11 UPLC–MS as an Analytical Tool for the Determination of Aflatoxins in Food

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11.1 INTRODUCTION

Aflatoxins, on a worldwide scale, are important mycotoxins in human foods and animal feedstuffs [1].

Aflatoxin contamination causes economic losses of corn, cottonseed, peanuts, sorghum, wheat, rice, and other commodities, as well as economic losses of processed food and feedstuffs. As commodities considered unsafe for human consumption can be incorporated into animal feedstuffs [2,3], there exists opinion that aflatoxicosis in domestic animals is considerably more prevalent than it is diagnosed. Health effects occur in companion animals, livestock, poultry, and humans because aflatoxins are potent hepatotoxins, immunosuppressants, mutagens, and carcinogens [4–6]. Aflatoxins are teratogenic [7].

Aflatoxicosis in the human population, especially in areas stricken by poverty and drought and other adverse growing conditions, is an important public health problem [1].

Most aflatoxins are chemically and structurally diverse. Since the majority of secondary metabolites are synthesized by simple biosynthetic reactions from small molecules (acetates, pyruvates, etc.), this is surprising, however, this leads to the compounds having such a diverse range of toxic effects, both acute and chronic [8].

11.2 AFLATOXINS AND FOOD CONTAMINATION

11.2.1 Mycotoxins

The contamination of food by the intentional use of chemicals, such as pesticides or veterinary drugs, is a worldwide public health concern. However, food contamination due to natural toxicants, such as mycotoxins, can also compromise the safety of food and feed supplies and adversely affect health (WHO/FAO, 2001) in humans and animals [9].

Mycotoxins are secondary metabolites of fungi. Many foods and feeds can become contaminated with mycotoxins since they can form in commodities before harvest, during the time between harvesting and drying, and in storage. Commodities and products frequently contaminated with mycotoxins include corn, wheat, barley, rice, oats, nuts, milk, cheese, peanuts, and cottonseed. The major fungal genera producing mycotoxins include *Aspergillus*, *Fusarium*, and *Penicillium*. The most common mycotoxins are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, T-2 toxin, and zearalenone (Table 11.1) [10]. Mycotoxins produce a wide range of adverse and toxic effects in animals in addition to being foodborne hazards to humans [11].

Among all mycotoxins, aflatoxins are the most toxic, widespread, and the strongest natural carcinogens (Table 11.2). The International Agency for Research on Cancer (IARC) has defined aflatoxin B1 (AFB1) as a carcinogen [11].

11.2.2 Aflatoxins

Aflatoxins, in the late 1950s and the early 1960s, were identified as the cause of the mysterious turkey "X" disease in Great Britain [4,5]. They have also been identified as carcinogens found in rainbow trout [11].

TABLE 11.1
Major Chemical Types of Mycotoxins

Mycotoxins	Main Producing Fungi
Aflatoxins B1, B2, G1, G2	*Aspergillus flavus, A. parasiticus, A. nomius*
Ochratoxin A	*Penicillium verrucosum, A. alutaceus, A. carbonarius*
Patulin	*P. expansum, A. clavatus, Byssochlamys nivea*
Fumonisins	*Fusarium moniliforme, F. proliferatum*
Deoxynivalenol (trichothecenes)	*F. graminearum, F. culmorum, F. crookwellense*
Zearalenone	*F. graminearum, F. culmorum, F. crookwellense*

In the United States, studies on aflatoxins incriminated aflatoxins as the cause of epizootic hepatitis in dogs and as the cause of moldy corn poisoning in pigs [12].

Aflatoxins (AFs) are a family of structure-related mycotoxins produced as secondary metabolites by the spoilage of fungi *Aspergillus*, particularly *A. flavus* and *A. parasiticus* [13–15].

The most important members are AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2. Among the major AFs of concern, AFB1 is the most frequent metabolite in contaminated samples and is clarified in group I as a human carcinogen, the carcinogenic mechanism of which is achieved by affecting the pericellular membrane, interfering with the inductive style of specific enzymes and inhibiting the synthesis of RNA [16–18].

AFB2, AFG1, and AFG2 are also clarified in group I as carcinogens to humans. Although the toxicity of AFM1 is lower than AFB1, it is known for its hepatotoxic and carcinogenic effects [19].

Aflatoxin in food is one of the most widely spread food contaminations. It can be found in over a hundred kinds of agro-products and foods, such as peanuts, corn, rice, soy sauce, vinegar, plant oil, pistachios, tea, Chinese medicinal herb, eggs, milk, feed, and so on. Some have also been detected in animal organisms. Besides

TABLE 11.2
Mycotoxins Notification

Hazard	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011
Aflatoxins	288	762	839	946	801	705	902	638	649	585
Deoxynivalenol (DON)						10	4	3	2	11
Fumonisins		15	14	2	15	9	2	1	3	4
Ochratoxin A	14	26	27	42	54	30	20	27	34	35
Patulin				6	7		3			
Zearalenone					1	6	3			
Total mycotoxins	**302**	**803**	**880**	**996**	**878**	**760**	**933**	**669**	**688**	**635**

Source: Adapted from European Commission. 2011. The Rapid Alert System for Food and Feed, Annual Report. http://ec.europa.eu/food/food/rapidalert/docs/rasff_annual_report_2011_en.pdf. With permission.

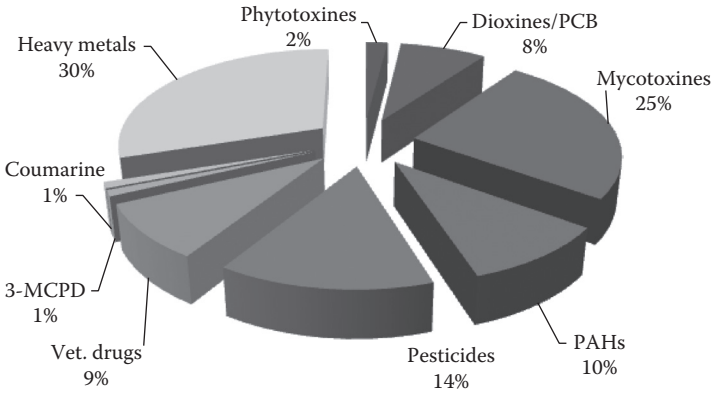

FIGURE 11.1 Illustration of the most common chemical hazards in food and feedstuff in European Union chemical alerts 2007.

these, aflatoxin can spread and be accumulated in the environment, for example, in rivers or agricultural fields [20].

