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Chapter 12

# A Focus on Aflatoxin in Feedstuffs: New Developments in Analysis and Detection, Feed Composition Affecting Toxin Contamination, and Interdisciplinary Approaches to Mitigate It

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Additional information is available at the end of the chapter

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**Abstract**

Aflatoxins are mold-synthetized secondary metabolites that are capable of causing disease and death in humans and other animals. Aflatoxins hold a prominent place in the discussion on feed safety as are the only mycotoxins with the regulatory framework. Feed ingredients and composition inevitably affect the susceptibility of feed to fungal and toxin contamination. To verify that legal thresholds are being complied, avoiding delivering contaminated feed to animals, and obtain correct prevalence data, analytical methods must be developed which are apt for application on a complex matrix such as animal feed. These methods should include simple screening assays and high-end confirmatory ones. Laboratories without expensive equipment can and should be able to implement methods and to analyze and detect aflatoxins. Aflatoxin contamination is a complex issue that should be assessed interdisciplinarily and farm-to-fork models should be integrated into vigilance. In this chapter, we have devoted some lines to each of the aspects mentioned above focusing on feed aflatoxin contamination.

**Keywords:** aflatoxins, analytical methods, sample preparation, feed composition, feed safety, farm-to-fork, One Health

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# 1. Introduction

## 1.1. Aflatoxins

The four major aflatoxins are called $B_1$, $B_2$, $G_1$, and $G_2$ (**Figure 1**) based on their fluorescence under UV light (blue or green) and retention factors during thin-layer chromatography. $AFB_1$ has been described as a potent natural carcinogen (classified in group 1; [1]) and is usually the major aflatoxin produced by toxigenic strains. However, other aflatoxins (e.g. $AFM_1$, $B_{2a}$, and $G_{2a}$) have been described, particularly since biotransformation products of the mammalian degradative enzyme metabolism, is based on cytochromes. This biosynthetic pathway is shared by norsolorinic acid, an anthraquinone, and sterigmatocystin (STE), a mutagenic and tumorigenic dihydrofuran toxin. STE is a late metabolite in the aflatoxin pathway and is also produced as a final biosynthetic product by some species such as *Aspergillus*, *Aspergillus chevalieri, Aspergillus ruber, Aspergillus amstelodami,* and *Aspergillus aureolatus* [2]. The reader is encouraged to consult the papers written by Bbosa and coworkers [3] and Dohnal and coworkers [4] that describe with detail aflatoxin metabolism.

Aflatoxins are difuranocoumarin derivatives produced by a polyketide pathway by many strains of *Aspergillus flavus* and *Aspergillus parasiticus*. Especially, *A. flavus* is a frequent contaminant in agricultural plants and commodities. Other aflatoxin-producing species have been encountered less frequently (**Table 1**). In fact, just in 2011, Varga and coworkers described two new aflatoxin-producing species, *Aspergillus pseudocaelatus* sp. (Argentina) and *Aspergillus pseudonomius* sp. (United States) [5]. Baranyi and coworkers [6] described the phylogenetic association among these strains based on partial calmodulin sequencing. We refer the reader

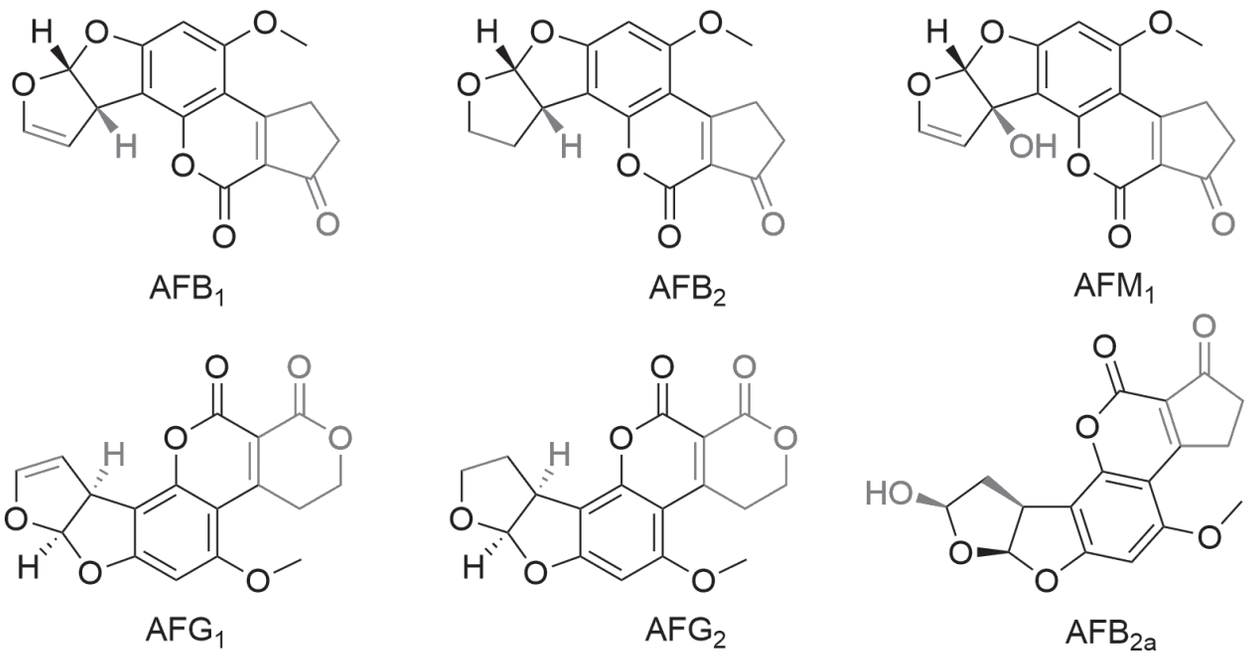



**Figure 1.** Chemical structure of the four major aflatoxins and two natural metabolites. Bonds colored in red showcase the main differences among them.

| Section *Flavi* | Section *Ochraceorosei* | Section *Nidulantes* |
|---|---|---|
| *Aspergillus arachidicola*[2] | *Aspergillus ochraceoroseus*[1] | *Aspergillus astellatus*[1] |
| *Aspergillus bombycis*[2] | *Aspergillus rambelli*[1] | *Aspergillus venezuelensis*[1] |
| *Aspergillus minisclerotigenes*[2] | | |
| *Aspergillus mottae*[2] | | |
| *Aspergillus nomius*[2] | | |
| *Aspergillus parvisclerotigenus*[2] | | |
| *Aspergillus pseudocelatus*[2] | | |
| *Aspergillus sergii*[2] | | |
| *Aspergillus pseudonomius*[1] | | |
| *Aspergillus pseudotamarii*[1] | | |
| *Aspergillus togoensis*[1] | | |

[1, 2] Blue and green colors represent the type of aflatoxins the strain is capable of producing.

**Table 1.** Aflatoxigenic fungi species capable of aflatoxin production [124].

to an excellent review by Samson and coworkers [7] regarding phylogeny, identification, and nomenclature of the genus *Aspergillus*, which is composed of more than 339 species.

From the mycological perspective, there are phenotypic and genetic differences in the strains within each aflatoxigenic species and each strain display various toxigenic abilities. For example, *Aspergillus* subgenus *Circumdati* section *Flavi* includes species with usually biseriate conidial heads, in shades of yellow-green to brown, and dark sclerotia [5]. On the other hand, several *afl* genes are involved in the biosynthesis of aflatoxins. Each strain produced toxins differentially (e.g. *A. flavus* and *A. parasiticus* are known to produce aflatoxins $B_1$ and $B_2$, aflatoxin fractions the latter only synthesizes $G_1$ and $G_2$). Meaning a genotypical difference as well since aflatoxin G producers have integral versions of genes *nad*A and *afl*F [8]. There is, in fact, a battery of molecular tests devoted to the genetical identification of *Aspergillus* section *Flavi* [7, 9]. Several *Aspergillus* strains have been isolated from feeds. For example, Iranian cattle feed [10], poultry feed from South Africa [11], chicken feed from Nigeria [12], and dairy goat feed from Brazil [13].

