Chapter

Determination of Aflatoxins by Liquid Chromatography Coupled to High-Resolution Mass Spectrometry

Natalia Arroyo-Manzanares, Natalia Campillo, Ignacio López-García and Pilar Viñas

Abstract

The most common mycotoxins are aflatoxins (AFs), which are produced by strains of various species of molds in the genus Aspergillus (A. flavus, A. parasiticus, A. nomius and A. tamarii) and can grow on many foods, mainly peanuts, maize and cottonseed. AFs are currently considered to be the most hazardous mycotoxins to health, in particular because of their hepatocellular carcinogenic potential. The main aflatoxins are B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2) although many other derivatives have been described. In addition, animals consuming contaminated feeds are able to metabolize them by hydroxylation in a certain position, yield for example aflatoxin M1 (AFM1) and aflatoxin M2 (AFM2) from AFB1 and AFB2, respectively. Nowadays, only the four main AFs and one hydroxylated metabolite (AFM1) are routinely analyzed. High resolution mass spectrometry (HRMS) using Orbitrap or time-of-flight (TOF) mass analysers is a trend for AFs determination, allowing to determine AFs and their derivatives for which there are no commercial standards available, in order to carry out metabolism studies, exposure assessment or monitoring modified AFs in food. The aim of this study is to show the recent trends in analytical methods based on LC-HRMS for determination of AFs.

Keywords: aflatoxins, high resolution mass spectrometry, liquid chromatography, biological samples, foods

1. Introduction

Aflatoxins (AFs) are highly toxic secondary metabolites produced by fungi belonging to several Aspergillus species, mainly found in hot and humid climates [1]. Currently, more than 15 different types of AFs have been identified; the naturally occurring are aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1) and G2 (AFG2). Aspergillus flavus colonizes mostly the aerial parts of plants (leaves, flowers) producing B AFs, while Aspergillus parasiticus produces B and G AFs, being more adapted to soil environments [2]. The structures of the main AFs and their derivatives are shown in Figure 1.
Raw materials usually used for human food and animal feed are contaminated by this type of fungi and their metabolites. Cereals (maize, wheat, rice, barley, soy, etc.), dried fruits, nuts, coffee and other foods could be contaminated during plant growth or postharvest, depending on different factors such as temperature, humidity, water activity, concurrent mycobiota, physical damage, and other storage conditions [2]. AFs are very stable and may resist cooking processes, resulting in a problem in processed foods. Human exposure to AFs can result directly from ingestion of contaminated foods, or indirectly from consumption of animal foods previously exposed to contaminated feeds. AFs have a great risk for human health, especially by their carcinogenic potential [3]. Degradation or enzymatic transformation of mycotoxins led to the appearance of modified mycotoxins, usually lesser toxic than the parent compounds. Thus, aflatoxin M1 (AFM1) is formed from the hydroxylation of AFB1 and eliminated in the milk of animals that consumed feed contaminated with this mycotoxin [4].

Therefore, it is important to develop reliable methods for the determination of AFs and their derivatives in foods and feeds, as well as toxicokinetic and toxicodynamic
studies for assessment of human or animal exposure. The target and non-target quantitative and quantitative analysis using high resolution mass spectrometry (HRMS) instruments, such as time-of-flight (TOF) and Orbitrap, brings great challenges for screening of AFs [5]. Main advantages include high sensitivity, accurate mass measurement, and retrospective data analysis, allowing both the target determination of AFs and the non-targeted screening of modified AFs or unknown metabolites.

2. Toxicity of aflatoxins

AFs are potent carcinogenic, mutagenic, teratogenic, and immunosuppressive agents. Their carcinogenicity has mainly been associated with liver and kidney, although the effect of AFs has also been reported in pancreas, bladder, bone, viscera or central nervous by some epidemiological and animal studies [6]. Their inhalation and direct contact could also cause lung and skin [7, 8] occupational cancers, respectively. In addition, feeds contaminated by AFs can involve high susceptibility to diseases, low productivity and low reproductive performance in animals [9].