Aflatoxin contributes around 25% of the total toxins in foods affecting human health (Figure 11.1).

11.2.3 Chemistry of Aflatoxins

Aflatoxins have a difuranocoumarin chemical structure. Approximately 18 aflatoxins have been chemically characterized. Aflatoxins are in two chemical groups, the difurocoumarocyclopentenone series (includes AFB_1, AFB_2, AFB_{2A}, AFM_1, AFM_2, AFM_{2A}, and aflatoxicol) and the difurocoumarolactone series (includes AFG_1 and AFG_2) (Figure 11.2).

The "B" group is fluorescent blue in long-wavelength ultraviolet light and the "G" group is fluorescent green. The primary aflatoxins of concern in feedstuffs are AFB_1, AFB_2, AFG_1, and AFG_2 (Figure 11.2). Analytical results for aflatoxins generally are the sum of the concentrations of these four toxins. AFB1 is the most potent aflatoxin and this chemical form is generally the most abundant in feedstuffs and foods. The order of toxicity is $AFB_1 > AFG_1 > AFB_2 > AFG_2$. Hydroxylated aflatoxin metabolites are excreted in milk and the important metabolites are AFM_1 and AFM_2 [21]. AFM_1 is the toxic metabolite of AFB_1 and AFM_2 is the hydroxylated form of AFB_2. Although AFM_1 and AFM_2 are commonly associated with milk and other edible animal products, these compounds can also be produced by aflatoxigenic fungi. The chemical methods are the most reliable for testing a wide variety of substances for aflatoxins.

11.2.4 Carcinogenicity

Aflatoxins are highly toxic, mutagenic, teratogenic, and carcinogenic compounds. AFB1, for example, has a toxicity 10 times that of potassium cyanide, 68 times that of arsenic, and 416 times that of melamine. Furthermore, their carcinogenicity is over 70 times that of dimethylnitrosamine and 10,000 times of benzene hexachloride

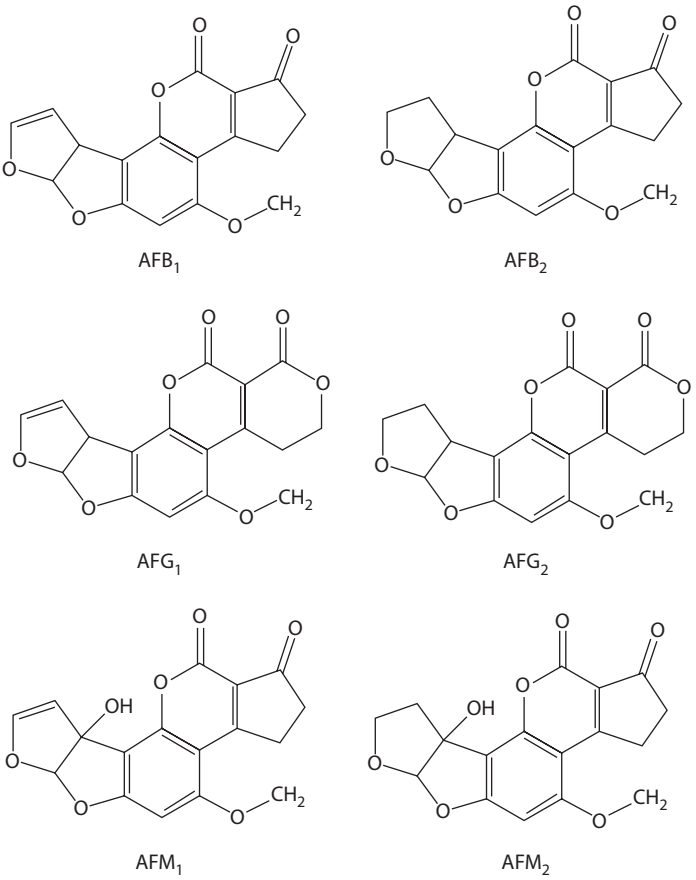

FIGURE 11.2 Structural formula of aflatoxins.

(BHC). The IARC of the World Health Organization (WHO) accepted that aflatoxin should be classified as a Group 1 carcinogen in 1987, and then AFB1 is classified as Group 1 (carcinogenic to humans) by the WHO–IARC in 1993 [20].

According to the most recent research conducted at the University of Pittsburgh, aflatoxin may play a causative role in 4.6–28.2% of all global hepatocellular carcinoma (HCC) cases [22].

11.2.5 Regulations

The toxicity of the aflatoxins has led many countries to set up regulations for their control in foods of plant origin that are intended for human or animal consumption (Table 11.3) (Commission Regulation, 2006; FAO, 2003; National Standard of PR China, 2005). In addition, in order to minimize the levels of mycotoxins in cereals, the European Union has also promoted several good agricultural practices from the cultivation to the distribution of cereals, such as crop rotation or dry storage. Regulations for major mycotoxins in commodities and food exist in at least 100

TABLE 11.3
U.S. FDA Action Levels and European Union Regulations on Maximum Levels for Aflatoxins in Foodstuffs and Animal Feedstuffs

United States		European Union[a]	
Product	**Level (ppb)**	**Product**	**Level (ppb)**
All foods	20	Groundnuts, nuts, and dried fruits, and processed products (direct human consumption)	4 (2)
Cottonseed meal intended for beef cattle/swine/poultry feedstuffs (regardless of age or breeding status)	300	Groundnuts (to undergo physical processing before human consumption)	15 (8)
Maize and peanut products intended for breeding beef cattle/swine or mature poultry	100	Nuts and dried fruit (to undergo physical processing before human consumption)	10 (5)
Maize and peanut products intended for finishing swine of 100 pounds or greater	200	Cereals (for direct human consumption or to undergo physical processing before human consumption)	4 (2)
Maize and peanut products intended for finishing beef cattle	300	Spices (*Capsicum* spp., *Piper* spp., *Myristica fragans*, *Zingiber officinale*, *Curcuma longa*)	10 (5)
		Feed materials with the exception of	(50)
		– Groundnut, copra, palm-kernel, cotton seed, babassu, maize, and products derived from the processing thereof	(20)
		Complete feedingstuffs for cattle, sheep, and goats with the exception of	(50)
		– Dairy cattle	(5)
		– Calves and lambs	(10)
		Complete feedingstuffs for pigs and poultry (except young animals)	(20)
		Other complete feedingstuffs	(10)
		Complementary feedingstuffs for cattle, sheep, and goats (except for diary animals, calves, and lambs)	(50)
		Complementary feedingstuffs for pigs and poultry (except young animals)	(30)
		Other complementary feedingstuffs	(5)

Source: Adapted from Zheng, M. Z., Richard, J. L., and Binder, J. 2006. *USA Mycopathol.* 161:261–273. With permission.