Several of the species above are important mycotoxin producers including aflatoxins, and like the genetic ability to make aflatoxin, contamination is highly variable. Crops can become contaminated with aflatoxin in the field before harvest, where it is usually associated with drought stress [14]; adding difficulty to this issue, storage conditions may favor mold growth. During storage, usually, the most important variables are the moisture content of the substrate and the relative humidity of the environment [15]. Aflatoxin contamination has been linked to increased mortality in farm animals and, thus, significantly lowering grain value as an animal feed and, thereafter, loss of productivity in the case of food-producing animals [16]. Milk products can also serve as a source of aflatoxin. When cows consume aflatoxin-contaminated feeds, they transform $AFB_1$ into a hydroxylated form called $AFM_1$. Cytochrome $P_{450}$ enzymes

convert aflatoxins to the reactive 8,9-epoxide form, which is capable of binding to both DNA and proteins [3, 4]. This metabolite is still considered carcinogenic and teratogenic and may affect young and newborn animals and reach human as a final consumption product. Unit operations during milk production usually have little to no effect over the $AFM_1$. Although we will not explore $AFM_1$ contamination in detail, we urge the reader to read a very thorough review regarding $AFM_1$ in bovine milk written by Becker-Algeri and coworkers [17].

Aflatoxin is associated with both toxicity and carcinogenicity in human and animal populations [16]. There are substantial differences in species susceptibility. On the other hand, within a given species, the magnitude of the response is influenced by age, sex, weight, diet, and exposure to infectious agents [16]. The presence of other mycotoxins (most common co-occurrence of AF and ochratoxin A or AF and fumonisins) and pharmacologically active substances may reflect antagonistic, additive, or synergistic effects [18]. Sufficient availability of feed is combined with regulations and continuous surveillance programs to monitor contaminant levels and protect animal populations from significant aflatoxin ingestion. The scarcity of resources (both economic and food supply) may play a role in the use of contaminated feed.

### 1.2. Feeds and feed ingredients and aflatoxin contamination

Feed is defined as any goods or materials which are consumed by animals and contribute energy and nutrients to the animal's diet [19]. Usually, it is divided into two categories, roughages and compound feed. Roughages comprise diets based on grass, silage, hay, legumes, bagasse and others. Equines and dairy cattle complete rations on occasion are complemented or based on roughages. In Costa Rica, for example, dairy cow diets are composed mainly of forage, including *Cynodon nlemfuensis* Vanderyst, *Pennisetum clandestinum* Hochst, and *Lolium perenne* L. Current global data regarding fungi and mycotoxins in silages have been described [20]. Aflatoxins and *Aspergillus* species have been found to be important especially in corn and sorghum silages.

On the other hand, compound feed is composed primarily of cereals (e.g. rice, wheat, barley, oats, rye, corn, sorghum, and millet), milling by-products (e.g. brans, hulls, pollards), and oil cakes (e.g. palm kernel, soybean, sunflower, rapeseed, peanut, linseed, cottonseed). Other feed ingredients include distillers dried grains. The components above (especially corn and corn by-products) are the most susceptible to aflatoxigenic fungi attack and therefore aflatoxin contamination [13]. The chemical composition, ingredients and nutritional quality of feed inherently influence the capability of fungi to inoculate and even make use of their genetic machinery available to produce aflatoxins within such a substrate. Hence, feed is especially susceptible to aflatoxin contamination.

A few papers have focused on this fact and examined some aspects relating aflatoxin contamination with nutritional analysis. For example, Hashimoto and coworkers analyzed 42 fish feed samples and found no association between pelletized and extruded feed and aflatoxin levels and no nutritional differences between both feed types [21]. However, they did acknowledge an aflatoxin/fumonisin co-occurrence of a 23.8%. Prabakaran and Dhanapal found that natural contamination observed in two Indian regions (220 and 15 $\mu g\ kg^{-1}$) were connected with those areas where feeds were prepared with higher moisture (11.29–11.70 g/100g) and crude fat (4.62–4.64 g/100 g) [22]. Interestingly, the

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authors also demonstrated that when feed undergo autoclaving and then inoculated with a toxin-producing *A. flavus* strain, higher concentration of toxin was attained when compared with the same non-autoclaved feed. The authors relate this finding to the release of zinc from phytic acid when pressure and temperature are applied, which results in the mineral being available for aflatoxin synthesis [23, 24]. Liu and coworkers found that defatted grains (i.e. soybean, peanut, corn, wheat corn endosperm, and corn germ) showed a significant decrease in aflatoxin concentration when compared to their full-fat counterparts [25]. On the other hand, when the same seeds were treated with corn oil, aflatoxin production capacity was regained by *A. flavus*. Hence, the removal of lipids contains $AFB_1$ production. The effects of starch, crude protein, soluble sugars (fructose, glucose, sucrose, maltose, raffinose, and stachyose), amino acids (aspartic acid, glutamic acid, glycine, arginine, and alanine), and trace elements (copper, iron, zinc, and manganese) on $AFB_1$ production and mycelial growth were examined. Maltose, glucose, sucrose, arginine, glutamic acid, aspartic acid, and zinc significantly induced $AFB_1$ production up to 1.7- to 26.6-fold. Stachyose promoted *A. flavus* growth more so than the other nutrients, playing a pivotal role in grain infection by *A. flavus*. These data provide new insights toward feed protection from contamination. Herzallah modeled aflatoxin carry over from feed to several tissues using a diet based mainly on corn meal (60.5 g/100 g), soybean meal (21.5 g/100 g), and vegetable oil (3.0 g/100 g) [26]. In this case, laying hens were exposed to aflatoxin concentrations up to 965.12 μg $kg^{-1}$ for 6 weeks, and aflatoxin levels were monitored. The author found worrisome tissue levels that rounded up to 0.63 and 2.12 μg $kg^{-1}$ in breast and liver, respectively. A very thorough and sophisticated study [27], applied to dairy cattle and milk, used Monte Carlo simulations to assess different scenarios which contemplated milk yield, feed composition, which considered normal aflatoxin levels found in the feed, and feed ingredients and their inclusion rates. This work reiterates the value of computational modeling to estimate possible contaminant exposure and is unique, as one of the variables used during modeling were aflatoxin levels found in real matrixes (e.g. a naturally contaminated batch of corn, with a maximum concentration of 168 μg $kg^{-1}$) extracted from the Dutch national surveillance program. The authors modeled diets based on high and low protein. And included relevant feed ingredients in different proportions, e.g. corn (10.24–15.06 g/100 g), soybean meal (14.96–0.23 g/100 g), sunflower seed meal (4.5–3.83 g/100 g), palm kernel (ca. 15 g/100 g), rapeseed meal (7.94–5.54 g/100 g), and corn gluten feed (3.67–1 g/100 g). All these raw materials with a differential potential of aflatoxin contamination. The $AFB_1$ analysis reflects that corn ingredients exhibit higher aflatoxin levels. In total, five different transfer equations of $AFB_1$ from feed to $AFM_1$ in the milk were included, and the results showed that in only 1% of the revised cases, milk toxin levels surpassed the legislative threshold. An increased contamination was found when contaminated feed ingredients were included in the formulation (i.e. contaminated corn), up to 28.5% of the iterations exceeded the threshold. The authors also observed that an increase in the milk production had a minimal effect on these data due to an apparent dilution effect. The same authors conclude that feeding regimes, including the composition of crude fiber and feeding roughages of dairy cows, should be carefully monitored and considered regarding their aflatoxin inclusion potential. Noteworthy, van der Fels-Klerx and Bouzembrak also used a similar approach to estimate the probability of $AFB_1$ contamination of compound feed for dairy cattle and to limit this contamination [28]. With the results obtained, the authors suggested an optimized feed composition, including a reduction of citrus pulp (10–0 g/100 g), sunflower seed meal (23–1.5 g/100 g), and soybean meal (10–5.1 g/100 g) and an increase in corn ingredients (20.5–29.4 g/100 g), palm kernel (16–22.5 g/100 g), and

wheat (2–30 g/100 g), with respect to usual formulations. The authors based their recommended diet on wheat which is relatively inexpensive but may not be available during some seasonal changes or inaccessible to some geographical regions. The authors claimed that 98.8% of the simulated diet would exhibit values below the legal threshold contrary to a 75.6% of cases assumed using a general formulation.