Among AFs, AFB1 is considered the highest risk. The Scientific Committee on Food has established that AFs are genotoxic carcinogens [10, 11], being the order of toxicity as follows: AFB1 > AFG1 > AFB2 > AFG2. Indeed, AFB1 has been shown to be carcinogenic in all experimental animals and has been classified since 1988 by the World Health Organization (WHO) as a human carcinogen. Consequently, the International Agency for Research on Cancer (IARC) [12] has classified AFB1 within the category of Group 1 substances based on the existence of sufficient evidence about its carcinogenicity to humans, both alone and in natural mixtures with the other AFs [13, 14].

The most common route of entry of AFs into the human body is the ingestion. In the case of AFB1, the best studied aflatoxin, is absorbed in the gastrointestinal tract, due to its liposolubility, and transported by red blood cells and plasma proteins to the liver. In the liver, it is metabolized producing intermediate metabolites that have been related with the toxic and carcinogenic effects of AFs [15]. Specifically, AFB1 is biotransformed in the liver by microsomal enzymes of the cytochrome superfamily P450. Microsomal biotransformation can result in the hydrolyzation of aflatoxin B1, producing less toxic metabolites such as AFM1, aflatoxin Q1 (AFQ1), aflatoxin P1 (AFP1) and aflatoxin B2a (AFB2a). In addition, AFB1 can produce aflatoxicol (AFL) via NADPH reductase. The formation of these compounds is considered a detoxification process although the protein binding of some of them can lead to additional toxicities [16]. They are excreted in urine and feces, although AFM1 is also commonly detected in breast milk.

The action of CYP450 enzymes can also metabolize AFB1 resulting in the appearance of a reactive intermediate metabolite, AFB1–8,9-epoxide (AFBO), which has two isomers (endo-8,9-epoxide and exo-8,9-epoxide). AFBO can be detoxified by glutathione S-transferase (GST) action, yield the adduct AFB1-glutathione (AFB1-GSH), that is eventually excreted as AFB1-mercapturic acid in the urine [17]. The formation of AFB-mercapturic acid is catalyzed by γ-glutamyltransferase (GGT), dipeptidase (DPEP), and N-acetyltransferase (NAT) [16]. However, due to its high electrophilic nature, AFBO can form covalent bonds with diverse macromolecules, such as nucleic acids and proteins. The protein binding is responsible for AFB1 toxicity, giving rise to adduct AFB1-lysine (AFB1-lys) with serum albumin. AFBO can also undergo rapid non-enzymatic hydrolysis to AFB1–8,9-dihydrodiol, which is in equilibrium with AFB1-dialdehyde. AFB1-dialdehyde can protein bind or be detoxified by the action of AF aldehyde reductase (AFAR) via conversion to the dialcohol [18].
The interaction AFB1-DNA causes AFB1-N7-guanine adduct, which is chemically unstable and undergoes rapid urinary excretion resulting in an apurinic (AP) site on the DNA backbone [16]. Alternatively, the adduct AFB-N7-guanine may be stabilized by rearranging to a ring-opened formamidopyrimidine structure (AFB1-FAPy) [17]. Both AP and AFB1-FAPy can produce mutagenesis. Figure 2 summarizes the action mechanism of AFB1.

From the action mechanism of AFB1 it can be deduced that AFB1-lys, AFB1-N7-guanine, AFB1-mercapturic acid or the hydroxylated forms (AFM1, AFQ1, AFP1, AFL and AFB2a) could be effective biomarkers for assessing AF exposure.

Due to the high lesions produced by AFs, especially cancer, the European Union has established maximum permitted levels of these contaminants in various foods through Regulation No. 1881/2006 [19]. Specifically, the maximum contents for AFB1, AFB2, AFG1, AFG2 and AFM1 in nuts, cereals, milk and baby foods are included in this regulation the maximum contents are between 4 and 15 μg kg⁻¹.

