarini et al, 2009), on piezoelectric quartz crystal immunosensor with gold nanoparticles (Jin et al, 2009).

Typical competitive ELISA formats are surface-based; in fact, they require either a toxin-protein conjugate or an antibody to be immobilized onto a surface (membrane, well, electrode, sensor surface, *etc.*) to facilitate the separation of the 'bound' and "unbound" tracer: assays of this nature are termed "heterogeneous" and encompass the vast majority of mycotoxin immunoassays. The separation can be achieved in various ways, from washing (as in ELISAs), chromatographically (as in lateral flow test strips), or reagent flowing over a surface (as in certain biosensors).

Fluorescence polarization immunoassay (FPIA) is a homogeneous assay conducted in solution phase. It is based on the different rate of rotation of smaller and larger molecules. A molecule like a toxin can be covalently linked to a fluorophore to make a fluorescent tracer. The tracer competes with toxin (eventually present in the sample) for a limited amount of toxin-specific antibody. In the case the toxin is absent in the sample, the antibody binds only the tracer, reducing its motion and causing a high polarization. In presence of the toxin, less of the tracer is bound to the antibody and a greater tracer fraction exists unbound in solution, where it shows a lower polarization (Maragos, 2009). The significant advantage of fluorescence polarization over traditional ELISA techniques is that it is measured without the need for separating the free and bound tracer. In particular, it does not require additional manipulations, such as the washing steps of competitive ELISAs, making it simple, rapid, also field portable and, therefore, useful for screening purpose. A homogeneous assay for determining the aflatoxin content in agricultural products based on the technique of fluorescence polarization has been described (Nasir & Jolley, 2002). The disadvantage of this technique is that the aflatoxin contents are underestimated, probably because of the low cross-reactivity of the antibody with AFB<sub>2</sub>, AFG<sub>1</sub> and AFG<sub>2</sub>.

The lateral flow device is one of the simplest and fastest immunoassay techniques have been developed. It is a screening test available in the format of strip or dipstick (Delmulle et al, 2005). Immunodipstick or lateral flow immunoassay has recently gained increasing attention because it requires simple and minimal manipulations and little or no instrumentations. Colloidal gold conjugated anti-aflatoxin antibodies are immobilised at the base of the stick. Aflatoxin present in the sample extract interacts with them; bound and

unbound antibodies move along the membrane-based stick, pass a test line containing aflatoxin, which binds free antibodies, forming a visible line, indicating that the level of eventual aflatoxin contamination of the sample is below the test cut-off value.

Recently, an immunoassay-based lateral flow device for the quantitative determination of four major aflatoxins in maize, that can be completed in 10 min, has been developed (Anfossi et al, 2011). Even quantification is possible by acquiring images of the strip and correlating intensities of the coloured lines with analyte concentration by means of a calibration curve in matrix. Very simple sample preparation is required, making the method reliable, rapid for application outside the laboratory as a point-of-use test for screening purposes.

The immobilization of the antibodies on nanoparticles with a silver core and a gold shell enhances the sensitivity of the assay (Liao & Li, 2010).

Similarly, the membrane-based flow-through device is a qualitative test: the test line is generated by an enzyme-substrate colour reaction (Sibanda et al, 1999). Thanks to the simplicity of the material required, these methods are fit for using as portable rapid field assay for the early detection of aflatoxin-contaminated lots.

Immunological methods, based on antigen/antibody specific interaction, can give false positive results: although antibodies are specific for their antigens, they can react with other substances, similar to those in analysis, binding them as it happens in the antigen/antibody reaction. For this reason, in the case of a suspected non-compliant result, it shall be confirmed by confirmatory method (LC-fluorescence or LC-MS for aflatoxins), as it has been set by the Commission Decision 2002/657/EC.

The recent development of biosensors has stimulated their application also to aflatoxin analysis: in literature many examples are reported, like DNA biosensor (Tombelli et al, 2009), electrochemical immunosensor (Paniel et al, 2010), electrochemical sensor (Siontorou et al, 1998; Liu et al, 2006), fluorimetric biosensor (Carlson et al, 2000).

