Chapter 8
Analytical Methods for Detection and Quantification of Aflatoxins

Alessandra V. Jager and Fernando G. Tonin

Abstract Aflatoxins are produced by filamentous fungi, primarily *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*. These fungi occur naturally and might infest several food commodities throughout the food chain. The four major aflatoxins are Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), and Aflatoxin G_2 (AFG_2). Aflatoxin M_1 (AFM_1) and Aflatoxin M_2 (AFM_2), the hydroxylated metabolites of AFB_1 and AFB_2, respectively, are excreted into the milk of mammals that have ingested contaminated feed. Aflatoxins are highly carcinogenic, teratogenic, and hepatotoxic to humans and animals; consequently, knowledge about their incidence and levels in food and feed is a matter of public health concern. Numerous countries have set specific regulations on the maximum permitted limits of these contaminants in foodstuffs. Therefore, identifying and quantifying aflatoxins by reliable analytical methods are paramount for compliance with these legal limits. This chapter presents the fundamentals and recent developments of sample extraction, cleanup procedures, and identification and quantification approaches for aflatoxins in food and feed.

Keywords Aflatoxins · Sample extraction · Immunoaffinity column · SPE · QuEChER · DLLME · Liquid chromatography · Thin-layer chromatography · Fluorescence · Mass spectrometry · ELISA · Method validation · Multiclass mycotoxin

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K. R. Hakeem et al. (eds.), *Aflatoxins in Food*, https://doi.org/10.1007/978-3-030-85762-2_8

8.1 Introduction

Before the 1960s, episodes of farm livestock intoxication due to consumption of moldy feed had been described, but back then these episodes were not further investigated and remained unexplained (Richard 2008). However, in May 1960, after the acute poisoning of turkeys named Turkey "X" Disease in the United Kingdom, the correlation between an unknown toxin produced by fungus in the feed and its toxic effects was finally recognized (Sargeant et al. 1961; Lancaster et al. 1961). Shortly afterward, the unknown toxin was demonstrated to be carcinogenic to rats; it was also linked with liver cancer in humans (Le Breton et al. 1962). Chemical and physical characterization of the toxin, termed aflatoxin, was only accomplished some years later (Van der Zijden et al. 1962; Asao et al. 1963; Wogan 1966). Actually, aflatoxins belong to a larger group of toxic substances known as mycotoxins, which are produced by diverse fungi. Since the aflatoxins were characterized, scientists worldwide have scrutinized them, particularly in studies related to their synthesis and incidence in food and feed, adverse effects on human and animal health, and mitigation strategies (Rushing and Selim 2019).

Aflatoxins are produced by filamentous fungi, primarily *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* (Olsen et al. 2008). *Emericella astellata, E. venezuelensis, A. bombycis, A. ochraceoroseus, A. pseudotamari,* and *A. tamarii* also produce aflatoxins; however, these fungi are less common in crops. All the aforementioned fungi occur naturally and might infest several food commodities throughout the food chain: before and during harvesting; during storage, transportation, or processing; and even during consumption (Kaale et al. 2021). Groundnuts, cereals, oilseeds, and spices grown in tropical and subtropical regions under hot and humid climate conditions are the most susceptible to contamination (Williams et al. 2004).

Presently, around twenty aflatoxins have been characterized. The four major aflatoxins are Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), and Aflatoxin G_2 (AFG_2). Aflatoxin M_1 (AFM_1) and Aflatoxin M_2 (AFM_2), the hydroxylated metabolites of AFB_1 and AFB_2, respectively, are also important: they are excreted into the milk of mammals that have ingested contaminated feed (Marchese et al. 2018). AFB_1 has been the most investigated and is the most prevalent in food and feed.

Aflatoxins are a group of difuranocoumarin derivatives (Fig. 8.1). AFB_1 and AFB_2 consist of a difuran ring fused to a coumarin nucleus with a pentenone ring; AFG_1 and AFG_2 contain a six-membered lactone ring instead of a pentenone ring (Dhanasekaran et al. 2011). Aflatoxins are insoluble in nonpolar solvents, very slightly soluble in water, and freely soluble in moderately polar solvents (e.g., methanol, acetone, chloroform, and dimethyl sulfoxide). AFB_1 and AFB_2 emit intense blue fluorescence under UV light, whereas AFG_1 and AFG_2 emit yellow-green fluorescence, hence the designations B and G, respectively (Antila et al. 2002). Ultraviolet (UV) light in the presence of oxygen, extreme pH values (<3 or >10), and oxidizing and chlorinating agents degrade aflatoxins (Budavari et al. 2001).

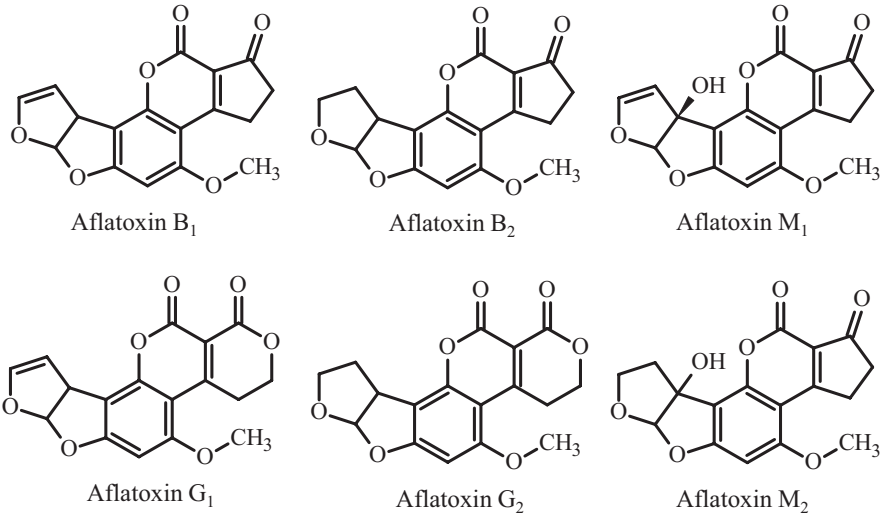

Fig. 8.1 Chemical structures of AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, and AFM_2

Table 8.1 Physical and chemical properties of AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1

Aflatoxin	Molecular weight (g mol^{-1})	Molecular formula	log K_{ow}	Solubility (mg L^{-1})[a]	Fluorescence emission λ (nm)	Ultraviolet absorption λ_{max} (nm)[b]
AFB_1	312.27	$C_{16}H_{12}O_6$	1.23	918.3	425	223 265 362
AFB_2	314.29	$C_{17}H_{14}O_6$	1.45	585.4	425	265 363
AFG_1	328.27	$C_{17}H_{12}O_7$	0.50	3152	450	243 257 264 362
AFG_2	330.29	$C_{17}H_{14}O_7$	0.71	2009	450	226 265 363
AFM_1	328.27	$C_{17}H_{12}O_7$	−0.27	45,840	425	226 265 357

[a]Estimated in water at 25 °C
[b]In ethanol

Aflatoxins have high thermal stability, which prevents them from being degraded in heat treatments during food processing. Table 8.1 summarizes some physical and chemical properties of aflatoxins (Zhang and Banerjee 2020).

In 1987, over two decades after AFB_1 was discovered and extensively investigated, the International Agency for Research on Cancer (IARC) classified it as

Group 1 carcinogen (Antilla et al. 2002). Furthermore, AFB_1 is the most harmful and lethal among naturally occurring carcinogens. Another reason for IARC classifying AFM_1 as Group 1 is its association with immunosuppression, genotoxicity, mutagenicity, teratogenicity, and carcinogenicity (Womack et al. 2016).

As knowledge about the adverse effects of aflatoxins on human health evolved over the years, many countries set specific regulations on the maximum permitted limits of these contaminants in foodstuffs. As a general rule, the limits established for animal feed are usually higher than the limits set for food intended for direct human consumption.