### 1.3. Aflatoxins, food chain safety, and the One Health approach

The One Health approach highlights the kinship of human, animal, and environmental health and the importance of transdisciplinary and interdisciplinary efforts [29]. Hence, collaborative efforts under this tactic aim to help promote animal and human health. The challenges posed by mycotoxicosis, a foodborne illness that results from consumption of aflatoxin-contaminated food and feed, are more likely to be understood and mitigated through a One Health approach. Shenge and LeJeune reported that it is estimated that a third of global food supplies are contaminated with aflatoxins [30]. This value alone should be cause for concern, as fungal contamination is not only a source of disease for crops but also generate poor harvests, and impact the well-being of animals and humans; creating, even more economic losses when food producing animals are involved. Several aspects of mycotoxins remain unclear, and research is still needed regarding all areas affected by mycotoxins. Although few articles tackle the issue from a holistically standpoint, at least one conference paper presented by Sirma is focused explicitly on using One Health in mycotoxin analysis [31]. On the other hand, Magnussen and Parsi published an article which encompasses a health issue such as hepatocellular carcinoma within the aflatoxin convoluted problematic [32]. More recently, two papers have more specifically considered the issue from the One Health stance. Frazzoli and coworkers contemplated aflatoxin contamination anticipating environment, animal, and human interaction, and the feed and food link with emphasis to the carryover that occurs from the presence of aflatoxins in a feed to milk (i.e. $AFM_1$) during the entire dairy chain [33]. On the other hand, Ogodo and Ugbogu considered the presence of aflatoxins in food industry, management and its relationship with hepatocellular carcinoma, linking a public health issue again with an agronomical one [34]. The latest effort in integrating the totality of the food chain is in the form of "MyToolBox", a European Commission funded initiative joining knowledge from different sectors to improve risk management, reduce crop losses and its impacts, and provide safe options to treat toxin-contaminated batches [35]. The final objective of the initiative is to offer recommendations and practical measures to the end users along the food and feed chain in a web-based platform.

## 2. Current methods for the analysis of aflatoxins in feedstuffs

### 2.1. Relevance of aflatoxin accurate determination

Food safety relies on the capability of laboratories to screen, detect, quantify, and confirm the presence of aflatoxins in different staple foods. Multiple methods have been designed over the years, the authors refer the reader to a good starting point to familiarize with the general principles and mechanisms involved in the main techniques used for aflatoxin analysis [36, 37]. For an ampler view regarding the recent developments in techniques for the detection of

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aflatoxins, we urge the reader toward the paper written by Yao and coworkers [38]. Herein, we will limit the discussion to techniques applied for the aflatoxin determination in feed, feed ingredients, and some selected related matrices as they lead the food chain.

Accurate mycotoxin analysis is paramount for feed and feed ingredients safety evaluation and epidemiology. Animal feed is at the beginning of the food chain, and any in-feed contaminants may reach the final consumer through food matrixes, such as eggs or meat products [39]. However, few methods are specialized for feeds, which can be noted by the sheer number of official methods for this kind of matrices. For example, AOAC only has three assays: 975.36 (Romer mini column), 989.06 (ELISA), and 2003.02 (liquid chromatography [LC]). Contrasting to the amount of approaches and principles available for other staple foods [40, 41].

## 2.2. Sampling and some sample preparation highlights

### *2.2.1. Feed sampling*

Aflatoxin sampling is especially complicated since mold growth (and hence toxin distribution), in feed and grain, may not be homogenous. For example, not all the conditions for the production of toxins will be met in the totality of a silo; a storage grain system will reduce the toxin production with $a_w$ values below 0.70 [42]. Errors in sampling methodology carry costs intrinsically. Assuming only a section of a feed batch is contaminated, the composite and homogenized sample is vital. Failure in detecting the mycotoxin will generate adverse effects on farm animals which will be fed with said foodstuff. Sampling directly and only from a "hot zone" will unchain legal events that usually ends in the elimination of a whole feed batch, which is costly for feed manufacturers or importers/exporters [43]. On the other hand, research has demonstrated that the bulk of the variability in mycotoxin analysis comes from sampling [44]. Some papers have focused specifically on aflatoxin sampling. For example, Mallmann and coworkers sampled eight lots of corn using two different plans: manual, using sampling spear for kernels; and automatic, using a continuous flow to collect corn meal [43]. The authors concluded that automatic sampling introduces less analytical variation and it is more accurate than manual sampling. In contrast, Herrman and coworkers sampled Texan grain elevator establishments and determined that while sampling contributes to variability in measuring aflatoxin in grain, aflatoxin analysis using commercially available test kits was a major contributor to variation in aflatoxin test results among commercial food handlers [45].

Several authorities have issued sampling guidelines. The American Association of Feed Control Officials (AAFCO) recommendations for mycotoxin test object collection is detailed in Feed Inspector's Manual for the member States [46]. Similarly, the European Commission has emitted the 2006/401/EC which lay down the sampling methods and analysis for the official control of the levels of mycotoxins in foodstuffs [47]. Food and Agriculture Organization of the United Nations (FAO) have developed a mycotoxin sampling tool (http://www.fstools.org/mycotoxins/). Berthiller and coworkers detailed other sampling and analysis methods that include other mycotoxins [48]. Lee and coworkers designed a statistically derived risk-based sampling plan for surveillance sample assignments of chemical and biological hazards using binomial probability distribution [49]. The authors found that the number of feed samples that exceeded legal thresholds for target analytes (aflatoxins, fumonisins,

*Salmonella*, and dioxins) in the validation data were lower than those of the average 3-year data in most feed products.

### *2.2.2. Commercially available tools for aflatoxin analysis*

Several companies have devoted their efforts in the fabrication of versatile products useful to isolate aflatoxins from samples. Different researchers have applied diverse approaches to obtain a clean extract, especially to inject into LC systems. For example, R-biopharm AG has two various methods available for the analysis of aflatoxins: RIDASCREEN® for total AF and $AFB_1$ single analysis and AFLAPREP® an immunoaffinity column which delivers limits of detection as low as 0.007 ng AF $mL^{-1}$ extract, and based on our laboratory experience, sample clean-up is capable and good recoveries are obtained. Noteworthy, R-biopharm also has an immunoaffinity column for sterigmatocystin.