In the field of animal nutrition, the specifications regarding the presence of mycotoxins in feed are reflected in Directive 2002/32/EC [20]. Only AFB1 has been legislated. The maximum levels ranged between 5 and 50 μg kg⁻¹. The lower limit was set for feed intended for milk-producing animals (5 μg kg⁻¹).

3. Applications of HRMS for determination of aflatoxins

3.1 HRMS: a useful tool for aflatoxins determination

Liquid chromatography (LC)-HRMS is a powerful tool for metabolomic approaches, allowing simultaneous quantitative and qualitative analysis of a wide variety of mycotoxins, as well as the search of related metabolites derived from mycotoxin biotransformation or degradation, enabling the detection and identification of unknown compounds. In addition, HRMS offers the ability to work in
various modes, such as target analysis and non-target screening, or retrospective analysis. The relative incompatibility of HRMS ion sources with the continuous liquid flow of LC limited the progress of LC-HRMS coupling for years, but the development of interfaces such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), where LC effluent is de-solvated, has allowed the proposal of a high number of LC-HRMS methods. Thus, complex mixtures can be separated in the chromatographic system and their components are unequivocally detected by HRMS with high sensitivity.

For AFs determination, quadrupole (Q)-TOF, Orbitrap and its hybrid Q-Orbitrap are the mass analysers most widely used. Comparing both instruments, Orbitrap shows better resolution and accuracy (Q-TOF: 60,000 full width at half maximum (FWHM) and between 1 and 10 ppm; Orbitrap: 240,000 FWHM and less than 1 ppm), and a greater range of m/z (Q-TOF: <4000 and Orbitrap: <6000), although this is not important for the aflatoxin determination, since the expected masses are around 300–350 Da and both mass spectrometers cover this range. However, Q-TOF instruments have shown to have a greater linear dynamic range (Q-TOF: >10^5 and Orbitrap: >10^4) [21]. Multiclass mycotoxin (including the main AFs) methods based on the hybrid ion trap (IT)-Orbitrap have also been developed and validated [22, 23]. In addition, AFB1 has also been monitored by TOF [24, 25].

ESI or its variant heated ESI (HESI) working in positive mode are the best options for AF determination. Although it should be noted that many of the methods described in this chapter are multiclass methods, i.e., they determine a greater number of mycotoxins, not just AFs, and in this case, authors usually prefer two independent runs using both positive and negative polarities.

Regarding LC instruments, ultra-high-performance LC (UHPLC) is normally coupled to HRMS. Although NanoLC coupled to Q-Orbitrap for the determination of AFB1-lys in human plasma [26] and high performance LC (HPLC)-TOF for the determination of AFB1 in beer [24] have also been proposed. In addition, Qi et al. used a multiple heart-cutting two-dimensional liquid chromatography (Heart-cutting 2D-LC) coupled to Q-Orbitrap for simultaneous determination of AFs and ochratoxin A in snus [27]. The utilization of Heart-cutting 2D-LC enables to reduce matrix effect, leading to better precision of the AF contents. The mobile phase is normally a mixture of water and methanol (MeOH) or acetonitrile (ACN). Formic acid (FA), acetic acid (AA), ammonium formate or ammonium acetate are used as additives. The stationary phase was mainly C_{18} although C_{8} has been also proposed [28]. Slobodchikova et al. [22] also used a pentafluorophenyl (PFP) column whereas Qi et al. [27] combined both C_{18} and PFP columns in the Heart-cutting 2D-LC system.

The analysis of biological samples focused on the monitoring of the four most important AFs (AFB1, AFB2, AFG1 and AFG2), although their adducts due to the interaction of AFB1 with proteins or DNA, AFB1-N7-guanine and AFB1-lys, respectively, as well as its hydrolysed derivative AFM1 have also been determined. In food samples, besides the four main AFs, some metabolites such as AFM1, AFM2, or AFL were also detected.