The advantages of biosensing techniques are: reduced extraction, clean-up analytical steps and global time of analysis (1 min or only few seconds); possibility of online automated analysis; low cost; skilled personnel not required. On the other side, sensitivity should be enhanced and their stability should be improved to allow long-term use.

Because of the ease of use of these devices, many commercial systems continue to be developed not only for aflatoxins, but also for all mycotoxins. For a long time many rapid assays were commercialized with no documentation on their performance characteristics. Since 2002, with Commission Decision 2002/657/EC, laboratories approved for official residue control can use for screening purposes only those techniques “for which it can be demonstrated in a documented traceable manner that they are validated”. As a consequence, many screening test are now commercially available with documentation enclosed with validation parameters, like detection limit and cut-off, sensitivity, specificity, false negative and false positive rate.

## 5. Conclusions

For aflatoxins analysis several methods have been developed over the last 30 years. Because of the advances in technology, the better clean-up procedures and the combination of both, a higher sensitivity has been registered, HPLC with fluorescence detection becoming the most used analytical methodology in laboratory. Moreover, highly sophisticated methods based on liquid chromatography coupled with mass spectrometry have been developed,

improving identification and accurate determination often without the need of sample preparation. Other advances have regarded the environment, as the replacing of chlorinated solvents, preferred in the past, by aqueous mixture of methanol or acetonitrile, that are also more compatible with antibodies, recently introduced in many applications. These reagents marked a turning point in the sample preparation step as well as in the identification phase, showing a high flexibility in many practical situations in which reliable, rapid and simple analyses are required to reduce costs. The choice of a method is made bearing in mind for what purpose aflatoxins analysis has to be performed. So, for example, if a yes/no or semi-quantitative response is considered satisfactory, the use of rapid test is suitable. On the other hand, official control laboratories, which are involved in the monitoring and risk-assessment studies and in official controls, have to apply methods that have been validated and adopted by AOAC International, CEN or ISO. As mycotoxins, not only aflatoxins, are a real problem for health, there will be always a big interest to them and, certainly, it is likely methods for their analysis will continue to improve.

Because of the potential co-occurrence of such contaminants, the challenge is to develop screening methods for their rapid simultaneous detection of multiple families of mycotoxins from the same sample. But the differences in their chemical and physical properties and of concentration range of interest have made simultaneous detection very difficult. In this regard HPLC technique coupled with mass spectrometry or multiple detectors has good prospects.

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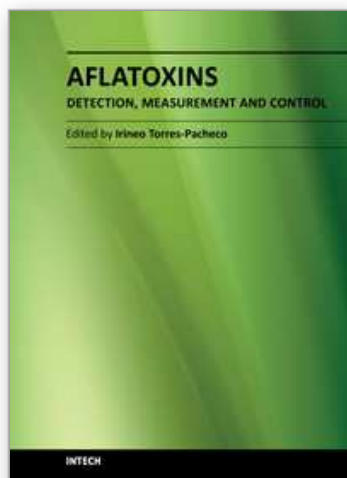


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## **Aflatoxins - Detection, Measurement and Control**

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This book is divided into three sections. The section called Aflatoxin Contamination discusses the importance that this subject has for a country like the case of China and mentions examples that illustrate the ubiquity of aflatoxins in various commodities. The section Measurement and Analysis, describes the concept of measurement and analysis of aflatoxins from a historical perspective, the legal, and the state of the art in methodologies and techniques. Finally the section entitled Approaches for Prevention and Control of Aflatoxins on Crops and on Different Foods, describes actions to prevent and mitigate the genotoxic effect of one of the most conspicuous aflatoxins, AFB1. In turn, it points out interventions to reduce identified aflatoxin-induced illness at agricultural, dietary and strategies that can control aflatoxin. Besides the preventive management, several approaches have been employed, including physical, chemical biological treatments and solvent extraction to detoxify AF in contaminated feeds and feedstuffs.

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