The European Community legislation is undoubtedly the strictest and the most comprehensive regarding aflatoxins (European Commission 2006a). This legislation sets maximum levels not only for the sum of AFB_1, AFB_2 AFG_1, and AFG_2 (ΣAF), as listed in most countries, but also for AFB_1 individually. Maximum AFB_1 and ΣAF levels of 2.0 and 4.0 μg kg^{-1}, respectively, are allowed for groundnuts, nuts, dried fruits, and all cereals intended for direct human consumption. If further treatment is applied, AFB_1 and ΣAF levels in groundnuts must not exceed 8.0 and 15 μg kg^{-1}, respectively. Higher AFB_1 and ΣAF maximum levels, 5.0 and 10 μg kg^{-1}, respectively, are acceptable for nuts, dried fruits, and maize that are processed before human consumption or used as a food ingredient. The most restricted limit of 0.1 μg kg^{-1} AFB_1 is set for dietary food for particular medical purposes, cereal-based food, and baby food intended for infants and young children. Concerning animal feed, only AFB_1 is regulated (European Commission 2003). The maximum limit of 20 μg kg^{-1} AFB_1 is obligatory for all feed components and complete feedingstuff for cattle, sheep, goats, swine, and poultry, while 10 μg kg^{-1} AFB_1 is set for complete feedingstuff for calves and lambs. Feed for dairy animals has the lowest permitted level of 5 μg kg^{-1} AFB_1.

Legal limits adopted by the US Food and Drug Administration (US FDA) are more general and specify only levels for the sum of aflatoxins (ΣAF). A range between 100 and 300 μg kg^{-1} ΣAF is defined for grains intended for beef cattle, swine, and poultry depending on the maturity stage. Up to 20 μg kg^{-1} ΣAF is acceptable for animal feed other than corn and cottonseed meal, which includes grains intended for immature and dairy animals and human food (Jimenez Medina et al. 2021).

Asian countries do not have a harmonized regulation, and the maximum limits adopted there may vary considerably among countries (Anukul et al. 2013). Maximum ΣAF limits might be as high as 30 μg kg^{-1} for all food, as defined in India and Sri Lanka, or 35 μg kg^{-1} in Indonesia and Malaysia. Japan sets the ΣAF limit of 10 μg kg^{-1} for all food, and the AFB_1 limit of 10 and 5 μg kg^{-1} for rice and other grains, respectively. China regulates AFB_1 in corn and corn products and peanuts and peanut products at 20 μg kg^{-1}. Also, Chinese regulation lays down maximum AFB_1 limits of 10 μg kg^{-1} for rice and vegetable oils, except peanut and corn oils, and of 5 μg kg^{-1} for wheat, barley, beans, and other grains. For condiments, such as soy sauce, vinegar, and fermented paste that use grains as primary materials, the maximum AFB_1 value is 5 μg kg^{-1}. The lower AFB_1 limit of 0.5 μg kg^{-1} is set for food intended for particular dietary uses, like formula food and complementary

food for infants and young children. South Korea sets the AFB_1 limit of 10 μg kg^{-1} for grains, cereal products, dried fruits, streamed rice, and dried fermented soybeans. The lower AFB_1 limit of 0.1 μg kg^{-1} is set for baby food. Only Indonesia regulates ΣAF at 50 μg kg^{-1} for corn feed.

In 2011, the Brazilian National Health Surveillance Agency (ANVISA) reviewed regulations for mycotoxin, and maximum limits for aflatoxins that had previously been stated only for peanut, peanut butter, corn, and its derivatives were extended to various grains and other food items (ANVISA 2011). ΣAF concentrations in peanut, peanut butter, corn, and some spices must not surpass 20 μg kg^{-1}. The maximum tolerable ΣAF limit of 5 μg kg^{-1} is set for beans, chocolate, cocoa products, and grains except for corn. Cereal-based food and infant formulas for young children must have a maximum ΣAF limit of 1 μg kg^{-1}.

Regulations worldwide consider not only food (e.g., groundnuts) that may frequently be more contaminated with known aflatoxin-producing fungi, but also foodstuffs that are mainly applied as staple food, which are the primary source of nutrients and energy. There is also a relationship with the culture and eating habits of each country or region. For instance, there are special regulations for rice in Japan and China; corn, rice, and beans in Brazil; and dried fermented soybeans in South Korea because these are everyday food items in these countries.

AFM_1 essentially contaminates milk and dairy products. Given that children widely consume these products, several countries have specific regulatory limits for this toxin. The European Community has the lowest permitted AFM_1 level – 0.050 μg kg^{-1} – for raw milk, heat-treated milk, and milk for manufacture of milk-based products (European Commission 2006a). An even lower limit – 0.025 μg kg^{-1} – is fixed for infant formulas and dietary food for particular medical purposes intended for infants. The United States, Brazil, China, Indonesia, Philippines, Singapore, South Korea, and Taiwan permit 0.5 μg kg^{-1} AFM_1 for milk (ANVISA 2011; Jimenez Medina et al. 2021).

Finally, numerous developing countries do not have regulations for aflatoxins. Consequently, the population is vulnerable and is likely to consume inappropriate food (Ayelign and De Saeger 2020). Besides variance in consumer exposure and safety, all the divergences discussed in the previous paragraphs might severely impair the international trade of food commodities. In this scenario, it is important to identify and to quantify aflatoxins for compliance with these legal limits.

8.2 Fundamentals of Analytical Methods

Analytical chemistry is fundamental in countless areas. With respect to food safety, analytical methods are indispensable to confirm adulterations and to identify and to quantify xenobiotics that are harmful to human and animal health. The first and most essential decision regarding quantitative analysis is selecting the method. This choice is frequently complex and requires experience on the part of the analyst.

Moreover, the required accuracy must be considered. Unfortunately, high reliability almost always demands considerable time and resources. The second decision concerns defining the number of samples, which also involves considering time and resources. Instrumental analysis might be an option if the number of samples is large, but reasonable time is spent on preliminary operations, such as assembling and calibrating instruments and preparing standard solutions. If the number of samples is small, an analytical method that dismisses the need for preliminary steps may be the best choice. Finally, the complexity of the sample and the number of components to be quantified also defines which method will be selected. Various analytical methods are available to determine aflatoxins. Because many countries have regulatory limits, analytical methods established by collaborative studies involving several laboratories or adopted by international organizations should be preferred, thus allowing analytical results to be compared. After validation according to international guidelines, new methods developed for analysis of aflatoxins are likely to become widely accepted in studies on the incidence of these toxins in food and human exposure to them (Berthiller et al. 2017).

The next decision regards sampling, which might be the most significant source of error. Sampling involves collecting a portion of the analyzed material; the composition of this portion must closely represent the entire material being sampled. The analyst must ensure that the laboratory sample is representative and must protect it from contamination and changes in composition before analysis. Contamination with aflatoxins originates from fungal metabolism and might not occur evenly throughout the sample, which poses an additional challenge (Wesolek and Roudot 2016). The European Commission regulation 401/2006 includes guidelines and plans for sampling mycotoxins, so that reliable qualitative and quantitative results can be achieved (European Commission, 2006b). Detailed discussion about sampling is beyond the scope of this chapter, but one must be aware of standard procedures before conducting a study (Galaverna and Dall'Asta 2012).

Processing the sample is another step in an analysis. Aflatoxins are determined in many solid samples, mainly grains, and a grinding step is mandatory before the analytical sample is removed. First, a solid sample is ground or milled, to reduce particle size. Then, it is mixed, to ensure homogeneity. After that, it is stored for some time before analysis. Zhang and Banerjee (2020) described dry, wet, and cryogenic grinding for analysis of aflatoxins in diverse food matrixes. The dry grinding protocol is extensively used to obtain homogeneous particle size for miscellaneous commodities like corn, wheat, peanuts, groundnuts, dried fruits, and spices (Spanjer et al. 2008). Although only dry grinding devices are available in most labs, and even though samples with bulky sizes must be handled or samples might contain high sugar or fat content, this type of grinding might cause obstruction or melting due to heat generated by the blades. An alternative is to turn to wet grinding, which provides samples with smaller particle sizes and uniform distribution of aflatoxins, but it is laborious and time-consuming (Spanjer et al. 2006). Wet grinding consists of mixing a sample with water or other solvents before the blending process, to form a slurry that provides test portions with better particle distribution and reproducibility than the test portions from samples processed by dry grinding. Lastly, cryogenic

milling allows heat-sensitive samples, typically fatty food, to be ground in teeny particle sizes (Liao et al. 2013). Cryogenic grinding requires that samples be frozen overnight and demands special milling devices and liquid nitrogen or dry ice as freezing agent. Compared to dry and wet milling, cryogenic grinding preserves the physical composition of samples. Many labs prefer using dry milling on a routine basis and only apply wet or cryogenic grinding to food matrixes that are unsuitable for dry milling.