Both data dependent (dd-MS^2) and data independent (DIA) acquisition have been proposed. For the analysis of biological samples, Full MS combined with dd-MS^2 by inclusion of a list of accurate masses of target or suspect compounds was more frequent. Although, Ogawa et al. [29] also proposed a dd-MS^2 by fixing an ion intensity threshold. Regarding food analysis, authors normally prefer Full MS and DIA, specifically, all-ion fragmentation (AIF) mode, where no precursor ion isolation is carried out. Other modes such as simple Full MS, selected ion monitoring (SIM) or parallel reaction monitoring mode (PRM) have also been investigated. Renaud et al. [30] compared three acquisition modes: dd-MS^2 with inclusion list,
Aflatoxins and AIF using targeted high energy collision dissociation (HCD) events across a mass range for MS/MS. Good linearity was achieved by AIF with different HCD events at low concentrations, demonstrating that the limits of detection (LODs) are much higher without a Q mass filtering. The potential of AIF with different HCD events has also been studied for the determination of five AFs in nutraceutical obtained from green tea [31].

Most authors who carried out a non-targeted acquisition opted for target processing, in order to quantify and/or confirm AFs. Only Jia et al. [31] carried out a non-targeted processing consisting of: non-target fourier peak picking, (ii) spectra automated componentization, (iii) suspicion spectral library searching, and (iv) marked fragments filtering. Finally, compounds were confirmed with reference standard. In addition, Castaldo et al. [32] and Renaud et al. [30] carried out a non-targeted processing using spectral library for the tentative identification of other fungal metabolites, although they processed AFs following a targeted approach.

Table 1 summarizes the separation and detection condition of HRMS methods for AF monitorization.