[a] Numbers in parentheses refer to a separate standard for aflatoxin B1 alone.

countries, most of which are for aflatoxins; maximum tolerated levels differ greatly among countries [23].

11.3 ANALYTICAL METHODS FOR AFLATOXIN DETERMINATION IN FOOD

Most aflatoxins are chemically stable, so they tend to survive storage and processing, even when cooked at quite high temperatures such as those reached during baking bread or breakfast cereal production. This makes it important to avoid the conditions that lead to aflatoxin formation, which is not always possible and not always achieved in practice. Aflatoxins are notoriously difficult to remove and the best method of control is prevention [24].

The presence of a recognized toxin-producing fungus does not, in fact, necessarily mean that the associated toxin will also be present, as many factors are involved in its formation. Equally, the absence of any visible mold will not guarantee freedom from toxins, as the mold may have already died out while leaving the toxin intact.

Fungi generally tend to develop in isolated pockets and are not evenly distributed in stored commodities. Therefore, it is important to develop a protocol to ensure that if a sample is taken for analysis, it is representative of the whole consignment. Grab samples have been reported to generally give very low estimates of mycotoxin content. In fact, nearly 90% of the error associated with aflatoxins assays could be attributed to how the original sample was collected. Since aflatoxins are not evenly distributed in grain or in mixed feeds, taking a feed or grain sample that will give a meaningful result in aflatoxins analyses is reported to be difficult [25,26].

The fact that most aflatoxins are toxic in very low concentrations requires sensitive and reliable methods for their detection. Sampling and analysis is of critical importance since failure to achieve a satisfactory verified analysis can lead to unacceptable consignments being accepted or satisfactory loads being unnecessarily rejected. Owing to the varied structures of these compounds, it is not possible to use one standard technique to detect all aflatoxins, as each will require a different method. What works well for some molecules could be inappropriate for others of similar properties, or for the same molecule in a different environment/matrix. Likewise, practical requirements for high-sensitivity detection and the need for a specialist laboratory setting create challenges for routine analysis. Therefore, depending on the physical and chemical properties, procedures have been developed around existing analytical techniques, which offer flexible and broad-based methods of detecting compounds. It would be desirable to have simple detection methods to be used by nonscientific personnel that are both fast and inexpensive. The application of simpler, cheaper, and effective solutions for the detection of aflatoxins is increasingly being required, due to their perceived importance, based around their toxicity and requirements of legislation for limits on amounts in foods. A successful detection method should be robust, be sensitive, and have a high degree of flexibility, over a wide range of compounds, but can also be very specific when required. All techniques should be reproducible to a high level, and the results gained must be relevant and easy to analyze. For fieldwork, the system should also be rapid and portable. There are many methods used, of which many are lab-based, but there is no

single technique that stands out above the rest, although analytical liquid chromatography, commonly linked with mass spectroscopy, is gaining popularity. Many of the techniques described below have been combined to form protocols, which are used in laboratories today [8].

11.3.1 Sample Pretreatment Methods

Most methods used for determination of aflatoxins must rely on the correct extraction and clean-up methods (with the exception of enzyme-linked immunosorbent assay (ELISA), which may not require clean-up) [27]. These steps are vital for a successful protocol, as they are time consuming (sample preparation is the main time factor in an analysis and takes approximately two-thirds of the total) and will affect the final choice of detection procedure. The extraction method used to remove the aflatoxins from the biological matrix is dependent on the structure of the toxin. Hydrophobic toxins such as AFT rely on use of organic solvents [28,29]. These can be direct extractions, or may be partitioned with other solvents, such as *n*-hexane for partial clean-up, to remove excess components of the biological matrix. The choice of extraction solvent is also dependent on the matrix from which the extraction is required, as the differing chemical mixtures can affect it [30]. The use of chlorinated chemicals for extraction is being gradually reduced, as they are proven to be ecological hazards [31]. The clean-up procedure used in a protocol is the most important step, as the purity of the sample affects the sensitivity of the results. Trace amounts of a target molecule may be masked by interfering compounds, found not only in the matrix but in the chemicals, materials, and solvents used in the technique. Glassware should also be free of contamination, such as alkaline detergents, which can form salts with the compounds and result in lower detection rates [29].

Several methods exist and have all been recorded for use with cleaning up aflatoxins samples [32]. Some of those that are widely used have been described below in this section.

11.3.1.1 Liquid–Liquid Extraction

Liquid–liquid extraction (LLE) involves exploiting the different solubilities of the toxin in aqueous phase and in immiscible organic phase, to extract the compound into one solvent, leaving the rest of the matrix in the other. Thus, solvents such as hexane and cyclohexane are used to remove nonpolar contaminants, for example, lipids and cholesterol. The procedure is effective for several toxins and works well in small-scale preparations [33].

However, it is time consuming, and is dependent on which matrix is being used and which compounds are been determined. Disadvantages lie with possible loss of sample by adsorption onto the glassware.

11.3.1.2 Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) uses a supercritical fluid, such as CO_2, to extract the required compound from the matrix. This works well due to the high solvating power and density of the solvating liquid. Supercritical fluid chromatography on

fused silica capillary columns has been applied previously for separating toxins [34] but it is not a successful technique owing to the problems related to SFE [35].

Further, this technique is not suitable for routine analysis due to high costs and the need for specialized equipment [36].

11.3.1.3 Solid-Phase Extraction

The basic principle of SPE technology is a variation of chromatographic techniques based around small disposable cartridges packed with silica gel, or bonded phases that are in the stationary phase. The sample is loaded in one solvent, generally under reduced pressure; rinsed, where most of the contaminants are removed; and eluted in another solvent [37].