Most analytical methods employ solutions of samples prepared in a suitable solvent. Ideally, the solvent should completely dissolve the analyte as fast as possible. The dissolution and extraction conditions must be sufficiently mild to prevent the analyte from being lost (Fifield and Kealey 2000). Many researchers have focused on the procedures and optimized conditions for efficiently dissolving and extracting aflatoxins from food and feed given that this is a decisive step for analysis of residual amounts of toxins. The choice of and the actual need for an extraction and purifica technique is closely related to the selected analytical method. If the analytical method is highly selective, purification may not be as extensive. On the other hand, if the analytical method cannot determine low concentrations like those found in samples, pretreatment must also pre-concentrate the analyte that will be later measured.

Once analytes are in solution, the next step eliminates any substances present in the sample that may interfere in the measurement, which frequently results in enrichment of the analyte. Interferent is any compound other than the analyte that affects the measurement. Few of the physical properties that are used to measure and to quantify an analyte are unique to a single chemical substance. In contrast, the measured properties are characteristic of a group of elements or substances. Initially, only absorption and fluorescence emission were employed to measure aflatoxins. Unfortunately, other substances from food matrixes also exhibit the same behavior. No fast and straightforward rules for eliminating interferents exist, and solving this problem may be the most critical aspect of analysis after sampling. Therefore, eliminating interferents to quantify aflatoxins has also been investigated. A crucial challenge is the diverse composition of the sample, which may contain fat, proteins, and carbohydrates as major components, comprising assorted substances that might interfere in the measurement. An ideal analytical method should determine many analytes in several matrixes while maintaining the same performance.

After interferents are eliminated, there is usually an intermediate step that is fundamentally represented by chromatographic separation techniques. Analysis of aflatoxins cannot be discussed without mentioning the first of all separation techniques, thin-layer chromatography (TLC). The years following the discovery of aflatoxins were also accompanied by the development of high-performance liquid chromatography (HPLC) and, lately, ultra-high-performance liquid chromatography (UHPLC). Chromatographic separation is not mandatory when it comes to identifying and quantifying aflatoxins, but it is undoubtedly available in most laboratories that determine these contaminants in food and feed.

Given that the analytical results depend on measuring a physical or chemical property of an analyte, this property must vary in a known and reproducible way as

a function of the concentration of the analyte. Early methods employed the natural ultraviolet absorbance or fluorescence emission of aflatoxins as a powerful identification and quantification tool. Indeed, these properties are still widely used and recommended by official protocols. In turn, combining HPLC or UHPLC with mass spectrometry (MS) provides analytical chemists with a remarkable technique to characterize and to quantify organic compounds.

Analytical results are incomplete without estimating reliability. Thorough method validation provides an analytical method with reliability and consistency (FDA 2019). Analytical validation is a quality assurance procedure that describes the conditions under which laboratory analysis are planned, performed, monitored, reported, and archived. Later, we will dedicate a topic to analytical validation parameters for identifying and quantifying aflatoxins in food and feed.

Knowledge about the presence of aflatoxins and their levels in food is a matter of public health concern. Therefore, one should not forget that many developing countries might lack resources for laboratory analysis and specialized personnel, maintenance, and supplies for high-tech instrumentation. On the other hand, resources for applying noninstrumental methods that can provide valuable data may be available. An important research area in analytical chemistry involves developing simple and inexpensive devices that can be used on-site and thus avoid that humans and animals consume contaminated and inappropriate food.

Developing analytical methods to determine aflatoxins goes hand in hand with knowledge about the presence of aflatoxins in food and their toxic effects. The greater the awareness and regulatory restrictions, the more selective and sensitive the analytical methods must be to meet these requirements. The general rule for analyzing almost any substance in food also applies to aflatoxins: extraction in a suitable solvent, elimination of interferents by a proper cleanup procedure, preconcentration when necessary, and identification or quantification on the basis of a physical or chemical property.

8.3 Sample Extraction and Cleanup for Determination of Aflatoxins

After a solid sample is properly sampled and powdered or ground, an extraction step is practically unavoidable (Reiter et al. 2009). Frequently, over 80% of the time required for an analysis is spent on sample preparation and cleanup. Several methods to extract aflatoxins from food and feed, like liquid-liquid extraction (LLE), solid phase extraction (SPE), immunoaffinity columns (IACs), dispersive liquid-liquid microextraction (DLLME), and QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe), have been established and extensively revised (Turner et al. 2009; Espinosa-Calderón et al. 2011; Zhang and Banerjee 2020; Miklós et al. 2020).

8.3.1 Liquid–Liquid Extraction

LLE is a classic and the oldest extraction technique. It is based on the solubility properties of the analytes in the aqueous or organic phase, or even in a system with two or more solvents. In the past, aflatoxins were primarily extracted by LLE because it was the only procedure available (Pons and Goldblatt 1965; Park and Melnick 1966). The first step entails extraction with methanol, acetone, chloroform, or a mixture of different polar organic solvents. Next, the extract is concentrated by evaporation. If necessary, a second extraction removes fats from the remaining residue by means of a polar and a nonpolar organic solvent such as hexane, cyclohexane, or petroleum ether. Some extractions include sodium chloride, which dissolves into the aqueous phase and increases its ionic force, facilitating extraction of aflatoxins into the organic phase, a process known as salting-out. A disadvantage of LLE is possible absorption of aflatoxins onto glassware, not to mention that LLE is a tedious, time-consuming procedure that requires large volumes of toxic solvents, which is not environmentally friendly.

Even though LLE is hardly sufficient to produce a clean extract to proceed with detection or quantification, some authors quantified aflatoxins in cereals, fish, spices, and beverages by using this extraction. Otta et al. (2000) extracted AFB_1, AFB_2, AFG_1, and AFG_2 from corn and wheat with a mixture of acetonitrile/water (9:1, v/v) and from fish with a mixture of methanol/water (7:3, v/v) on a vibrating shaker. The extracts were filtered, evaporated to dryness, and dissolved in dichloromethane/acetone (9:1, v/v), and the analysis was continued without any additional cleanup. Sheijooni-Fumani et al. (2011) quantified AFB_1 in rice, chickpea, and lentil by employing a two-step LLE. Well-milled solid samples were extracted with methanol/water (8:10, v/v); after vigorous shaking for 30 min, the supernatant was extracted again with chloroform. Then, after centrifugation for 5 min, the settled extraction phase was dried; the residue was dissolved in methanol; and the analyte was quantified. García-Moraleja et al. (2015) extracted aflatoxins from coffee beverages by LLE. The samples were freeze-dried, and the residues were extracted with ethyl acetate/formic acid (95:5, v/v) in three 5-min cycles. The supernatants were evaporated to dryness and reconstituted in methanol/water (1:1, v/v) before analysis. Sailaja et al. (2018) reported that a single extraction with chloroform at room temperature was adequate to extract aflatoxins from red chili. The chloroform extracts were filtered, washed with distilled water, and dried with anhydrous sodium sulfate. After being concentrated almost to dryness, the residues were dissolved in chloroform. Kokkonen et al. (2005) extracted AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1 from cheese with acetonitrile acidified with 0.1% formic acid and defatted with hexane. The mixture was filtered, and an aliquot of the acetonitrile phase was evaporated to dryness and dissolved in methanol for later quantification.