Other laboratories have recently applied these concrete columns to monitor aflatoxin in food and feed [50, 51]. Multiple columns (e.g. AOF MS-PREP®) based on this same principle are also available. However, care must be taken as usually recoveries vary with regard with that of the single toxin column as competition for active sites may arise causing lower recoveries. Romer Labs® has also developed a wide range of detection and sample treatment techniques, including AgraStrip®, which are rapid, ready-to-use (qualitative total aflatoxin or quantitative WATEX) lateral flow devices ideal for on-site or surface testing with a limit of detection of 3.31 μg $kg^{-1}$. AgraQuant® Aflatoxin/Aflatoxin $B_1$ which are ELISA tests with sensitivities ranging from 1 to 3 μg $kg^{-1}$ and FluoroQuant® quantitative fluorometric tests based on a solid-phase or immunoaffinity column clean-up. Romer Labs® has two immunochemical columns: Aflastar™ FIT and Aflastar™ R, the latter have been used in our laboratory and applied to feed aflatoxin monitoring with excellent results. Vicam has similar products ranging from strip tests (Afla-V, Afla-V aqua which has removed the use of hazardous organic solvents) to LC clean-up immunoaffinity columns (Afla B, AOZ HPLC [high-performance liquid chromatography]) approached vary from quick response, qualitative to quantitative. Immunoaffinity columns are a very attractive option for sample clean-up and concentration; however, it is important to consider that this approach not only has inherent drawbacks [52, 53] but also may increase laboratory analysis costs considerably.

Although the use of immunoaffinity approaches is appealing, LC-MS or LC-MS-MS techniques usually require chemically based solid phase extraction as several structurally different analytes are analyzed simultaneously. For example, as early as in 2006, Garon and coworkers developed an HPCL-MS/$ESI^+$ approach to analyzing up to 11 mycotoxins (including $AFB_1$) in corn silage using an Oasis® HLB cartridges and eluting with a mixture of methyl *tert*-butyl ether/methanol (9:1) [54]. This matrix deserves particular attention since many ruminants' diets are—at least partially—based on silages and forages. Finally, solid phase extraction sorbents based on molecularly imprinted polymers (AFFINIMIP®) have also been developed and are commercially available for mycotoxin analysis. On the other hand, Pickering Laboratories mainly offer two different technologies to enhance aflatoxin sensitivity: i. the Pinnacle PCX derivatization instrument, which is used as a second pulse-free pump, and reaction system and can be coupled to an LC before the fluorescence detector (FLD). The system pumps (with

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a predetermined flow) a reagent, e.g. iodine/iodide or a pyridinium hydrobromide perbromide (PBPB) solution. And ii. a photochemical reactor equipped with a 254 nm lamp and a knitted reactor coil (UVE™) (which transforms aflatoxins into stable fluorescent hydroxylated counterparts, e.g. AFB1 is converted into $AFB_{2a}$). The latter approach was used by Soleimany and coworkers which developed a RP-HPLC multiple toxin analysis for cereals with the use of a photodiode array and fluorescence detectors and a photochemical reactor for enhanced detection [55]. The authors found the limits of detection for $AFB_1/AFG_1$ and $AFB_2/AFG_2$ to be 0.025 and 0.012 ng $g^{-1}$, respectively.

Likewise, only a few methods have been described elsewhere, e.g. Shakir Khayoon and coworkers detailed an assay for the determination of aflatoxins in animal feeds and ingredients by LC with multifunctional column clean-up [56]. Biotage® Isolute Multimode® Columns were used to assess aflatoxins successfully. These particular columns have three mechanisms of action: strong cation exchange ($R\text{-}SO_3^-\ H^+$), hydrophobic-based retention [$-(CH_2)_{17}CH_3$], and weak anion exchange [$(CH_2)_3N^+(CH_3)_3Cl^-$]. Based on structural analysis of aflatoxins, not all these mechanisms play a role during their extraction. The authors report great results, i.e. the sensitivity of 0.10 and 0.06 ng $g^{-1}$ for $AFG_1/AFG_2$ and $AFB_1/AFB_2$, respectively. Acetonitrile:water (9:1) mixture gave satisfactory recoveries for all aflatoxins (>85%).

### *2.2.3. Recent approaches for the extraction of aflatoxins from feeds*

Depending on the method and analytical instrumentation chosen for aflatoxin analysis, the extraction step can become a limiting stage of the overall assay. For example, liquid chromatography coupled with extensive treatment to obtain clean extracts before injection. MS-based approaches have an inherent advantage over classic ones. The detector can differentiate between two different mass/charge units even if chromatographic signals are overlapped. Hence, less intensive and straightforward sample preparation techniques, such as QuECheRS (quick, easy, cheap, effective, rugged and safe), dispersive liquid-liquid microextraction, or "dilute-and-shoot", are employed [57].

Dzuman and coworkers optimized a QuEChERS method for the determination of 56 *Fusarium*, *Alternaria*, *Penicillium*, *Aspergillus,* and *Claviceps* mycotoxins in animal feeds by UHPLC-MS/MS [58]. The authors demonstrated that the pH of extraction solvents was the most critical factor during the preparative step. Silages represent an attractive matrix because the organic acids produced by fermentation acidify and buffer any aqueous media, such conditions, if not considered, may interfere with solvent extraction, chemical or immunological sorbents interaction and may change injection micro-conditions affecting retention times. The same authors applied a dispersive SPE using $C_{18}$ sorbent to avoid coextraction of triacylglycerols and thus prolonging the life of the analytical column. León and coworkers also used QuEChERS to assess 77 banned veterinary drugs, mycotoxins, ergot alkaloids and plant toxins, and a post-target screening for 425 substances, including pesticides and environmental contaminants in feed [59]. Although not specifically in feed, Sirhan and coworkers developed and applied an QuEChERS-based method that included as samples, seeds ($n$ = 51), nuts ($n$ = 78), and several cereals ($n$ = 274/669 samples), that have been also used as feed ingredients (e.g. peanuts, sunflower, almond), and could very easily be applied to other matrices [60]. The authors

compared their method to a classic fluorimetric one and found the former to be superior in precision and less biased.

Dispersive liquid-liquid microextraction was applied by Campone and coworkers to the determination of aflatoxins in cereals such as corn, rice, and wheat [61]. Chloroform was selected as transfer solvent, whereas a methanol:water (8:2) was selected as an extraction mixture and a 2.5 enrichment factor was reported. Afzali and coworkers developed a method using dispersive liquid-liquid microextraction for the preconcentration of ultratrace amounts of $AFB_1$, $AFB_2$, $AFG_1$, and $AFG_2$; the authors validated several parameters as extraction solvent (chloroform), disperser solvent (acetonitrile), sample pH, and centrifugation time finally settling for a two-step approach [62]. Lai and coworkers used a microextraction method to concentrate 1.25 times aflatoxin $B_1$, $B_2$, and ochratoxin A with acetonitrile/water/acetic acid mixture as extraction solvent and chloroform as a disperser in rice samples [63]. Noteworthy, it is usual to these microextraction methods to be coupled with immunoaffinity column extraction as an additional step or to compare performance results among methods. Amirkhizi and coworkers used a dispersive liquid-liquid microextraction as a clean-up method before the quantitation of $AFB_1$ in eggs ($n = 150$) and chicken livers ($n = 50$) obtaining incidences of 72% and 58%, respectively [64]. A review by Spietelun and coworkers treat, in general, miniaturized analytical pretreatment options (e.g. single-drop microextraction, hollow fiber liquid-phase microextraction, dispersive liquid-liquid microextraction) with emphasis in green chemistry [65]. In fact, Zhao and coworkers used ionic liquid-based dispersive liquid-liquid microextraction specifically on feeds, obtaining enrichment factors from 22 to 25 for aflatoxins [66].

Two multi-mycotoxin methods, a dilute-and-shoot LC-MS/MS method and a method based on multi-toxin immunoaffinity columns before LC-MS/MS, were used for the determination of mycotoxins in corn samples, which included integral and moldy grains, harvested in South Africa [67]. Arroyo-Manzanares and coworkers used acetonitrile as an extraction solvent for a "dilute-and-shoot" method for the determination of AFs in animal feed in combination with matrix-matched calibration [68].