This system can be used "on" and "off" line. These cartridges have a high capacity for binding of small molecules and contain different bonding phases, ranging from silica gel, C-18 (octadecylsilane), florisil, phenyl, aminopropyl, ion exchange materials (both anionic and cationic), to affinity materials such as immunoadsorbents and molecular imprinted polymers (MIPs) [38–49].

In addition to cleaning the sample, they can also be used to preconcentrate the sample, providing better detection results. SPE has found widespread use and is an integral part of many extraction and detection protocols [50].

11.3.2 Chromatographic Technique

11.3.2.1 TLC Technique

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 thin layer chromatography (TLC): since 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 [51], and cotton seed [52–54], and it has been considered the Association of Official Agricultural Chemists (AOAC) official method for a long period. This technique is simple and rapid and the identification of aflatoxins is based on the evaluation of fluorescent spots observed under a UV light. AFB1 and AFB2 show a blue fluorescent color, while it is green for AFG1 and AFG2. TLC allows qualitative and semiquantitative determinations by comparison of sample and standard analyzed in the same conditions. Many TLC methods for aflatoxins were validated more than 20 years ago and again more recently, though 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 the much-improved modern clean-up stage offers the possibility to be a simple, robust, and relatively inexpensive technique [55] that after validation can be used as a 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 also be used in laboratories of developing countries as an 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 aflatoxins analysis [56].

Over pressured-layer chromatographic technique (OPLC), developed in the 1970s, has been used for quantitative evaluation of aflatoxins in foods [57] , as well as in fish, corn, wheat samples that can occur in different feedstuffs [58].

11.3.2.2 Capillary Electrophoresis

For a short time, capillary electrophoresis has been a technique of interest in aflatoxin separation, in particular its application as micellar electrokinetic capillary chromatography with laser-induced fluorescence detection [59], but it has not found application in routine analysis.

11.3.2.3 High-Performance Liquid Chromatographic Technique

Because of its higher separation power, higher sensitivity, and accuracy, and the possibility of automating the instrumental analysis, HPLC is now 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 a suitable confirmatory method for aflatoxin 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 it may also use as screening for the analysis of a large number of samples to limit the HPLC analysis only to positive samples. Liquid chromatographic methods for aflatoxin 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 fluorescent detection. Reverse-phase eluents quench the fluorescence of AFB1 and AFG1 [60]; for this reason, to enhance the response of these two analytes, chemical derivatization is commonly required, using pre- or postcolumn derivatization with suitable fluorophore, improving detectability.

The precolumn approach uses trifluoroacetic acid (TFA) with the formation of the corresponding hemiacetals [61–63] that are relatively unstable derivatives. The postcolumn derivatization is based on the reaction of the 8,9-double bond with halogens. Initially, the postcolumn reaction used iodination [64], but it has several disadvantages, such as peak broadening and the risk of crystallization 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 the addition of bromide or pyridinium hydrobromide perbromide (PBPB) to a mobile phase and using a short reaction coil at ambient [65–69]. The bromination methods offer the advantage to be rapid, simple, and easy to automate, improving reproducibility and ruggedness and reducing analysis time. A postcolumn derivatization 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 [70,71].

A method based on the formation of an inclusion complex between aflatoxins and cyclodextrins (CDs) has been recently developed [72], and specific CDs are added to mobile phase (water–methanol), including aflatoxins in their cyclic structure, enhancing AFB1 and AFG1 fluorescence [73].

11.3.3 Bioassay Technique

11.3.3.1 Enzyme-Linked Immunosorbent Assay

ELISA methods for aflatoxins assay have been available for more than a decade. The technology is based on the ability of a specific antibody to distinguish the three-dimensional structure of specific aflatoxins. The direct competitive ELISA is commonly used in aflatoxin analysis [74].

A conventional microtiter plate, ELISA requires equilibrium of the antibody–antigen reaction that would require an incubation time of approximately 1–2 h. Currently, most of the commercially available ELISA test kits for aflatoxins are working in the kinetics phase of antibody–antigen binding, which reduces the incubation time to minutes. Although reduction of incubation time may lead to some loss of assay sensitivity, the test kit can provide accurate and reproducible results [75].

A typical principle of direct competitive ELISA is shown in Figure 11.3. After an aflatoxin is extracted from a ground sample with solvent, a portion of the sample

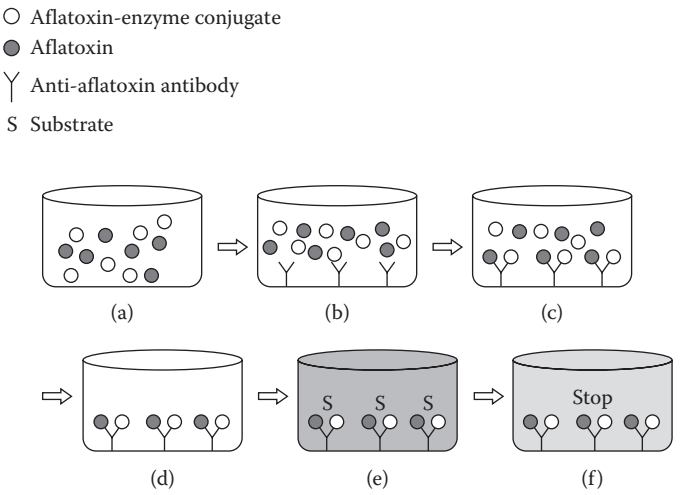

FIGURE 11.3 Principle of competitive ELISA for aflatoxin analysis. (a) Sample mixed with conjugate; (b) mixed content added to antibody-coated well; (c) aflatoxin binds to antibody in the first incubation; (d) unbound materials are rinsed away in the washing step; (e) substrate is added to develop color; (f) stop solution is added to stop the reaction. (Adapted from Zheng, M. Z., Richard, J. L., and Binder, J. 2006. *Mycopathologia* 161:261–273. With permission.)

extract and a conjugate of an enzyme-coupled aflatoxin are mixed and then added to the antibody-coated microtiter wells. Any aflatoxin in the sample extract or control standards is allowed to compete with the enzyme-conjugated aflatoxin for the antibody binding sites. After washing, an enzyme substrate is added and a blue color develops. The intensity of the color is inversely proportional to the concentration of aflatoxin in the sample or standard. A solution is then added to stop the enzyme reaction. The intensity of the solution color in the microtiter wells is measured, optically using an ELISA reader with an absorbance filter of 450 nm. The optical densities (ODs) of the samples are compared to the ODs of the standards and an interpretative result is determined [75,76].