Although LLE is a low-cost, widely applicable method, LLE extracts are not entirely free of interferents, and additional cleanup is frequently required (Kamimura et al. 1985). Given that further cleanup becomes inevitable, some methods include a purification step on a minicolumn filled with silica, florisil, or alumina, to purify

the extract (Waltking 1970; Maia and Siqueira 2002). This has led to the well-recognized and commonly used SPE for analysis of aflatoxins

8.3.2 Solid-Phase Extraction

Samples can be purified by SPE, a process that has been available since the mid-1980s and which was developed as an alternative or supplement to LLE (Otles and Kartal 2016). SPE aims to reduce interference from components of the sample matrix and to improve detection sensitivity. The principle of purification is similar to that of solid-liquid chromatography. Sample separation, purification, and enrichment are mainly achieved by selective adsorption and desorption of components in the sample by a solid phase, placed inside a column or a disposable cartridge, which are widely commercially available. Commonly, two modes of SPE are employed: retention and elution of analytes or removal of interferents. The most usual method, which involves more steps, entails eluting the sample extract through the solid phase (adsorbent), to retain the analyte, followed by elution with a solvent of appropriate strength, to wash away impurities. Then, the analyte is eluted with a small volume of solvent, to achieve separation, purification, and concentration. Interfering impurities can also be selectively adsorbed while the analyte is allowed to elute, with cleanup comprising a single step. In addition, the SPE technique is a valuable tool for many purposes because it is versatile and might be optionally used in combination with other extraction techniques. Given that samples of food and feed designed for analysis of aflatoxins are solid, a single LLE usually precedes the SPE step, but purification and enrichment obtained in the latter step are much superior. Adsorbents that are used to purify extracts to determine aflatoxins are mostly prepared with C_{18}, florisil, silica gel, or alumina, and countless other adsorbents for SPE are commercially available (Zhang and Banerjee 2020).

Successful applications of SPE to clean up diverse samples aiming at determining aflatoxins have been reported. Several parameters such as type and amount of sorbent, elution solvent, and previous extraction or dilution with proper solvents are constantly being reevaluated for each type of sample. Romero-Gonzales et al. (2009) analyzed AFB_1, AFB_2, AFG_1, and AFG_2 in beer by just eluting 10 mL of degassed samples through a C_{18} commercial cartridge. After washing the column with water, aflatoxins were eluted with acetonitrile/methanol (60:40, v/v). Minicolumns prepared in-house and filled with aluminum oxide efficiently purified extracts of various grains including cornmeal, cottonseed, peanuts, almonds, and pistachios in one step. Ground samples were extracted with methanol/water (80:20, v/v), filtered, and diluted (1:1, v/v) with acetonitrile. The previous mixture was eluted through the columns. The purified extract was collected and directly injected into a LC system. Compared with other available commercial cartridges, these minicolumns allowed substantial savings (Sobolev and Dorner 2002). The same authors also showed that florisil adsorbed aflatoxins selectively in polar solvents (Sobolev 2007). Raw almonds, Brazil nuts, walnuts, hazelnuts, brown and white

rice, cornmeal, and dry-roasted pistachio nuts were first extracted in methanol/water (80:20, v/v). After a sequential wash with methanol/water (60:40, v/v), methanol, and chloroform/methanol (90:10, v/v), to remove impurities, aflatoxins were released from the column with acetone/water/formic acid (96:3.7,0.3, v/v). Sep-Pak Silica and Oasis HLB Waters™ were used to clean up extracts of cooked rice and medicinal herbs, respectively (Park and Kim 2006; Ventura et al. 2004).

8.3.3 Immunoaffinity Columns

IACs might be considered a subtype of SPE columns. IACs contain antibodies immobilized on inert support beads that exclusively retain aflatoxins (Ertekin et al. 2019). Due to their high specificity, IACs produce cleaner extracts with lower level of interfering matrix components than the less selective solid phases of SPE sorbents. IACs have been extensively applied because they are well established for cleaning up and concentrating sample extracts for analysis of aflatoxins (Scott and Trucksess 1997; Patey et al. 1991).

IACs are undoubtedly handy when a less selective detector is used, which is the case of ultraviolet or fluorescence detectors, and they have emerged as one of the most important sample preparation techniques. The processes involved in IACs are essentially the same as those described for SPE: the sample extract is eluted on the IAC, and the antibody retains aflatoxins. A sequential wash with water or buffer removes impurities, and aflatoxins are released by elution with an appropriate solvent, usually methanol, which breaks the aflatoxin-antibody bond. Several commercial IACs are available for AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1, but, unlike regular SPE cartridges, IACs are filled with phosphate-buffered saline (PBS) solution and kept refrigerated, to maintain the functionality of the antibody.

Another critical point in IAC development is the high content of solvents, such as acetonitrile, methanol, or acetone, in sample extracts: antibodies are not tolerant to high concentrations of organic solvents. Before being eluted through the column, extracts must be diluted with water or buffer. The problem is that this dilution increases the volume that has to be eluted and may yield insoluble compounds that interfere in the binding of aflatoxins to the antibodies (Uchigashima et al. 2009).

Despite the higher selectivity of IACs, special attention must be given to them when they are applied to food matrixes that have not been previously evaluated. Castegnaro et al. (2006) highlighted that some drawbacks of IACs must not be overlooked. Complex matrixes might contain unknown substances that obstruct the binding site of antibodies, thereby reducing the absorption of toxins and yielding inaccurate results. Furthermore, interaction with other substances might alter the structure of toxins, so antibodies will not recognize them.

IACs are manufactured for single extraction, and instructions clearly recommend that they be discarded after use. Unfortunately, many laboratories cannot afford their high cost, mainly when they are imported and purchased with foreign currency. This led numerous researchers to assess the reuse of different commercial

columns, but results regarding aflatoxins and the composition of samples are not consistent among manufacturers. Liao et al. (2020) evaluated regenerating columns for raw malt extracts. They found that washing the IAC with PBS and stocking it at 4 °C overnight maintained IAC performance for nine additional extractions of AFB_2, AFG_1, and AFG_2, but only two additional extractions of AFB_1. Iha et al. (2017) also attempted to reuse IAC by immediately washing it with PBS and storing it at 8 °C overnight for re-equilibration, for use on the following day. A naturally contaminated peanut sample was extracted six times with the same column, and the results obtained for aflatoxins were statistically equivalent, except for AFG_2. If only AFB_1 was quantified, the same selectivity was maintained for ten consecutive extractions. Liu et al. (2012) used reconditioned IACs twice to extract aflatoxins from medicinal herbs. Nevertheless, they highlighted that if the column was dirty with pigments or ingredients that did not elute with water, it should not be reused due to poor analyte recovery.

Divergences among assessments clearly demonstrate that caution must be taken when reusing IACs from different manufacturers and applying them to analyze food or feed extracts with different compositions from the previously assessed compositions.

8.3.4 Dispersive Liquid–Liquid Microextraction

To reduce solvent consumption in traditional LLE substantially, DLLME has been used to extract aflatoxins from milk, dairy products, fruit juices, oils, wheat, and eggs. DLLME is a miniaturized extraction technique that offers advantages such as simplicity, rapid operation, high throughput, and low cost. In a usual DLLME protocol, an appropriate mixture of extraction solvent and organic dispersant is rapidly injected into the aqueous sample with a syringe (Rezaee et al. 2006). This generates a cloudy solution consisting of microdroplets of the extraction solvent, which is dispersed entirely into the aqueous phase. After centrifugation, the fine particles of the extraction solvent are settled on the bottom and are removed for further analysis. Thus, the extraction solvent must have higher density and low solubility in water. Because the contact area between the organic solvent and the water sample is large, extraction is fast and efficient. The extent of enrichment of DLLME enhances with dispersion of the extraction solvent. The finer the droplet of the extraction solvent, the higher the enrichment performance. Some parameters affecting the extraction efficiency must be optimized, including the volume of extraction solvent and disperser solvent, extraction, and centrifugation time.