Although less complex sample clean ups are very attractive to offer a swift response on a relatively low budget, care must be taken as high matrix interference (when injecting crude extracts) represent a limitation, so some sample treatment methods are usually a requirement. New approaches are continually being developed such as the method selected by Ates and coworkers which injected extracts directly into an automated turbulent flow sample clean-up system, coupled to an LC-HRMS (high-resolution mass spectrometry [Orbitrap]) system to screen up to 600 fungal metabolites to generate feed contaminant profiles [69]. On the other hand, Fabregat-Cabello and coworkers used multi-level external calibration using isotopically labeled internal standards, multiple and single level standard addition, one point isotopic internal calibration and isotope pattern deconvolution to compensate sample extracts, such as those from a feed, that demonstrate powerful matrix effects [70].

On the other hand, Hu and coworkers simplified immunoaffinity column analysis reducing sample extraction and toxin purification to one step and using microbeads coupled with monoclonal antibodies against $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, zearalenone, ochratoxin A, STE,

and T-2 toxin [71]. Eighty feed samples were successfully tested using this tactic. Zhao and coworkers described a method for analyzing 30 different mycotoxins (e.g. aflatoxins, ochratoxin A, trichothecenes, zearalenone, fumonisins, and citrinin) in animal feed, animal tissue, and milk [72]. The authors compared three extraction mixtures, different SPE cartridges, including Oasis HLB®, an amino cartridge, Oasis MAX®, and MycoSep® 226 multifunctional cartridge, and sorbents, including $C_{18}$, chitin, carbon nanotubes, and florisil. The reader is referred to the review by Arroyo-Manzanares and coworkers who cite new techniques in sample preparation for mycotoxins [73].

## 3. Immunoaffinity-based techniques for aflatoxin detection

Other technologies have helped perform easier and faster toxin analysis. Though, they are limited as to the amount of information that can be drawn from a sample. Recently, the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) developed the first portable low-cost (up to 2 USD) device designed for rapid detection of aflatoxins. On the other hand, this technology seems to need little to no technical experience to use. This development means that feed producers and farmers may take decisions on location saving unnecessary exposure to toxins and limit economic loss. Another cost-effective approach is based on a lateral flow device (immunodipstick) to assess as little as 5 μg $kg^{-1}$ of $AFB_1$ in swine feed. We consider this type of approach to be considerably useful to assess aflatoxin cross-contamination in surfaces at feed manufactories, farms, or even dedicated laboratories. Lee and coworkers developed a semi-quantitative one dot lateral flow immunoassay for $AFB_1$ using a smartphone as a reading system with a sensitivity of 5 μg $kg^{-1}$ [74]. The authors applied this method to whole corn and feed with great results.

With the widespread use of immunochemical based techniques, the development of new toxin-specific monoclonal antibodies with a very high selectivity are in need. Zhang and coworkers reported a new $AFB_1$ monoclonal antibody (MAb) 3G1 obtained by immunizing Balb/c mice with aflatoxin $B_{2a}$-Bovine serum albumin [75]. The approach rendered a highly sensitive immunochromatographic assay, a detection limit of 1 ng $mL^{-1}$, showed no cross-reactivity with other aflatoxins and avoided providing false-positive results. The authors included during validation among other matrices, feedstuffs. Several conjugates and antibodies have been commercially developed for sample preparative purposes. Recently, ImmuneChem® has developed $AFB_1$, and $AFM_1$ bovine serum albumin and horseradish peroxidase immobilized antigens for anti-aflatoxin antibody assays. Rabbit and mouse antibodies-based sorbents are also available and can be utilized for detection and quantization of food-borne $AFB_1$. The standard application of these antibodies is in ELISA test. The usage of monoclonal ELISA test was introduced to research practice early on assessing aflatoxin concentrations in the feed. For example, Banerjee and Shetty applied this technique to poultry feed [76]. Recent approaches have incorporated improvements on ELISA tests. For example, Rossi et al. developed an indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) based on an anti-aflatoxin $B_1$ monoclonal antibody [77]. The authors reported that the method was validated for aflatoxin screening in poultry feed samples obtaining detection limits and recoveries of 1.25 ng $g^{-1}$ and 98% for broiler

feed and 1.41 ng $g^{-1}$ and 102% for laying hen, respectively, on both accounts. The method was also compared with HPLC results, and the authors found a high correlation with HPLC of 0.97 (broiler feed) and 0.98 (laying hen feed). Another research group developed an indirect competitive electrochemical ELISA for the determination of $AFB_1$ in barley. The method used disposable screen-printed carbon electrodes and anti-$AFB_1$ monoclonal antibodies (MAb) for immunosensor development. Cross-reactivity of $AFG_1$ was found, and the authors demonstrated that the coated electrodes could be used for up to 1 month after their preparation when stored at 4°C. The limit of detection was found to be 90 pg $mL^{-1}$, which translates to 0.36 μg $kg^{-1}$.

Gold colloid strip tests have also become available for some matrixes and are somewhat popular. For example, Ateko Masinde and coworkers developed a colloidal gold-based immunochromatographic strip which they applied to the analysis of corn and rice [78]. In our context, these matrixes are relevant since both are common feed ingredients. More recently, Sun and coworkers developed a green method using anti-$AFB_1$ antibody-coated gold colloids as probes in plant oils [79]. Noteworthy, the extraction is attained using water as a solvent. The authors reported a successful visual detection under 5 min with a sensitivity of 1.5 mg $kg^{-1}$. The methods above are interesting since no professional training needs to be involved in applying them efficiently and can be used in the field.

## 4. Chromatography coupled with fluorescence detection-based methods for aflatoxin derivatization

AOAC HPLC-based assays are for aflatoxins are scarce. The one method available is 2003.02, which is designed for the determination of $AFB_1$ in cattle feed. Although it can easily be used to quantitate each AF fraction and other feeds. This last method uses post-column derivatization (a standard approach for AF HPLC-based methods to enhance sensitivity; [80]) using a R-biopharm's KOBRA® CELL, which principle is based on the electrochemical *in situ* $Br_2$ formation (from potassium bromide) and hence the formation of fluorescent AF derivatives. Similar methods have been reported earlier in the literature [81]. An additional approach is the use of PBPB as another derivatizing agent. Manetta and coworkers already used this method to quantitate a chemically related compound, $AFM_1$, in milk and cheese [82]. Interestingly, Woodman and Zweigenbaum compared the use of PBPB with the derivatization obtained with a KOBRA® CELL and reported better results using the former [83]. Remarkably, Ramirez-Galicia and coworkers described that $AFB_1$ suffered fluorescence enhancement when forming $AFB_1$:β-cyclodextrin inclusion complexes [84]. Hence, β-cyclodextrin could very well be a novel reagent for derivatization.

In our laboratory, we have implemented an accredited assay (according to ISO/IEC 17025 requirements) based on the derivatization of AF using an aqueous $I^-/I_2$ solution with excellent results ([85], see **Figure 2**). We base our method on the fact that iodine/iodide is less oxidizing than other agents and easier to manipulate. The drawback of this approach is that high temperatures (95°C) must be used to obtain AF derivates swiftly (using a 0.14 mL reaction loop); this is not the case for bromine.