ELISA test kits are favored as high-throughput assays with low sample volume requirements and often less sample extract clean-up procedures compared to conventional methods such as TLC and HPLC. The methods can be fully quantitative. They are rapid, simple, specific, sensitive, and portable for use in the field for the detection of aflatoxins in foods and feeds [77]. Although the antibodies have the advantage of high specificity and sensitivity, because the target compounds are aflatoxins but not the antigens, compounds with similar chemical groups can also interact with the antibodies. This so-called matrix effect or matrix interference commonly occurs in ELISA methods, resulting in underestimates or overestimates in aflatoxin concentrations in commodity samples [78]. Additionally, insufficient validation of ELISA methods causes the methods to be limited to those matrices for which they were validated [79]. Therefore, an extensive study on the accuracy and precision of an ELISA method over a wide range of commodities is needed and a full validation for an ELISA method is essential and critical [80].

11.3.4 Chromatography and Mass Spectrum Combination Technique

11.3.4.1 GC and GC–MS Techniques

GC is regularly used to identify and quantify the presence of aflatoxins in food samples, and many protocols have been developed for these materials. Normally, the system is linked to MS, flame ionization detector (FID), or Fourier transform infrared spectroscopy (FTIR) detection techniques in order to detect the volatile products [81–83]. Most aflatoxins are not volatile and therefore have to be derivatized for analysis using GC [83]. Several techniques have been developed for the derivatization of aflatoxins. Chemical reactions such as silylation or polyfluoroacylation are employed in order to obtain a volatile material [32].

The GC–MS detection allowed monitoring of up to four compounds simultaneously during a 23-min GC run. The volatile fungal metabolites were measured in grain as indicators of fungal contamination [84]. The GC–MS system was compared with electronic nose, showing superior performance of the first one, since the GC–MS misclassified only three of 37 samples and the electronic nose, seven of 37 samples.

While as shown above, a number of examples do exist on the successful application of GC for analysis of aflatoxins, there are several disadvantages. First, the samples that need to be analyzed are those that are volatile or those that can be converted into volatile samples. Further, thermal stability is a problem because heating

sometimes degrades the samples. In some cases, injection of a sample has been shown to be a problem that needs addressing. This is mainly because the sample gets lost when it comes into contact with the heated areas of the injector, leading to loss in vaporization. However, the use of GC detection is not expected for commercial protocols due to the existence of cheaper and faster alternatives such as HPLC.

11.3.4.2 LC–MS Technique

The introduction of mass spectrometry and the subsequent coupling of liquid chromatography to this very efficient system of detection has 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 of mass spectral fragmentation patterns.

Some of them comprise a single-liquid extraction and direct instrumental determination without a clean-up step [85–87]. This assumption relies on the ability of the mass analyzer to filter out by mass any coeluting impurities. However, many authors assert that further sample preparation prior to LC–MS analysis would benefit analysis [88–90] because ionization suppression can occur by matrix effects. A number of instrument types have been used: single quadrupole, triple quadrupole, and linear ion trap [15,88–90].

Atmospheric pressure chemical ionization (APCI) is the ionization source that provides lower chemical noise and, subsequently, lower quantification limit than electrospray ionization (ESI) which 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.

In Table 11.4, some analytical methods for aflatoxin determination have been included with their performance characteristics.

11.4 UPLC–MS ANALYSIS OF AFLATOXINS IN FOOD

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 μm 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: aflatoxin 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 a traditional detector or, using a mobile phase of water/methanol with 0.1% formic acid, to a mass spectrometry detector. The aim of this chapter is to summarize a number of the most important recent research on using UPLC coupled with MS for aflatoxin analysis in different food matrices. Table 11.5 shows some examples of recent studies on aflatoxin analysis in food using UPLC/MS [91].

TABLE 11.4
Some Analytical Methods for Aflatoxins Determination and Their Performance Characteristics

Aflatoxin	Matrix	Method	Sample Preparation	LOD (μg/kg)	LOQ (μg/kg)	R%	RSD_R (%)	Reference
B1	Corn	HPLC/fluorescent (fluor). precolumn der. trifluoroacetic acid (TFA), postcolumn pyridinium hydrobromide perbromide (PBPB)	IAC	–	–	82–84	19–37	[68]
B1, B2 G1, G2	Corn, raw peanut, peanut butter	Thin layer chromatography (TLC)/densit.	SPE	–	–	95–139	26–84 (B1)	[51]
B1, B2 G1, G2	Corn, raw peanut, peanut butter	HPLC/fluor. postcolumn der. (iodine)	IAC	–	–	97–131	11–108	[92]
B1, B2 G1, G2 M1	Mold cheese	LC–MS/MS triple quadrupole (electrospray ionization (ESI) source)	Only extraction	0.3 (M1) 0.8 (B-G)	0.6 (M1) 1.6 (B-G)	96–143	2–12	[86]
B1, B2 G1, G2	Fish, corn, wheat	Over pressure layer chromatography (OPLC)	Extraction and L–L partition	2	–	73–104	7–13 (RSDr)	[58]
B1	Corn	Capillary electrophoresis/ laser-induced fluor.	SPE or IAC	0.5	–	85	–	[59]
B1, B2 G1, G2	Peanuts	HPLC/fluor.	MSPD	–	0.125–2.5	78–86	4–7 (RSDr)	[15]
M1	Milk	HPLC/fluor. precolumn der. (TFA)	SPE or IAC	0.027–0.031	–	82–92 (RSDr)	15–19	[15]
M1	Milk	Colorimetric ELISA	None	0.006	–	100 (RSDr)	11	[62]
M1	Milk, soft cheese	HPLC/fluor. postcolumn der. (PBPB)	SPE	0.001– 0.005	–	76–90	3–9 (RSDr)	[66]