Most analytical methods that use this technique require previous extraction of the sample with a polar solvent given that the basic principle of DLLME is to extract substances from an aqueous or immiscible phase. Table 8.2 contains some examples showing that previous extraction with an aqueous solvent is always performed before DLLME.

Table 8.2 Dispersive liquid-liquid microextraction-based methods to extract aflatoxins from food and feed

Matrix	Toxin	Previous extraction	DLLME	Analytical method	Reference
Edible oils	AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample + methanol/water (6:4, v/v) (2) Centrifugation at 4000 rpm/10 min 3) Dilution with water 4) IAC Afla Clean™	(1) 5.0 mL IAC extract in PBS + 500 μL ACN (disperser) + 120 μL $CHCl_3$ in (extractant) (2) Centrifugation 4000 rpm/3 min	HPLC – fluorescence detection LOD: 1.1 × 10^{-4} – 5.3 × 10^{-3} ng mL^{-1}	Afzali et al. (2012)
Dairy products	AFB_1 AFM_1	QuEChERS: (1) Sample + (water, ACN, NaCl, sodium citrate) (2) Centrifugation at 5000 rpm/45 min 3) Supernatant + ($MgSO_4$, PSA, C_{18}) 4) Centrifugation at 2700 rpm/5 min	(1) 3.0 mL previous extract (disperser) + 500 μL $CHCl_3$ (extractant) injected in 7.0 mL of deionized water (2) Centrifugation: 2700 rpm/10 min	HPLC – fluorescence detection LOD: 0.1 μg kg^{-1} AFB_1 0.1 μg kg^{-1} AFM_1	Karaseva et al. (2014)
Plant-based milk	AFB_1 AFB_2 AFG_1 AFG_2	(1) 5 mL sample + 6 mL ACN + NaCl (2) Centrifugation at 6000 rpm/5 min	(1) Previous extract (disperser) + 1500 μL $CHCl_3$ (extractant) injected in 5.0 mL of deionized water (2) Centrifugation: 6000 rpm/5 min	HPLC – fluorescence detection LOQ: 0.5 μg kg^{-1}	Hamed et al. (2019)
Egg	AFB_1	(1) Sample + ACN/water (80:20, v/v) + diatomaceous earth (2) Ultrasound/2 min (3) Filtration (4) Aqueous extract defatted with hexane	(1) 1.2 mL previous extract (disperser) + 240 μL $CHCl_3$ (extractant) injected in 3.0 mL of deionized water (2) Centrifugation: 4000 rpm/3 min	HPLC – fluorescence detection LOD: 0.12 μg kg^{-1}	Amirkhizi et al. (2018)
Fish	AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample + 5 mL (60:40 ACN/PBS) (2) Ultrasound/7 min (3) Filtration	(1) 400 μL $CHCl_3$ (extractant) injected in 5.0 mL previous extract + 25 mg NaCl (disperser) (2) Centrifugation: 2500 rpm/10 min	LC – MS/MS LOD: 0.07–0.036 μg kg^{-1}	Jayasinghe et al. (2020)

(continued)

Table 8.2 (continued)

Matrix	Toxin	Previous extraction	DLLME	Analytical method	Reference
Peanuts	AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample + methanol/water (80:20, v/v) (2) Ultrasound/30 min (3) Centrifugation at 2795 × *g*/5 min	(1) 1.0 mL previous extract (disperser) + 200 μL $CHCl_3$ (extractant) injected in 5.0 mL deionized water (2)Centrifugation: 2795 × *g*/5 min	HPLC – fluorescence detection LOD: 0.03–0.1 μg kg^{-1}	Chen et al. (2017)
Vegetable oils	AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample + ACN (84% in water) (2) Shaken/30 min (3) Centrifugation at 4000 rpm/3 min	(1) 0.8 mL oil extract + 200 μL TFA (disperser) + 400 μL CH_2Cl_2 (extractant) injected in 3.2 mL deionized water (2) Centrifugation: 4000 rpm/5 min	HPLC – fluorescence detection LOD: 0.005–0.03 μg kg^{-1}	Wang et al. (2019)
Yogurt	AFM_1 AFB_1 AFB_2 AFG_1 AFG_2	(1) Sample centrifuged at 6000 rpm/5min (2) Sample + NaCl + ACN (3) Centrifugation at 6000 rpm/5 min	(1) 5.0 mL previous extract (disperser) + 1500 μL $CHCl_3$ (extractant) injected in 5.0 mL of deionized water (2) Centrifugation at 6000 rpm/5min	HPLC – fluorescence detection LOD: 0.0015–0.0055 μg kg^{-1}	Hamed et al. (2017)

LOD detection limit, *ACN* acetonitrile

8.3.5 QuEChERS

Although the previously described extraction procedures selectively isolate aflatoxins from a complex matrix and use much less solvent than LLE, modern analytical chemistry is continuously developing rapid, easy-to-perform, and low-cost analytical procedures (Perestrelo et al. 2019). QuEChERS is a simple dispersive solid-phase extraction (d-SPE) procedure that can be applied to numerous samples. QuEChERS consists of extracting the homogenized sample with an appropriate solvent, normally acetonitrile, by vortex-shaking for a few minutes. A liquid-liquid partition is created by adding excess salts or buffers to the mixture. After centrifugation, water might be removed with anhydrous $MgSO_4$, and the extract is further cleaned in an additional step in which the extract is mixed with a sorbent, such as primary-secondary amine (PSA), silica gel, C_{18}, or graphitized carbon black (Juan et al. 2017). QuEChERS was initially developed to simplify screening of pesticide residues in large numbers of agricultural samples, but now it has been applied for analysis of many other food contaminants, and aflatoxins are surely among them (Anastassiades et al. 2003; Michlig et al. 2016; Choochuay et al. 2018).

With a classic procedure QuEChERS, AFB_1 was extracted from wheat, rice, oat, rye, maize, and barley (Zhao et al. 2017). First, aqueous acetonitrile solution (95:5, v/v) was added to milled samples; then, the mixture was vortexed for 1 min and subjected to ultrasound for 3 min before NaCl and $MgSO_4$ were added. The extract was vortexed for 1 min and centrifuged for 5 min. Next, the supernatant was transferred to another tube containing PSA. After vortexing and centrifugation, the extract was ready for analysis.

Likewise, all other extraction procedures, solvent type, extraction time, and need for adsorbents or not must be optimized before the extraction procedure is effectively established. Also, analysts must always bear in mind that the extraction procedure is unquestionably committed to the detection system that will be used subsequently.

Lastly, even though research has focused on reducing extraction steps, most methods for analysis of aflatoxins still combine two or three approaches to achieve the best results.

8.4 Detection and Quantification of Aflatoxins

8.4.1 Thin-Layer Chromatography

TLC with fluorescence detection was the only chromatographic technique available in the 1960s. Although it was the method of choice of AOAC International (Association of Official Analytical Collaboration International) for an extended period, it is no longer widely used (Trucksess 2000). Despite being a low-cost procedure, the separation efficiency of TLC is low, and identification of aflatoxins might be susceptible to interferences from the fluorescence of other components with similar migration patterns in the sample. Eventually, when combined with a selective cleanup method, such as IACs, TLC is a robust and straightforward screening method (Stroka et al. 2000). Aflatoxins can be quantified if TLC is coupled to densitometry, becoming an alternative for labs that cannot afford more expensive chromatographic methods (Stroka and Anklam 2000; Marutoiu et al. 2004). In two-dimensional thin-layer chromatography (2D-TLC), the plate is rotated 180° after the first separation, and aflatoxins and other components of the sample are eluted once more with a second solvent (Durakovic et al. 2012). High-performance thin-layer chromatography (HPTLC) and bidirectional HPTLC are derived from the previous TCL and have been successfully applied for quantification of aflatoxins in food and feed (Tomlins et al. 1989; Ramesh et al. 2013).