| AF studied | Matrix | Instrument | LC conditions | Acquisition | Ref. |
|------------|--------|------------|---------------|-------------|------|
| AFB1, AFB2, AFG1, AFG2, AFM1 | Breast milk | UHPLC-Orbitrap HESI | Hypersil GOLD C18 (100 × 2.1 mm, 1.9 μm) H2O/MeOH with FA and HCOONH4 | Full MS (m/z 100-1000) | [33] |
| | | | | | |
| AFB1, AFB2, AFG1, AFG2 | Isoflavone supplements | UHPLC-Orbitrap HESI | Hypersil Gold C18 (100 × 2.1 mm, 1.9 μm) H2O/MeOH with FA and HCOONH4 | Full MS (m/z 100-1000) and AIF (m/z 65-700) | [34] |
| | | | | | |
| AFB1, AFB2, AFG1, AFG2 | Ginkgo biloba supplements | UHPLC-Orbitrap HESI | Hypersil Gold C18 (100 × 2.1 mm, 1.9 μm) H2O/MeOH with FA and HCOONH4 | Full MS and AIF (m/z 100-1000) | [35] |
| | | | | | |
| AFB1, AFB2, AFG1, AFG2 | Green tea and royal jelly supplements | UHPLC-Orbitrap HESI | Hypersil Gold C18 (100 × 2.1 mm, 1.9 μm) H2O/MeOH with FA and HCOONH4 | Full MS and AIF (m/z 100-1000) | [36] |
| | | | | | |
| AFB1, AFB2, AFG1, AFG2 | Coix seed | UHPLC-Orbitrap HESI | C18 (100 × 2.1 mm, 1.6 μm) H2O/MeOH with FA and CH3COONH4 | Full MS (m/z 100-800) | [37] |
| | | | | | |
| AFB1, AFB2, AFG1, AFG2, AFM1, AFL | Feed | UHPLC-Orbitrap HESI | Hypersil Gold C18 (100 × 1.6mm,1.9 μm) H2O/MeOH/ACN with AA | Full MS and AIF (m/z 55-1000) | [38] |
| | | | | | |
| AFB1, AFB1-lys | Human serum | UHPLC-Q-Orbitrap HESI | C18 (100 × 2.1 mm, 1.7 μm) H2O/MeOH with FA and HCOONH4 | Full MS (m/z 100-700) and dd-MS2 (list dependent) | [39] |
| | | | | | |
| AFB1, AFM1, AFB1-N7-guanine | Human urine | UHPLC-Q-Orbitrap HESI | Acquity BEH C18 (100 × 2.1 mm, 1.7 μm) H2O/MeOH with FA and HCOONH4 | Full MS (no range) and dd-MS2 (list dependent) | [40] |
| AF studied | Matrix | Instrument | LC conditions | Acquisition | Ref. |
|------------|--------|------------|---------------|-------------|------|
| AFB1, AFM1 | Milk   | UHPLC-Q-Orbitrap HESI + | Luna Omega C$_{18}$ (50 × 2.1 mm, 1.6 μm) H$_2$O/MeOH with FA and HCOONH$_4$ | Full MS and AIF (m/z 100-1000) | [41] |
| AFB1, AFM1 | Milk   | UHPLC-Q-Orbitrap HESI + | Accucore C$_{18}$ (150 x 2.1 mm, 2.6 μm) H$_2$O/ACN with FA and CH$_3$COONH$_4$ | Full MS (m/z 50-1000) and dd-MS$^2$ (list dependent) | [42] |
| AFB1, AFB2, AFG1, AFG2, AFM1, AFM2 | Maize | UHPLC-Q-Orbitrap HESI + | Zorbax Eclipse Plus RRHD C$_{18}$ column (50 × 2.1 mm, 1.8 μm) | Full MS, dd-MS$^2$ (list dependent), AIF and AIF with HCD events | [30] |
| AFB1, AFB2, AFG1, AFG2 | Cashew nut | UHPLC-Q-Orbitrap HESI + | H$_2$O/MeOH with FA and HCOONH$_4$ | PRM | [43] |
| AFB1, AFB2, AFG1, AFG2 | Cereals | UHPLC-Q-Orbitrap HESI + | Kinetex C$_{18}$ (50 × 3 mm, 1.7 μm) H$_2$O/MeOH with AA and CH$_3$COONH$_4$ | Full MS and AIF (m/z 50-1000) | [44] |
| AFB1 | Durum wheat pasta and baby food pasta | UHPLC-Q-Orbitrap HESI + | Accucore C$_{18}$ (100 × 2.1 mm 2.6 μm) H$_2$O/MeOH with FA and HCOONH$_4$ | Full MS (m/z 90-1000) | [45] |
| AFB1, AFB2, AFG1, AFG2 | Green tea supplements | UHPLC-Q-Orbitrap HESI + | Hypersil Gold C$_{18}$ (100 × 2.1 mm, 1.9 μm) H$_2$O/MeOH with FA and HCOONH$_4$ | Full MS (m/z 100-800), AIF, and AIF with HCD events | [31] |
| AFB1, AFB2, AFG1, AFG2 | Medicinal herbs | UHPLC-Q-Orbitrap HESI + | Kinetex C$_{18}$ column (100 × 2.1 mm, 1.7 μm) H$_2$O/MeOH with FA | SIM | [46] |
| AFB1, AFB2, AFG1, AFG2 | Pet foods | UHPLC-Q-Orbitrap HESI + | Luna Omega C$_{18}$ (50 × 2.1 mm, 1.6 μm) H$_2$O/MeOH with FA and HCOONH$_4$ | Full MS and AIF (m/z 100-1000) | [32] |
| AFB1, AFB2, AFG1, AFG2 | Waters | UHPLC-Q-Orbitrap HESI + | C$_{18}$ (325 × 2 mm, 5 μm) H$_2$O/ACN with FA | Full MS (no data) and dd-MS$^2$ (list dependent) | [47] |
| AFB1-lys | Human plasma | NanoLC–Q-Orbitrap HESI + | Acclaim C$_{18}$ (15 cm, 75 μm) H$_2$O/ACN with FA | Full MS (m/z 50-900) and PRM | [26] |
| AFB1, AFB2, AFG1, AFG2 | Snus | Heart-cutting 2D-LC-Q-Orbitrap HESI + | Hypersil Gold C$_{18}$ (100 × 0.5 mm, 3 μm) and ACQUITY HSS PFP (100 × 2.1 mm, 1.7 μm) H$_2$O/MeOH/ACN with FA and HCOONH$_4$ | PRM | [27] |
| AFB1, AFB2, AFG1, AFG2 | Human plasma | UHPLC–IT-Orbitrap HESI + | PFP (50 × 2.1 mm, 2.6 μm) H$_2$O/MeOH with AA | Full MS (m/z 280-500) | [22] |
AFs can grow on many foods, mainly peanuts, maize and cottonseed, although they have also been found in all types of nuts, copra, cereals, sunflower and soya beans, unrefined vegetable oils, spices, dried fruits, coffee, cocoa and animal feed [1, 16].