M1	Hard cheese	HPLC/fluor. postcolumn der. (PBPB)	SPE	0.008	0.025	67	4–7 (RSDr)	[69]
M1	Milk	HPLC/fluor.	IAC	–	0.005	74	21–31	[93]
M1	Milk	HPLC/fluor.	IAC	0.006	0.015	91	8–15	[94]
M1	Milk	Chemiluminescent enzyme-linked immunosorbent assay (ELISA)	None	0.00025	0.001	96–122	2–8	[95]
M1	Milk	LC–MS/MS linear ion trap (ESI and APCI source)	Carbograph-4 cartridge	–	0.006–0.012	92–96	3–8	[89]
M1	Milk	Membrane-based flow through enzyme immunossay	IAC	0.05	–	97	–	[96]
M1	Milk	Electrochemical biosensor	None	0.01	–	–	–	[97]
M1	Milk, milk powder	LC–MS/MS triple quadrupole (ESI source)	IAC	0.59–0.66	–	78–87	–	[88]
M1	Milk, milk powder	LC–MS/MS triple quadrupole (ESI source)	Multifunction column	9–14	–	7–16	–	[88]

Source: Adapted from Manetta, A. C. 2011. Aflatoxins: Their measure and analysis. In: *Aflatoxins—Detection, Measurement and Control,* ed. Dr Irineo Torres-Pacheco, InTech Publisher, http://www.intechopen.com/books/aflatoxins-detection-measurement-and-control/aflatoxins-their-measureand-analysis. With permission.

TABLE 11.5
Examples for Aflatoxins Analysis in Food Using UPLC/MS

Aflatoxin	Matrix	UPLC	Mass Spectrometry	Column	Sample Preparation	LOD (μg/kg)	LOQ (μg/kg)	R%	RSD%	Reference
B_1, B_2, G_1, G_2, M_1	Corn, peanut butter	Acquity (Waters, USA)	Micromass Quattro Ultima triple-quadrupole (Micromass, UK)	BEH C18 (Waters, USA) (100 mm–2.1 mm–1.7 μm)	SPE	0.003, 0.006(G_2)	0.01, 0.02(G_2)	91–104	3.56–5.18	[131]
B_1, B_2, G_1, G_2, M_1	Maize, walnut, biscuit, breakfast cereals	Acquity (Waters, USA)	Acquity TQD tandem quadrupole (Waters, UK).	BEH C18 (Waters, USA) (100 mm–2.1 mm–1.7 μm)	SLE	0.02(B_1), 0.2(G_1, G_2), 0.01(M_1), 0.1(B_2)	–	71–108	5.8– 21.9	[132]
B_1, B_2, G_1, G_2, M_1, M_2	Chinese medicines	Acquity (Waters, USA)	Micromass Quattro Ultima triple-quadrupole (Micromass, UK)	HSS T3 (Waters, USA) (100 mm–2.1 mm–1.8 μm)	SPE (Homemade cartridge)	0.13(B_1), 0.16(B_2), 0.17(G_1), 0.14(G_2), 0.13(M_1), 0.15(M_2)	0.16(B_1), 0.33(B_2), 0.25(G_1), 0.18(G_2), 0.18(M_1), 0.24(M_2)	85– 113	1.2–15.9	[133]
B_1, B_2, G_1, G_2, M_1, M_2	Fresh peanuts, musty peanuts, peanut butters	Acquity (Waters, USA)	Micromass Quattro Ultima triple-quadrupole (Micromass, UK)	HSS T3 (Waters, USA) (100 mm–2.1 mm–1.8 μm)	SPE (Homemade cartridge)	0.009(B_1), 0.056(B_2), 0.085(G_1), 0.212(G_2), 0.017(M_1), 0.106(M_2)	0.012(B_1), 0.084(B_2), 0.182(G_1), 0.273(G_2), 0.021(M_1), 0.138(M_2)	80–88	1.9–9.4	[134]

B_1, B_2, G_1, G_2	Wheat, cucumber, red wine	Acquity (Waters, USA)	Acquity TQD tandem quadrupole (Waters, UK).	BEH C18 (Waters, USA) (100 mm–2.1 mm–1.7 µm)	QuEChERS	–	5.4(B_1), 4.4(B_2), 3.8(G_1), 4(G_2)	71–110	3–20	[125]
B_1, B_2, G_1, G_2, M_1	Baby food, milk	Acquity (Waters, USA)	Acquity TQD tandem quadrupole (Waters, UK).	BEH C18 (Waters, USA) (50 mm–2.1 mm–1.7 µm)	IAC	4(B_1), 5(B_2), 3.5(G_1), 3(G_2), 2(M_1)	12(B_1), 25(B_2), 11(G_1), 10.5(G_2), 6.5(M_1)	79–112	3–10	[126]
B_1, B_2, G_1, G_2	Cereals	Finnegan TSQ quantum ultra mass (Thermo, USA)	Finnegan TSQ quantum ultra mass (Thermo, USA)	C18 (Thermo, USA) (50 mm–2.1 mm–1.9 µm)	SLE	0.3(B_1), 0.5 (B_2), 0.08(G_1), 0.7(G_2)	0.55(B_1), 0.9(B_2), 0.15(G_1), 1.25(G_2)	83–107	6.8–9.9	[127]
B_1, B_2, G_1, G_2	Nonalcoholic beverages (Waters, USA)	Acquity (Waters, USA) (Micromass, UK)	Micromass Quattro Ultima triple-quadrupole (Micromass, UK) (Waters, USA)	BEH C18 (Waters, USA) (100 mm–2.1 mm–1.7 µm)	LLE 0.003(B_2),	0.003(B_1), 0.003(B_2), 0.01(B_2), 0.001(G_1), 0.002(G_2)	0.01(B_1), 0.01(B_2), 0.004(G_1), 0.007(G_2)	86–95	0.01–2.3	[128]
B_1, B_2, G_1, G_2	Barley	Accela (Thermo Fisher, USA)	Orbitrap (Thermo Fisher, USA)	HSS T3 (Waters, USA) (100 mm–2.1 mm–1.8 µm)	MSPD Modified QuEChERS SLE	–	–	73–81 75–82 80–85	14–18 9–12 12–17	[129]
B_1, B_2, G_1, G_2	Cereals	Acquity (Waters, USA)	Acquity TQD tandem quadrupole (Waters, UK)	BEH C18 (Waters, USA) (50 mm–2.1 mm–1.7 µm)	SLE	–	–	–	–	[130]

11.4.1 Instruments

HPLC combined with fluorescence detection is proven to be more accurate and has been studied extensively in different materials [98–101]. However, conventional HPLC methods often cost a lot of time to separate the target analytes and, additionally, in order to improve detection limits of AFB1 and AFG1, a tedious pre- or post-column derivatization must be done [98,102]. These problems have been successfully solved in the present study by introducing UPLC–MS/MS method. Reduction of the particle diameter from 5 μm (HPLC) to 1.7 μm (UPLC) results in greatly increased speed, while the introduction of the MS/MS detection avoids the tedious derivatization process. Despite the high sensitivity and selectivity of the LC–MS/MS method, the variable matrix effects limit its application. As a result, the previously established LC–MS/MS method could not be applied to determine AFs in different medicinal materials [103,104].