8.4.2 High-Performance Liquid Chromatography Coupled to Fluorescence Detector

The most widely used strategy to determine aflatoxins in food and feed is separation by HPLC followed by fluorescence detection. However, although AFB_1 and AFG_1 exhibit natural fluorescence, it is less intense than the fluorescence of AFB_2 and AFG_2. Moreover, water, acetonitrile, and methanol, used as mobile phases in reversed-phase liquid chromatography, also quench the fluorescence of AFB_1 and AFG_1. Pre- or post-column derivatization methods are recurrently used to circumvent this issue, to increase the signal during analysis. Pre-column derivatization with trifluoroacetic acid (TFA) hydrolyzes AFB_1 and AFG_2, to form the more fluorescent hemiacetals AFB_{2a} and AFG_{2a}. Even though the time required for complete reaction is short, derivatization represents an additional step. Nevertheless, it is worth performing because derivatization enhances detection (Saito et al. 2020; Wang et al. 2020). Detection limits ranging from 0.1–0.2 μg kg^{-1} or tenfold lower are easily achieved and entirely meet the requirements of analytical methods for surveillance. Post-column derivatization is accomplished by adding pyridinium bromide-perbromide (PBPB) to the mobile phase after separation. Two atoms of the bromide are added at the 8,9-double bond of the dihydrofuran moiety of aflatoxin, enhancing the fluorescence signal (Stroka et al. 2001). Such bromination is more cost-effective, but bromine can also be produced by an electrochemical cell (Kobra™ Cell) in the post-column step by adding KBr to the mobile phase (Kok 1994; Omotayo et al. 2019). Extraction by IAC and quantification by reversed-phase high-performance liquid chromatography-fluorescence detector (HPLC-FD) without or with pre- or post-column derivatization are among the official methods adopted by AOAC International (AOAC International 2002). Although the mass spectrometry detector has continuously replaced the fluorescence detector, the latter is still widely employed for various purposes because it is spread among analytical chemistry laboratories, as shown in Table 8.3.

8.4.3 Liquid Chromatography Coupled to Mass Spectrometry Detector

Since the mid-1990s, when atmospheric pressure ionization (API) interfaces were developed and overcame the low sensitivity and ionization efficiency of thermospray, particle beam, and fast atom bombardment interfaces, liquid chromatography-mass spectrometry has emerged as the most powerful technique for identification and quantification of contaminants in food (Miklós et al. 2020).

Commercially available atmospheric pressure ion sources include ESI (electrospray), APCI (atmospheric pressure chemical ionization), and APPI (atmospheric

Table 8.3 Overview of HPLC-fluorescence detection-based methods for determination of aflatoxins

Matrix	Toxin	Pretreatment	Analytical method	LOD μg kg^{-1}	Reference
Milk (pasteurized and UHT)	AFM_1	(1) Water bath at 37 °C (2) Centrifugation (3) AflaM1 IAC	Column: Nucleosil C_{18} (250 × 4.6 mm, 5 μm) Mobile phase: water/acetonitrile: methanol (6:2:3, v/v/v), isocratic Detection: direct fluorescence detection	0.01	Mannani et al. (2021)
Milk, yogurt, milk powder, and ice cream	AFM_1 AFM_2	(1) LLE, methanol/water (7:3, v/v) + NaCl (2) Dilution with water (3) IAC	Column: agilent XDB C_{18} (250 × 4.6 mm, 5 μm) Mobile phase: water/methanol: acetonitrile (6:2:2, v/v), isocratic Detection: post-column derivatization, Kobra® cell	0.125 0.151	Lee and Lee (2015)
Surk cheese	AFB_1 AFB_2 AFG_1 AFG_2 AFM_1	(1) LLE, Celite + chloroform + NaCl (2) Dilution with PBS (3) IAC	Column: Inertsil ODS-3 (150 × 4.6 mm, 5 μm) Mobile phase: water/acetonitrile/acetic acid (49.5:49.5:1, v/v/v), isocratic Detection: post-column derivatization, electrochemical cell	0.033–0.061	Sakin et al. (2018)
Cereal flour (wheat, maize and rice)	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (8:2, v/v) (2) Dilution with PBS (3) AflaTest IAC	Column: Inertsil ODS-3 (250 × 4.6 mm, 5 μm) Mobile phase: water/acetonitrile: methanol (6:2:3, v/v/v), isocratic Detection: post-column derivatization, Kobra® cell	0.014–0.028	Kara et al. (2015)

(continued)

Table 8.3 (continued)

Matrix	Toxin	Pretreatment	Analytical method	LOD µg kg^{-1}	Reference
Coffee (beans and powder)	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (8:2, v/v) (2) Dilution with PBS (3) Easi-Extract IAC	Column: Novapack C_{18} (150 × 3.9 mm, 4 µm) Mobile phase: water/methanol: acetonitrile (64:18:18, v/v), isocratic Detection: pre-column derivatization with TFA	0.09–0.17	Al-Ghouti et al. (2020)
Dried figs	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (3:2, v/v) + NaCl (2) Dilution with PBS (3) Aflatest IAC	Column: C_{18} (250 × 4.6 mm, 5 µm) Mobile phase: water/acetonitrile: methanol (6:2:3, v/v/v), isocratic Detection: post-column derivatization, Kobra® cell	0.13–0.46	Bakirci (2020)
Spices (turmeric, red pepper, black pepper, cinnamon)	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (8:2, v/v) + NaCl (2) Dilution with water (3) Aflatest IAC	Column: Zorbax Eclipsed XDB (250 × 4.6 mm, 5 µm) Mobile phase: water/acetonitrile: methanol (5:2:3, v/v/v), isocratic Detection: post-column derivatization, electrochemical cell Libios-K01	0.1–0.3	Zareshahrabadi et al. (2020)

(continued)

Table 8.3 (continued)

Matrix	Toxin	Pretreatment	Analytical method	LOD μg kg^{-1}	Reference
Feed ingredients (corn, wheat bran, soybean, dried distillers grains with solubles)	AFB_1	(1) LLE, methanol/water (8:2, v/v) (2) Dilution with PBS (3) AokinImmunoClean IAC	Column: C_{18} (150 × 4.6 mm, 5 μm) Mobile phase: water/methanol (50:50, v/v), isocratic Detection: post-column derivatization, electrochemical cell AURA	0.03	Li et al. (2014)
Feed samples (mustard cake, cotton seed cake, soybean cake, groundnut cake, wheat bran, crushed wheat/maize)	AFB_1 AFB_2 AFG_1 AFG_2	(1) LLE, methanol/water (7:3, v/v) + NaCl (2) Dilution with water (3) AflaPure IAC	Column: Spherisorb C_{18} (250 × 4.6 mm, 5 μm) Mobile phase: water/methanol: acetonitrile (6:2:2, v/v), isocratic Detection: pre-column derivatization with TFA	0.06–0.92	Patyal et al. (2021)

pressure photoionization). Any of them can analyze aflatoxins; however, the ESI source is undoubtedly the most frequently found in laboratories and the most employed (Capriotti et al. 2010). After exiting the API source, the molecules enter the vacuum chamber and reach the mass analyzer through an ion transporting and focusing region. Single-stage or multistage (MS/MS) mass analyzers are commercially available. Except for in-source CID, there is no collision-induced dissociation (CID) in a single-stage mass analyzer, and molecular ions cannot be fragmented. Thus single-stage mass analyzers do not meet the European Union recommendations for analysis of residues in food because a precursor ion and two product ions are required to confirm a contaminant (European Commission 2002). This can be achieved by multiple reaction monitoring (MRM) of selected ions, a parameter that is experimentally optimized for each compound.

Aflatoxins can be analyzed on a MS/MS mass analyzer like triple quadrupole (QqQ), quadrupole time-of-flight (Q-TOF), quadrupole-linear ion trap (Q-TRAP), and Orbitrap. Although triple-quadrupole is the most regularly used for determining mycotoxins, not all analytical standards are commercially available. As a result,

interest in high-resolution mass spectrometry (HRMS), such as Orbitrap and time-of-flight (TOF) mass analyzers, has increased. HRMS has essential advantages: it records full scan spectra by measuring the accurate mass of analytes, screens untargeted compounds, and reviews analysis data, allowing analytes that had not been considered at the time of spectral acquisition to be investigated (Tittlemier et al. 2021).