Noteworthy, at 365 and 455 nm as excitation and emission wavelengths, respectively, $AFB_2$ and $AFG_2$ show natural fluorescence when no derivatization is used, while the signal for

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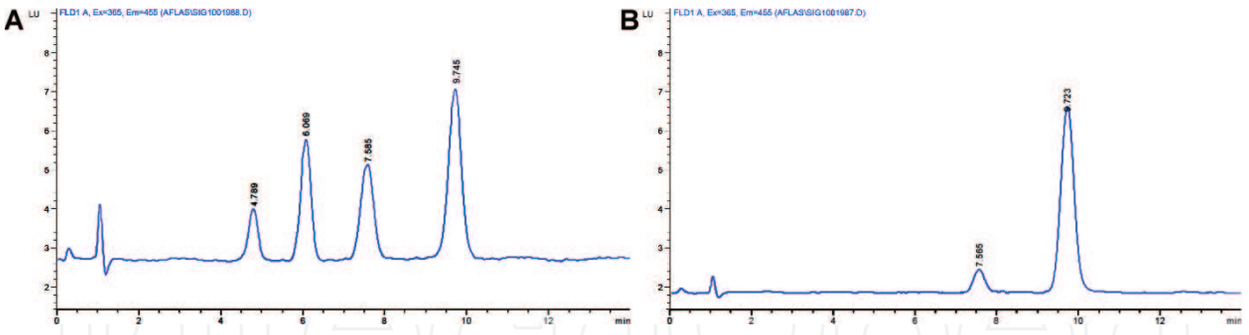



**Figure 2.** RP-HPLC analysis of aflatoxins using iodine/iodide-based derivatization. (A) 40 μg $L^{-1}$ standard in methanol and 10 μL injection AF fractions in order of elution: $AFG_2$(4.789), $AFG_1$(6.069), $AFB_2$(7.585), and $AFB_1$(9.745). (B) Same method used to analyze a naturally contaminated corn sample with $AFB_2$(7.565) and $AFB_1$(9.723).

the other two fractions is negligible. Both iodine and bromine generate fluorescent derivatives using an addition reaction, which introduces a halogen atom on the double bond of the dihydrofuran ring. The steric hindrance and electronic repulsion conferred by the halogens, which are spatially opposite to each other, render an aflatoxin molecule with far more torsion [−0.2812 ($AFB_1$) vs. 2.9320 ($AFB_1I_2$), using an MM2 energy minimization]. Hence, favoring a more rigid structure, a fluorescence prone one (**Figure 3**).

Machado Trombete and coworkers validated a fluorescence-based method and compared three methods of extraction; the authors found chloroform to be the most efficient solvent [86]. Pre-column derivatization with trifluoroacetic acid was used to increase sensitivity (reaching 0.6 μg $kg^{-1}$ as the limit of detection). Although the authors intended this method for wheat projected for human consumption, this matrix is a widely used as a feed ingredient in some countries. Horizon technologies introduced to the market a thermostatically controlled heated water chamber that facilitates the pre-column derivatization of aflatoxins with trifluoroacetic acid (XcelVap®). Cortés and coworkers also used a fluorescence-based method without derivatization using 360 nm as an excitation wavelength and 418–700 nm as excitation [87]. The method included aflatoxicol (a reduced derivate from AFB1; cyclopentanone moiety is converted to cyclopentanol) and also assessed the recovery of aflatoxins and aflatoxicol in

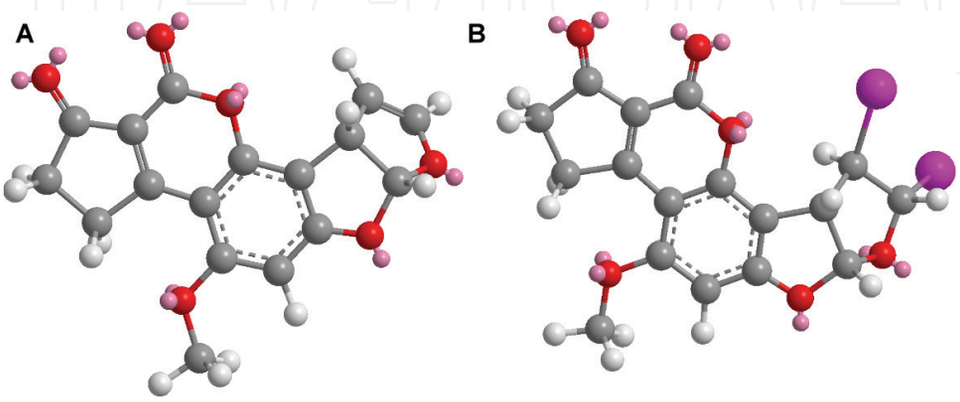



**Figure 3.** 3D structure minimized energy using MM2 calculations of (A) aflatoxin $B_1$ (total energy of 48.2584 kcal $mol^{-1}$) and (B) $AFB_1$ after iodine addition (total energy of 53.8536 kcal $mol^{-1}$); pink-colored beads represent non-bonding electron pairs.

poultry litter finding non-trivial levels of these contaminants. A relevant feature since poultry litter (urea, uric acid, and ammonium-rich by-products) has served on occasion as livestock feed. Interestingly, in a Waters Corporation application note, Benvenuti and coworkers used a fluorescence detector-based approach to quantitate aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, and $M_1$ without derivatization [88]. The authors used 365 nm as excitation wavelength and emission wavelengths 429 and 455 nm for $AFM_1/AFB_2/AFB_1$ and $AFG_1/AFG_2$, respectively. Pickering Laboratories developed a fluorometric method using photochemical derivatization and $\lambda_{ex}$ = 365 nm and $\lambda_{em}$ = 455 nm for the detection of aflatoxins in dried distillers grains (DDGs). This process was conceived as a multiple toxin analysis using just a fluorescence detector.

Pirestani and coworkers measured aflatoxins both in dairy cattle feeds and milk samples from the province of Esfahan, Iran [89]. The authors compared results from HPLC (bromine post-column derivatization) and ELISA. It was concluded that there was no significant difference between the values obtained by the two procedures. However, sensitivity and specificity were determined to be superior to that of ELISA. Gomes Pereira and coworkers also did analyze dairy cattle feed and milk from the Lavras, Minas Gerais region of Brazil [90]. In the case of cattle feed samples, the authors state they used an AOAC method but failed to specified which.

## 5. Mass spectrometry coupled chromatography multiple toxin approaches (including aflatoxins)

With the advent of confirmatory and multi-analyte techniques such as tandem MS (mass spectrometry) coupled to LC, a whole new span of methods has been described which include the "classic" toxins and other not-so-known ones into feed vigilance schemes. In 2012, Warth and coworkers described a multiple-toxin method based on LC-MS/MS that included several metabolites, a total of 63 analytes were tested in corn, groundnut, sorghum, and feed produced in Burkina Faso and Mozambique [91]. De Souza and coworkers reported a LC-MS/MS using $ESI^+$ with a QTrap 4000 system, which was used to analyze 119 samples collected from poultry feed factory [92]. The researchers analyzed $n$ = 74 whole corn samples, $n$ = 36 chicken feed, and $n$ = 9 feed mill residue. Limits of detection ranged from 0.5 ($AFG_1$) to 1 ($AFG_2$) μg $kg^{-1}$, and recoveries ranged from 71 to 87% for corn and 65 ($AFB_2$) to 77% ($AFB_1$) for feed. This analysis is relevant since most feed formulations recurred to corn products to supply energy and carbohydrates. Contamination in feed ingredients will concurrently have an adverse impact on feed safety.