The term "UPLC" is a trademark of the Waters Corporation, but is often used to refer to the more general technique. The Waters Acquity Ultra-High-Performance LC system (Waters, Milford, MA, USA) seems to be the most popular UPLC system in aflatoxins analysis in food [105–111]. Other UPLC systems have been used successfully, such as the Finnegan TSQ quantum ultra mass (Thermo Scientific, CA, USA) system [112], and the An Accela U-HPLC system (Thermo Fisher Scientific, San Jose, CA, USA). The hyphenated MS/MS detectors were Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK) [23,108,109,111], tandem quadrupole mass spectrometer (Micromass, Manchester, UK) [105–107,110], Finnegan TSQ quantum ultra mass (Thermo Scientific, CA, USA) system [113] and single-stage Orbitraps mass spectrometer (Exactive; Thermo Fisher Scientific, Bremen, Germany) [112].

Chromatographic separations were achieved on an Acquity UPLC HSS T3 column (1.8 μm, 100 × 2.1 mm I.D., Waters, Milford, MA, USA) [106,107,112], UPLC BEH C18 column (1.7 μm, 100 × 2.1 mm I.D., Waters, Milford, MA, USA) [23,105,108,110], UPLC BEH C18 column (1.7 μm, 50 × 2.1 mm I.D., Waters, Milford, MA, USA), [109,111], and C18 column (1.9 μm, 50 × 2.1 mm I.D., Thermo Scientific, CA, USA) [113]. Zheng Han et al. [106] compared four candidate columns with different lengths and particle sizes, that is, (1) Agilent SB-C18 column (2.1 × 150 mm, 1.8 μm particle size), (2) Acquity UPLC HSS T3 column (2.1 × 100 mm, 1.8 μm particle size), (3) Atlantics RC18 column (2.1 × 150 mm, 1.8 μm particle size), and (4) UPLC BEH Shield RP18 column (2.1 × 100 mm, 1.7 μm particle size), in the pilot test to get a complete separation of the AFs. The separation efficiency of columns 1, 2, and 3 was obviously better than that of column 4. The sensitivity was greatly improved when choosing column 2 compared to other candidate columns.

11.4.2 Sample Pretreatment

Pretreatment of the sample (protein precipitation, defatting, extraction, and 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 such as methanol, acetone, or acetonitrile. The aqueous mixture should be the one most recently used because it will be more compatible not only with the environment but also with the antibodies involved in the subsequent step of clean-up with immunoaffinity columns that are increasingly utilized.

Clean-up is another very critical step. It is necessary to remove many of the coextracted 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 of conventional glass columns packed with silica, are time and solvent consuming. Nowadays, new sample preparation technologies, based on extraction by adsorbent materials, are available.

After extraction using acetonitrile aqueous solution, homogenization and filtration take place [105], then cleaned-up an aliquot of 15 mL of filtrate by passing through the Mycosep 226 Aflazon+ Multifunctional cartridges (PN. COCMY 2226, Romer Labs, Tulln, Austria). The sample was then dried, redissolved by a mixture of methanol and ammonium acetate, and shaken briefly for about 30 s by vortex to mix the content of the tube. Finally, the solution was passed through a 0.22 µm nylon filter and ready for injection. A Chinese group [106,107] has prepared a homemade cartridge and used it for the clean-up of the food samples for aflatoxins analysis in two different research. They prepared their cartridge simply as two layers of silica gel and alumina in a 6 mL hollow SPE cartridge, then covered by a cribriform plate to ensure the supine surface is smooth and flat.

R. Romero-Gonzalez et al. [108] compared three different pretreatment methods:

Method A: The well-known QuEChERS methodology [114] (quick, easy, cheap, effective, rugged, and safe). QuEChERS-based methodologies have been applied for the extraction of compounds with a wide range of physicochemical properties from different samples [115] using an acetate buffer [116]. For cucumber and red wine samples, 10 g of sample was weighed in a 50 mL polypropylene centrifuge tube. For wheat, 5 g of homogenized sample was weighed and 5 mL of water was added, soaking for 1 h. Subsequently, 10 mL of 1% acetic acid in acetonitrile (v/v) was added, and the tubes were shaken for 1 min with a vortex. Then, 4 g of anhydrous magnesium sulfate and 1.5 g of sodium acetate were added and the tubes were shaken immediately for 1 min. After centrifugation at 5000 rpm ($4136 \times g$) for 5 min, the supernatant was taken and filtered through a Millex-GN nylon filter (0.20 µm, Millipore, Carrightwohill, Ireland) prior to UHPLC–MS/MS analysis.

Method B: Sonication extraction. A sample of 5 g was weighed into a 50 mL polypropylene centrifuge tube and 10 mL of a mixture of acetonitrile/water 80:20 (v/v) was added. The mixture was vortexed for 2 min and then the tube was kept in an ultrasonic bath for 30 min. Then, the mixture was centrifuged for 10 min at 5000 rpm ($4136 \times g$), and the supernatant was filtered through a Millex-GN nylon filter and transferred into an autosampler vial prior to UHPLC–MS/MS analysis.

Method C: Generic extraction procedure, developed by Mol et al. [117]. Analytes were extracted using a method based on the procedure previously described by Mol et al. [112], where 2.5 g of sample was weighed into a 50 mL

polypropylene centrifuge tube and 5 mL of water was added. The mixture was shaken with a vortex for 1 min. If wheat matrix was studied, the mixture was allowed to soak for 1 h, then 15 mL of acetonitrile (1% formic acid, v/v) was added, and the sample was extracted by end-over-end shaking for 1 h at 50 rpm. After that, the mixture was centrifuged for 10 min at 5000 rpm ($4136 \times g$) and the supernatant was filtered through a Millex-GN nylon filter and transferred into an autosampler vial prior to UHPLC–MS/MS analysis.