One of the main advantages of LC-MS/MS systems is their remarkable sensitivity and selectivity, dismissing the need for extensive cleanup of sample extracts. Nevertheless, for some substances in very complex samples, cleanup cannot be entirely neglected when high sensitivity is desired. When it comes to the practical use of LC-MS/MS methods, co-eluting matrix components might significantly suppress or enhance the signals of the analytes. In other words, analyte ionization is prevented by competition of charges from the other components of the sample. This is the well-recognized "matrix effect" in mass spectrometry detection, and it harms the performance of LC-MS/MS methods, mainly in terms of the accuracy of quantification (Truffeli et al. 2011a, b). For instance, if a solvent calibration curve is prepared with the analytical standards for the quantification, differences in the signals of the analytical standards and sample extracts will directly impact the result. There is no universal approach, but understanding why signals are suppressed or enhanced might support the analyst's decision.

The most straightforward approach to avoid the matrix effect is to dilute the sample extract in an appropriate solvent (Stahnke et al. 2012). However, diluting the extract will also dilute aflatoxin, and the mass spectrometer might not be sensitive enough to overcome the loss in concentration. If calibration curves are prepared in blank extracts, a similar suppression or enhancement effect is predicted both for standards and sample extracts. This approach is named the matrix-matched calibration curve. Nevertheless, the availability of samples free from the contaminant should be verified before deciding on this method. The standard addition method is laborious and time-consuming, especially when more than one aflatoxin must be quantified. Another valuable approach is the stable isotope (SI) dilution assay. Deuterated and ^{13}C-aflatoxins are commercially available and considered the ideal Internal Standards. SI solutions can be added to sample extracts and calibration curves or incorporated in the sample before extraction and cleanup (Varga et al. 2012). This procedure provides enormous flexibility under the conditions of sample extraction and significantly improves the precision of the method, but SIs are expensive, which should be borne in mind before choosing the methodology.

Due to its outstanding performance, it is not surprising that LC-MS/MS has been widely and successfully employed to determine aflatoxins in food and feed. Table 8.4 depicts several applications of LC-MS/MS in diverse samples together with the extraction and cleanup procedures.

Table 8.4 Overview of LC-MS/MS-based methods for determination of aflatoxins

Matrix	Toxin	Pretreatment	Analytical method	LOD ($\mu g\ kg^{-1}$)	Reference
White cheese	AFM_1	(1) Extraction with Celite, CH_3Cl, and NaCl (2) Extract defatted with hexane (3) IAC	LC (Q-TRAP) Column: Optima ODS-H (150 × 2.0 mm, 5 µm) Mobile phase: (A) 10 mmol L^{-1} ammonium acetate, (B) 10 mmol L^{-1} ammonium acetate in methanol, gradient elution	0.0625	Kamel et al. (2017)
Milk and Jujube	AFM_1 AFB_1	Milk: (1) Dilution with deionized water, (2) Filtration (3) C_{18} Micro-SPE Jujube: filtration (1) Extraction with methanol/water (7:3, v/v) and sonication/20 min (2) Centrifugation at 4000 rpm/3 min (3) C_{18} Micro-SPE	LC (Q-TOF) Column: SB-C_{18} (50 × 4.6 mm, 1.8 µm) Mobile phase: (A) 0.1 % aqueous formic acid, (B) 0.1% formic acid in ACN, gradient elution	AFM_1: 0.049 AFB_1: 0.023	Du et al. (2018)
Milk, powder milk, and yogurt	AFM_1	(1) Extraction with acetonitrile, formic acid, and NaCl (2) Centrifugation at 13,000 rpm/5min	Online SPE-LC (QqQ) Online cartridge: BioBasic C18 Column: PFP (100 × 2.1 mm, 2.6 µm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in methanol, gradient elution	0.0005–0.0007	Campone et al. (2016)
Whole milk, milk-based infant formula, and animal feed	AFB_1 AFB_2 AFG_1 AFG_2 AFM_1	(1) Extraction with acetonitrile/water (50:50, v/v) (2) Centrifugation at 4500/15 min (3) Filtration	LC (Q-TRAP) Column: Kinetex XB-C_{18} (100 × 2.1mm, 2.6 µm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in methanol, gradient elution	0.005–0.0038	Zhang et al. (2018)

(continued)

Table 8.4 (continued)

Matrix	Toxin	Pretreatment	Analytical method	LOD (μg kg^{-1})	Reference
White rice and sorghum	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water (50:50, v/v) containing 0.1% formic acid (2) Centrifugation at 4500/5 min (3) Filtration (4) SPE with ISOLUTE Myco	LC (QqQ) Column: XB bridge C_{18} (100 × 2.1, 1.7 μm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in acetonitrile, gradient elution	0.28–0.90	Ok et al. (2016)
Baby food and feeds	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water (78:22, v/v) (2) Centrifugation at 5000/8 min (3) Evaporation of acetonitrile by rotoevaporation (4) Dilution with water (5) IAC	LC (QqQ) Column: BEH C_{18} (100 × 2.1, 1.7 μm) Mobile phase: 0.1 % formic acid methanol/ water (75:25, v/v), isocratic	0.003–0.008	Alfaris et al. (2020)
Ground maize	AFB_1 AFB_2 AFG_1 AFG_2	QuEChERS: (1) Extraction with methanol/water (60:40, v/v) (2) Addition of $MgSO_4$ and NaCl (3) Centrifugation at 4000/5 min (4) Filtration	LC (QqQ) Column: Zorbax Eclipse C_{18} (50 mm × 3.0, 1.8 μm) Mobile phase: (A) 5 mmol L^{-1} ammonium acetate, (B) 5 mmol L^{-1} ammonium acetate in methanol, gradient elution	0.11–0.36	Ouakhssase et al. (2019)
Corn powder, edible oil, peanut butter, and soy sauce samples	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water (50:50, v/v) (2) Sonication/20 min (3) Addition NaCl (4) Centrifugation at 8000/5 min (5) Filtration	Online TFC-SPE-LC (QqQ) TFC: Cyclone (50 × 0.50 mm, polymer type) Column: Phenomenex C_{18} (100 × 2.1 mm, 1.7 μm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in methanol, gradient elution	0.20–2.0	Fan et al. (2015)

(continued)

Table 8.4 (continued)

Matrix	Toxin	Pretreatment	Analytical method	LOD (μg kg^{-1})	Reference
Dark tea	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water (86:14, v/v) + NaCl (4) Pressure filtration (5) MFC (PriboFast MFC260) + IAC	LC (Q-TOF) Column: C_{18} (100 × 2.1 mm, 1.8 μm) Mobile phase: (A) water, (B) methanol, gradient elution	0.024–0.21	Ye et al. (2019)
Fish feed	AFB_1 AFB_2 AFG_1 AFG_2	(1) Extraction with acetonitrile/water, 0.1 mol L^{-1} KH_2PO_4 (60:40, v/v) (2) Sonication/7 min (3) Matrix Imprinted Polymer	LC (Q-TRAP) Column: Zorbax C_{18} (100 × 4.6 mm, 3.5 μm) Mobile phase: (A) 0.1% aqueous formic acid, (B) 0.1% formic acid in methanol, gradient elution	0.42–1.15	Jayasinghe et al. (2020)

TFC turbulent flow column, *MFC* multifunctional column

8.4.4 Screening and Rapid Methods

Enzyme-linked immunosorbent assay (ELISA) is the most common and extensively used screening method for determination of aflatoxins in agricultural raw materials. ELISA is easy to perform, is not as expensive as chromatography, does not use organic solvents, and is essential to provide on-site results about aflatoxins in a short period. Therefore, it is a standard and well-accepted method for monitoring aflatoxins. ELISA kits are commercially available and also provide quantitative results. Several samples can be analyzed without any extensive cleaning step. However, ELISA is highly matrix-dependent, so analysis of more complex samples requires that the manufacturer be consulted or the method be individually validated for the specific sample (Miklós et al. 2020).