Recently, Njumbe and coworkers reported a LC-MS/MS method that included 23 mycotoxins in different sorghum varieties, all analytes eluted under 14 minutes and stated a high sensitivity for all mycotoxins, specifically 2.5 and 5.0 μg $kg^{-1}$ for $AFB_1/AFB_2$ and $AFG_1/AFG_2$, respectively [93]. Although sorghum, in some regions, has been substituted by other grains such as corn, it has seen a resurgence as a crop for feed in several parts, which is relevant since some grain production is not continuous throughout the year, and feed ingredient supplies are in

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high demand, and their availability is constrained. More interestingly, when the method was applied to a small subset of retailed samples from Belgium and Germany, 90% were positive for aflatoxin $B_1$. Regarding food and feed monitoring, a very comprehensive review was written by Zhang and coworkers [94]. This report is unique since it cites the techniques used by the US FDA to assess mycotoxins in different staple foods including LC-FLD (fluorescence detector), MS, tandem MS, and HRMS. For example, a LC-MS/MS method including 11 different mycotoxins (e.g. aflatoxins), using stable isotope dilution, has been developed and validated in various matrixes (including cat/dog food, corn, feeds, and wheat flour). Samples were fortified using $^{13}$C-IS and prepared by solvent extraction. In general, the recoveries ranged from 70 to 120%, with RSDs < 20%. Limit of quantitation was calculated to be 0.005 μg $kg^{-1}$ for $AFB_1$. The method above applied in our laboratory based on LC-FLD with post-column derivatization renders a similar acuteness for $AFB_1$ (limit of detection and quantification 0.005 and 0.15 μg $kg^{-1}$, respectively) nonetheless our method had to be modified to include a ca. 200-fold concentration step. Lattanzio and coworkers used a similar approach to analyze cereal-based foods using as a clean-up strategy SPE [95]. Zhang and coworkers opted for a LC/MS/MS approach to analyze mycotoxins in feed using isotope dilution and circumventing the clean-up step altogether [96]. For a thorough review of chromatographic and spectrometric techniques used for mycotoxin analysis, we suggest the paper wrote by Li and coworkers [97]. Ok and coworkers recently opted to include aflatoxins and sterigmatocystin in the same analysis using tandem MS for their assay in sorghum and rice [98].

DDGs is an essential matrix since the shortage and costs of other corn-based feed ingredients have pushed toward their extensive use [99, 100]. As this is a residue from ethanol production, any mycotoxins initially found in the raw material may be concentrated. On the other hand, Oplatowska-Stachowiak and coworkers developed a UPLC/MS/MS method capable of analyzing as much as 77 mycotoxins and other fungal metabolites [101]. The method analyzed 169 DDGs samples produced from wheat, corn, barley and other grains. Aflatoxin contamination was frequently encountered in corn DDGs. In contrast, wheat and mixed DDGs showed none or very few contaminated samples. In a very exhaustive analysis of European feedingstuffs, Zachariasova and coworkers used a UHPLC–QtrapMS/MS. The authors found that forages showed the lowest mycotoxin incidence while the most diversity of detected mycotoxins. In contrast, the highest concentrations, was quantified in DDGs [99]. For example, $AFB_1$ was found with a mean value and a maximum of 0.6 and 6.4 μg $kg^{-1}$, respectively.

Another important feed ingredient is palm kernel cake, which is used as a source of protein and energy for livestock and occasionally used as poultry feed supplement. Yibadatihan and coworkers developed a LC/MS/MS $ESI^+$ to analyze several toxins in palm kernel cake, including aflatoxins [102]. Recoveries ranged from 84 to 110, and the method sensitivity was calculated as 0.16 and 0.54 for $AFB_2$/$AFG_2$ and $AFB_1$/$AFG_1$, respectively. Twenty-five samples were analyzed using this approach, and a very high prevalence for aflatoxins (>85% samples tested positive for any of the fractions) was found. The lowest and highest concentrations found were 1.31 (for $AFG_1$) and 78.38 (for $AFG_2$) μg $kg^{-1}$. As with DDGs, any toxin found in palm kernel raw material will probably be concentrated as the palm kernel is mechanically pressed to extract vegetable oil.

Finally, in a provoking research, Escrivá and coworkers assessed mycotoxin (including aflatoxins) contamination of rat feed [100]. Twenty-seven commercial Spanish rat feed was analyzed using a liquid chromatography equipped with a 3200 QTrapVR mass spectrometry system with a Turbo electrospray ionization interface. Considerable mycotoxin burden was found in feeds. For example, concentrations of $AFB_2$ (21.61 μg/kg bw/day), and $AFG_2$ (15.09 μg/kg bw/day) were calculated for the assayed feeds. Since laboratory animals are used as models in other research, these contaminants' toxic effects may cause artifacts and confounding results. The authors detailed the feed composition listing each ingredient use during formulation, data that is usually overlooked during contaminant analysis. Feed composition plays a significant role in toxin pollution as the main ingredients may guide which contaminants will be more likely to be present [103]. McElhinney and coworkers developed a method for the determination of mycotoxins in grass silage [104]. In this case, they used a modified QuEChERS approach with almost no clean-up and an UHPLC/MS/MS technique. Polarity switch during the analysis permitted to assess both positive and negative ions. $AFB_1$ detection limit was calculated to be 3 μg $kg^{-1}$ DM. This relatively low sensitivity is usually the cost of a swift sample preparation and avoiding thorough clean-up steps. A similar approach was used by Dzuman and coworkers for the analysis of cereals, complex compound feeds, extracted oil cakes, fermented silages, malt sprouts, or DDGs using U-HPLC-HRMS [105].

## 6. Novel approaches for aflatoxin determination in feed

An interesting earlier report made by Babu and Muriana stated that AF recovery was enabled by the use of primary polyclonal antibodies for $AFB_1$ [106]. Said antibodies, were covalently attached to 2.8 μm diameter magnetic beads using a cross-linking agent and a secondary antibody for the toxin covalently linked to DNA oligonucleotides based on the *luc* gene as a reporter DNA molecule which, in turn, was amplified using real-time immune quantitative polymerase chain reaction after aflatoxin capture if present. The authors prepared toxin suspensions in methanol:water solution. This mixture can also serve as an extraction solvent; the sensitivity of this method was calculated to be 0.1 μg $kg^{-1}$. The same authors [107], later on, applied a modified version of this approach and applied it to poultry, dairy and horse feed, and a whole kernel corn, corn gluten feed, and yellow corn meal. The authors conclude that the technique is useful for quantifying low natural aflatoxin levels in animal feed samples without the requirement of additional sample cleanup. However, samples artificially contaminated with high levels of aflatoxin (i.e. 200 μg $kg^{-1}$) exhibited recoveries of 60% which are considered poor.

Another novel approach for the extraction, preconcentration, and determination of aflatoxins in animal feedstuffs was carried on recently by Zhao and coworkers who developed a novel two-step extraction technique combining ionic liquid-based dispersive liquid-liquid microextraction with magnetic solid-phase before HPLC coupled with FLD [66]. The ionic liquid 1-octyl-3-methylimidazolium hexafluorophosphate was used as the toxin-retrieval agent, and hydrophobic pelargonic acid modified $Fe_3O_4$ magnetic nanoparticles as an active sorbent.

Ramesh and coworkers used a high-performance thin-layer chromatography method that uses a stationary phase based on silica gel 60G F254 and a mobile phase that consisted of acetone:chloroform (1: 9), $n$ = 59 samples of feed were analyzed by this method

that reported 0.5 μg $kg^{-1}$ as the limit of detection [108]. A similar approach was used by Kotinagu and coworkers to assess $AFB_1$ in a total of 97 livestock feed samples. In this case, a chloroform:acetone:water mixture (28:4:0.06) was used to elute [109]. The toxin was revealed using a 366 nm wavelength. Finally, Zhang and coworkers developed an on-site analysis for aflatoxin $B_1$ in food and feed samples using a chromatographic time-resolved fluoroimmunoassay that offered a magnified positive signal and low signal-to-noise ratio [110]. Wang and coworkers developed a fluorescence based on europium nanospheres and monoclonal antibodies for the determination of total aflatoxin in the feed with a 0.16 μg $kg^{-1}$ limit of detection [111]. Interestingly, the authors designed this method to use with a portable reader. A good association was found among the assay and HPLC results for corn, wheat bran, peanut meal, soybean meal, cottonseed meal, DDGs, alfalfa forage, silage, swine feed, and poultry feed.