In order to evaluate the performance of the three selected methods, wheat blank samples spiked at 50 μg/kg were treated, applying the three procedures showing the obtained results. It can be observed that the best results were obtained when QuEChERS procedure was used, allowing the extraction of more than 80 compounds with suitable recoveries (70–120%) and relative standard deviation (RSD) lower than 20%. When the ultrasound method was applied, more than 80 compounds were extracted, but only 36 compounds, including all the mycotoxins and biopesticides assayed in this study, were quantitatively extracted, whereas this approach was not suitable for most of the selected pesticides. Finally, an intermediate situation was obtained when the procedure described by Mol et al. was applied. More than 50 compounds were extracted with recoveries ranging from 70% to 120% and RSD values lower than 20%.

Rubert et al. [118] also compared different procedures for Barley sample pretreatment for aflatoxins analysis via UPLC–MS/MS.

Matrix solid-phase dispersion (MSPD): Barley samples were homogenized by mixing them thoroughly. Homogenized and representative 1 g portions were weighed and placed into a glass mortar (50 mL) and gently blended with 1 g of C18 for 5 min using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100×9 mm i.d. glass column, and eluted dropwise with 1 Mm ammonium formate in 10 mL of acetonitrile/methanol (50/50, v/v) by applying a light vacuum. Then, an aliquot (1 mL) of extract was filtered through a 22 μm nylon filter prior to injection into the UPLC–Orbitrap MS.

Modified QuEChERS: This procedure was employed to extract aflatoxins from the examined matrix [118,119]. Homogenized and representative portions of 2 g were weighed into a 50 mL PTFE centrifuge tube (conical-bottom centrifuge tube), and then 10 mL of 0.1% formic acid in deionizer water was added. The mixture was mixed for 3 min and waited for the next step for 10 min. Afterward, 10 mL acetonitrile were added, and consecutively, the mixture was vigorously shaken (3 min). The following step, 4 g MgSO4 and 1 g NaCl were added, and then the mixture was shaken for 3 min again. Once the extraction was completed, the sample was centrifuged (5 min, 11,000 rpm, 20°C). Then, an aliquot (1 mL) filtered through a 22 μm nylon filter before their injection into the UPLC–Orbitrap MS.

Solid–liquid extraction (SLE): Representative portions of 2 g samples were accurately weighed and transferred to a PTFE centrifuge tube (50 mL). Samples were extracted by shaking with 10 mL acetonitrile/water/acetic acid (79:20:1, v/v/v) on an automatic shaker for 90 min, and then centrifuged (5 min, 11,000 rpm, 20°C). Afterward, the supernatant extract was twofold diluted with HPLC-grade water, taking an aliquot of 0.5 mL and diluting to 1 mL. After that the sample was filtered through a 0.22 μm filter, consecutively the sample was injected into the UPLC–MS/MS.

SPE clean-up method: The previous SLE extract was used for clean-up. The extraction procedure was used according to Vendl et al. [120]. C18-SPE clean-up procedure was performed with Oasis HLB cartridges (150 mg) from Waters. The 2 mL of SLE extract was diluted with 30 mL of water in order to obtain a required maximum concentration of 5% organic solvent. The columns were prewashed with 10 mL of acetonitrile, and further conditioned with 10 mL of 5% acetonitrile in deionized water. Consequently, the diluted sample was loaded onto the C18 cartridge. After that, SPE columns were washed with 10 mL of 5% acetonitrile in water. The cartridges were then dried for 30 min. In the last step, the aflatoxins were eluted by adding 5 mL of acetonitrile. Then, the extract was transferred into a 15 mL conical tube and evaporated to dryness at 35°C with Buchi Rotavapor. The residue was reconstituted to a final volume of 1 mL with methanol/water (50:50, v/v) and filtered through a 0.22 μm Millex-G nylon filter, before the injection. To sum up, modified QuEChERS was selected for further studies in order to take advantage of its potential for simultaneous extraction of selected compounds. The data comparison showed that QuEChERS offered an acceptable range of recoveries and low RSDs. Furthermore, QuEChERS took very little time during the extraction procedure, and it was also easier and cheaper than MSPD, SLE, and SPE clean-up. For these reasons, QuEChERS was the most efficient and effective extraction procedure evaluated.

Immunoaffinity has been used by Eduardo Beltrán et al. [109] as a clean-up procedure to analyze aflatoxins in baby food and milk. In order to prepare the extracts for the immunoaffinity clean-up, acetonitrile of the extract was removed by using a turbo evaporator system (water bath at 50°C under gentle nitrogen stream). Then, the extracts were diluted with water up to 20 mL final volume. The 20 mL aqueous extracts were passed through an AflaOchra HPLC™ column at 1–2 drops per second. Then, the column was washed with 5 mL of HPLC water. Aflatoxins were eluted from the column with 4 mL methanol. To ensure complete elution of the bound toxin from the antibody, the solvent remained in contact with the column at least 1 min before starting the elution. The methanolic elutes were dried under gentle nitrogen stream at 50°C and reconstituted with 1 mL of HPLC-grade water. Finally, 20 μL extracts were injected into the UHPLC–ESI–MS/MS system. The use of a mixed-mode antibodies column has made possible the determination of all targeted aflatoxins in one single analysis. Their results showed that immunoaffinity columns allowed the simultaneous clean-up and analyte preconcentration, obtaining satisfactory chromatograms, with recoveries in the range of 79–112%.

11.5 CONCLUSION

In conclusion, a broad range of detection techniques used for practical analysis and detection of aflatoxins are available. This chapter highlighted some recent developments and new techniques about aflatoxins analysis in food via UPLC/MS. As shown, though there have been several recent successes in detection of aflatoxins, new methods are still required to achieve higher sensitivity and address other challenges that are posed by these toxins.

UPLC provides an efficient, fast, and high-resolution separation, and the application of MS in conjunction with other tools for decreasing limits of detection has been

of increased interest in recent times. Future trends will focus on rapid assays and tools that would measure multiple toxins from a single matrix. Since matrix interferences were detected during the UHPLC–MS/MS analysis of the sample extracts, additional analyte identification suitable for extensive multianalyte methods needs to be investigated.

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