To quantify AFM_1 in raw milk, Maggira et al. (2021) validated a commercial ELISA kit among three commercially available kits against an HPLC-FL method. The authors found that the ELISA kit was a faster and equally reliable alternative method to HPLC in routine analysis.

A calibration curve implanted enzyme-linked immunosorbent assay (C-ELISA) was developed to determine aflatoxin B_1 in wheat, corn, soybean, and peanuts (Wu et al. 2020). The new development implanted an optimized standard curve data into a matched analysis software, programmed by the researchers, to make data processing more convenient and faster. The new method proved rapid and sensitive and provided equivalent results to HPLC for all the AFB_1 concentrations in real samples.

AFB_1 detection by electrochemical immunoassays is sensitive and fast. Kong et al. (2018) used 2-aminoethanethiol to increase the speed and sensitivity of a conventional electrochemical immunoassay by assembling the thiol on the surface of a

gold electrode, to form self-assembled monolayers. Then, non-competitive immunoassays occurred on the surface, to give an electrochemical immunoassay sensor. The detection limit achieved by the new development was 0.1 ng mL^{-1} AFB_1.

Among new developments, Fan et al. (2020) established a time-resolved fluorescence immunoassay based on fluorescent microspheres containing a Eu^{3+} chelate named AFM_1-POCT. They used a portable fluorimeter, and the reaction took 5 min. The results were equivalent to the results of UHPLC-MS determination in the range of 0.0121–2.0 μg kg^{-1}, so the method met the detection limits of 0.05–0.5 μg kg^{-1} required by the regulatory organization and enables on-site sampling.

8.5 Analytical Method Validation

Analytical method validation essentially involves evaluating whether a new or a modified literature method applies to the routine of a certain laboratory. The main objective is to demonstrate that the analytical method is appropriate, ensuring that it is accurate, reproducible, and applicable to the substance intended for identification and quantification. The method is considered validated when it is evaluated according to a series of at least some preestablished parameters. The evaluated performance parameters will vary depending on the intended use of the method, its type (quantitative or qualitative), and the degree to which it has been previously validated (FDA 2019). For example, new quantitative methods should include at least the following performance characteristics: selectivity, detection limit, quantification limit, linearity, or other calibration models, range, accuracy, precision, measurement uncertainty, ruggedness, confirmation of identity, and spike recovery (FDA 2019).

Vast literature is available to assist the validation process for those who intend to proceed with an in-house validation. The most comprehensive and maybe widely accepted guidance is the European Commission Council Directive concerning the performance of analytical methods and the interpretation of results (European Commission 2002). National guidelines might also be compulsory and might be accessed by region or country, if necessary. Raposo and Ibell-Bianco (2020) presented a valuable discussion about general analytical method validation, which was extremely useful because the authors presented discrepancies and controversies among different guidelines for obtaining and interpreting the most required parameters in analytical method validation.

Considering how aflatoxins are formed in food and feed, validation should be best evaluated with Certified Reference Materials (CRM), if available. Matrix reference materials with naturally occurring mycotoxins are preferred over fortified ones because the incurred mycotoxins are incorporated deeper within the matrix (Tittlemier et al. 2021). Using a spiked matrix with standard solutions to determine recovery might yield unrealistic values (Dzuman et al. 2014). Finally, proficiency tests or interlaboratory studies are crucial. Numerous collaborative studies have been carried out by AOAC International (Bao et al. 2012; Stroka et al. 2001).

Thus, assuming the importance of this topic associated with strong regulations of some segments, laboratories must attend to their analytical methods and their adequate validation whenever appropriate.

8.6 Multiclass-Mycotoxin Methods of Analysis

Unfortunately, aflatoxin-producing fungi are not the only fungi that infest crops, and even fungi related to the synthesis aflatoxins might produce other mycotoxins. Besides aflatoxins, the most investigated mycotoxins in food and feed are Fumonisin B_1 (FB_1), Fumonisin B_2 (FB_2), Ochratoxin (OTA), Deoxynivalenol (DON), Nivalenol (NIV), Zearalenone, (ZEA), T-2, and HT-2, among others.

Current mycotoxin regulations and acceptable levels in food and feed apply to many different individual mycotoxins in a single food, which emphasizes the importance of establishing methods that can simultaneously determine multiple mycotoxins in a single analysis (European Commission 2006a). To achieve these goals, methods for determining a group of mycotoxins became common (Lago et al. 2021). Due to the various structures and physical and chemical properties of different mycotoxins, extraction and analysis parameters must be adapted to the intended matrixes, to meet the minimum requirements of recovery, sensitivity, and selectivity. Routine determination of multiple mycotoxins is only possible, thanks to significant advances in extraction and purification techniques and notable increase in the sensitivity of triple quadruple mass spectrometers frequently coupled to liquid chromatography, as already mentioned. Diverse multi-mycotoxin analytical methods are described in the specialized literature (González-Jartín et al. 2021)

8.7 Multi-Residue Methods of Analysis

Besides the multiple mycotoxins that are likely to contaminate food and feed, other harmful substances exist. Other toxic substances such as pesticides, veterinary drugs, hormones, and plant alkaloids might also be incorporated along the food production chain. Multi-residue methods for several analytes differing in polarity, structural formulas, and physicochemical properties are becoming regularly accessible (Steiner et al. 2021a, b). Because the surveillance of aflatoxins in food and feed is mandatory and well-recognized, these contaminants are recurrently included in the list of multiclass methods. When a method for multi-residue determination is employed, almost all efforts are directed to increasing the number of identified or quantified analytes and reducing the time required for analysis. Analyzing more analytes in a shorter time means saving resources and making faster decisions, especially decisions related to accepting a raw material batch or not or releasing a final product to consumers.

With single methods, aflatoxins can be quantitatively extracted after proper cleanup and determined with a suitable detector. In contrast, a compromise among all the components is needed when developing multiclass methods, especially for complex samples, in which the applicability of analyte-specific extraction is not practicable (Steiner et al. 2020). Multi-residue analytical methods also expand the possibility of screening before a more specific method is applied. The numerous investigations and efforts in this research area brought impressive advances in approaches for sample preparation, identification, and quantitation (Dzuman et al. 2015). Diverse applications are easily found in the specialized literature (Steiner et al. 2021a, b).

8.8 Conclusions

Since aflatoxins were discovered, they have attracted a lot of attention, effort, and resources. The search for new analytical methods to determine mycotoxins is undoubtedly a fertile field of research. Protocols for sample preparation have been continuously improved and optimized for analysis of aflatoxins in various food and feed matrixes, having progressed from laborious liquid-liquid extractions to solid-phase extraction and modern immunoaffinity columns, culminating in the simple dilute and shoot and QuEChER approaches. Faster cleanup has allowed significant advances in high throughput analysis. Different analytical protocols for determining aflatoxins in food commodities have advanced and gradually improved. Despite drawbacks such as low sensitivity and poor accuracy, TLC was the most used chromatographic technique for quantification of aflatoxins until the 1980s. Thereafter, it was steadily replaced with HPLC coupled to ultraviolet and fluorescence detectors. IAC cleanup with liquid chromatography separation and fluorescence detection is widely used as a gold analytical method in laboratories worldwide because it has been extensively validated in collaborative studies and recognized by regulatory boards. Mass spectrometry detectors impressively enhanced the selectivity and sensitivity of methods for the determination of aflatoxins and multiple mycotoxins. Liquid chromatography coupled to mass spectrometry is the most potent tool for monitoring and controlling the levels of aflatoxins in food and feed. Due to its vast applicability, LC-MS/MS instruments have become the mainstream device in almost all research and routine laboratories. However, its inclusion in several laboratories is hampered by the high costs for acquiring, maintaining, and training personnel for the instrument. Finally, screening and fast methods, represented mainly by ELISA, are essential for on-site monitoring of aflatoxins. Constant developments to improve immunoassays and biosensors promise to bring new cost-effective, reliable, and straightforward methods to determine aflatoxins.

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