Ren and coworkers used an immunochromatographic assay based on CdSe/Zn quantum dot beads, reaching values as low as 0.42 pg $mL^{-1}$ $AFB_1$ [112]. Quantum dots were prepared using poly(methyl methacrylate), poly(maleicanhydride-*alt*-1-octadecene), and *N*-(3-(dimethylamino)propyl)-*N*′-ethylcarbodiimide hydrochloride. The method results were compared with ELISA and LC-MS/MS, exhibiting great association with both. Feed ingredients that included corn, soybean meal, rapeseed meal, cottonseed meal, distillers dried grain, and wheat, were tested. Setlem and coworkers generated high-affinity single-stranded DNA aptamers that specifically bind to $AFB_1$ by a modified Systemic Evolution of Ligands by Exponential Enrichment procedure [113]. The two aptamers with lower Gibbs energy threw 20–40 ng $mL^{-1}$ sensitivity. Coupled with HPLC, the aptamers were able to recover and quantify 82.2–96.21%. He and coworkers constructed $AFB_1$-BSA conjugated "nanobody" from immunized alpacas [114]. The most interesting part about the outcome is that the authors describe the resulting nanobody to be thermally and organic solvent resistant. Recovery from spiked peanut, rice, corn, and feedstuff ranged from 80 to 115%. Xiong and coworkers reported an improved magnetic bead-based immunoaffinity extraction method, for the highly efficient purification of $AFB_1$ from corn samples, that circumvents common inherent disadvantages of this approach [115]. The method involves the expression of anti-$AFB_1$ nanobodies, with degeneration resistance, to replace conventional antibodies. Magnetic beads, carrying poly(acrylic acid) "brushes", expand significantly adsorption capacity (i.e. 623 μg $g^{-1}$) and reusability (10x without obvious loss of the capture efficiency for $AFB_1$). The reliability of the proposed method for $AFB_1$ extraction was further evaluated using $AFB_1$-spiked corn samples.

Finally, new efforts to quantitate and detect mycotoxins should include emerging analytes, such as other *Aspergillus* metabolites, such as STE and emodin, neither analyte routinely screened for in feed nor regulated by legislation [116].

## 7. Bearing of aflatoxigenic molds isolation from feed

Isolation and identification of fungi, especially those with aflatoxigenic capabilities, is an analytical feature, during aflatoxin determination, which is seldom considered. These data may easily be contrasted with concentrations obtained by any of the analysis methods aforementioned. However, a few papers have indeed tackled the issue. Suganthi and coworkers

used both ELISA (based on urea peroxide and the chromogen tetramethylbenzidine) and thin-layer chromatography (using a mixture of chloroform and acetone) to detect aflatoxins in animal feed and also isolated molds using Czapek Dox Agar medium [117]. In this case, the authors concluded that 80% *Aspergillus* strains were *A. flavus* and none of the strains were toxigenic. Finally, they also showed an antifungal effect of *Lactobacillus* species. Chandra and coworkers assessed $AFB_1$ in corn from Indian markets using competitive ELISA [118]. The authors also determined mold count that ranged from $1.0 \times 10^2$ to $3.6 \times 10^6$ CFU/g. Relevant data since isolation of aflatoxigenic fungi from contaminated samples are seldom done. Although no association was found between microbial analysis and contamination, other more selective media can be used to isolate more specific *Aspergillus* species (e.g. AFPA Base Oxoid™), this may help to determine which species are responsible for the contamination. For example, Queiroz and coworkers used ELISA to assess aflatoxin contamination in three quality type of bird feeds from Brazil. Furthermore, they used Dichloran Rose Bengal Chloramphenicol agar, as a general medium used to estimate mold counts and dichloran glycerol 18% agar, a low $a_w$ medium that facilitates the growth of xerophilic fungi [119]. The authors found that *Aspergillus* (82%), *Cladosporium* (50%), and *Penicillium* (42%) were the predominantly isolated genera. *Aspergillus niger* aggregate (35%), *Aspergillus fumigatus* (28%) and *A. flavus* (18%) had the highest relative densities. Finally, aflatoxins have rarely been detected in feeds and foods in Hungary. However, Sebök and coworkers analyzed several corn fields alongside Hungary between the years 2013 and 2014 and found the presence of aflatoxigenic fungi in corn and soil samples with isolation ratios of 26.9 to 16.1% and 42.3 to 34.7%, respectively, on both accounts [120]. The authors evaluated on the isolates the presence of partial calmodulin gene demonstrating the identity of the strains to be *A. flavus* ($n$ = 110/114) and *A. parasitcus* ($n$ = 4/114). Based on the strain genotoxic response, 45.5% of the 110 *A. flavus* strains were toxin producers. Carvalho and coworkers not only found a prevalence of 77.7% for aflatoxin in tropical corn silages (Minas Gerais, Brazil) but also identified *A. fumigatus* in all silages that presented growth of molds [121]. Yeast species including those from the *Candida* genera were isolated. As an additional example, Kaya-Celiker, in an interesting paper, successfully used Fourier transform mid-infrared and photoacoustic spectroscopy to identify and separate infected peanuts based on spectral characteristics [122]. Ibrahim and coworkers recently screened 102 feed samples (including poultry feed, cotton seed meal, and corn) for the presence of aflatoxin biosynthetic pathway genes *ver*-1, *apa*-2, and *omt*-1 using PCR assay, and thin-layer chromatography was performed to confirm the synthesis of aflatoxin in PCR-positive strains [123]. Nine samples exhibited the simultaneous presence of the three genes and all were capable of producing $AFB_1$ and $AFB_2$.

## 8. Conclusions and perspectives

As stated before, research based on animal feeds is somewhat lacking and usually, the importance of this matrix within the food chain sometimes omitted. Although this chapter is devoted to aflatoxins and regulatory standards, till this day, target only specific toxins, evidence suggests that other mycotoxins, contaminants, residues, and xenobiotics interact

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with aflatoxins sometimes even enhancing their carcinogenic potential. We consider that aflatoxin monitoring programs should be implemented and reinforced to minimize the impact of aflatoxins on animals and humans. On the other hand, policy makers and officials should concentrate efforts and prioritize the incorporation of country-wise feed monitoring systems where currently there are none. Based on the data recollected here-in, sampling and surveying should focus especially on corn and corn products and pet food and full ruminant rations including balanced feed. From the zootechnical standpoint, evidence indicates that general nutritional formulations can be modified to minimize fungal and toxin contamination and hence animal health impacts and still cover the traditional nutritional needs. On the other hand, farmers may equivocally attribute productivity loss to toxin presence where none is found. As occasionally farm feed practices are based on the exploitation of residue from other agricultural activities (e.g. fruit processing wastes, poultry litter, alcohol product by-products [DDGs]), strict control of this type of samples should be kept. Aflatoxin toxicity occurs at very low concentrations. Therefore, sensitive and reliable methods for their detection are required. Sampling and analysis of aflatoxins are paramount. Failure to achieve a verifiable analysis can lead to erroneous conclusions or judgments; contaminated feedlots being accepted or satisfactory batches unnecessarily rejected. Thanks to technological advancements method for aflatoxin detection are continuously improving in sensitivity, repeatability, accuracy, efficiency, and with less and less waste. Data herein demonstrate that even in countries where expensive technology (e.g. LC-MS/MS) is scarce or not readily available, feed monitoring is possible. Efforts have been made to provide proficiency testing for laboratories (e.g. American Association of Feed Control Officials [AAFCO] and Laboratory of Government Chemist [LGC Standards]), which improve method accuracy bias and reliability. However, feed-based certified materials available are still few. Finally, considering the relevance of feed in the food chain safety, countries should implement and improve monitoring programs for aflatoxin in foodstuffs; these programs should contemplate risk management, One Health or "MyToolBox" approaches, and farm-to-fork models that include all stakeholders to mitigate the economic and health burden that aflatoxin contamination generates.

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