Milk is the most consumed beverage in the world, and it is the primary source of nourishment for the normal growth of infants and children. The maximum permissible level of AFM1 in milk is set at 0.5 μg kg\(^{-1}\) in China and USA, and 0.05 μg kg\(^{-1}\) in Europe. Zhao et al. [42] proposed the determination of AFB1, AFB2, AFG1, AFG2, AFM1, AFM2 and other mycotoxins in liquid milk using UHPLC-Q-Orbitrap. Limits of quantification (LOQs) were in the range of 0.015–0.150 μg kg\(^{-1}\). AFM1 was detected in four samples in a range from 0.026 to 0.039 μg kg\(^{-1}\). Rodríguez-Carrasco et al. [41] proposed to study the contamination of milk with AFM1 and its parent compound, AFB1, using also UHPLC-Q-Orbitrap. In this case, LOQs were 0.001 and 0.002 μg L\(^{-1}\) for AFM1 and AFB1, respectively. The validated method was applied to 40 Italian milk samples and neither AFB1 nor AFM1 were found above the LOD in any of the analyzed samples.

### 3.2 HRMS applications for food analysis

| AF studied | Matrix | Instrument | LC conditions | Acquisition | Ref. |
|------------|--------|------------|---------------|-------------|------|
| AFB1, AFB2, AFG1, AFG2 | Beer | UHPLC-IT-Orbitrap | Gemini C\(_{18}\) (150 × 2 mm, 5 μm) H\(_2\)O/MeOH with FA and HCOONH\(_4\) | Full MS (m/z 90-900) and dd-MS\(^2\) (list dependent) | [23] |
| AFB1 | Beer | HPLC-TOF | Kinetex C\(_{18}\) (50 × 3 mm, 1.7 μm) H\(_2\)O/MeOH with FA and CH\(_3\)COONH\(_4\) | Full MS (m/z 50-1000) | [24] |
| AFB1 | Beer | UHPLC-TOF | Kinetex C\(_{18}\) (50 × 3 mm, 1.7 μm) H\(_2\)O/ACN with FA and CH\(_3\)COONH\(_4\) | Full MS (m/z 50-1000) | [25] |
| AFB1, AFB2, AFG1, AFG2 | Human plasma | UHPLC–Q-TOF | ODS C\(_{18}\) (150 × 1.5 mm, 5.0 μm) H\(_2\)O/MeOH with HCOONH\(_4\) | Full MS (no data) and dd-MS\(^2\) (Ion intensity-dependent) | [29] |
| AFB1-lys | Human serum | UHPLC–Q-TOF | Acquity BEH C\(_{18}\) (50 × 2.1 mm, 1.7 μm) H\(_2\)O/ACN with FA | Full MS (m/z 280-650) and dd-MS\(^2\) (list dependent) | [48] |
| AFB1, AFM1 | Plasma, urine, feces (pig, broiler) | UHPLC–Q-TOF | Acquity HSST3 C\(_{18}\) (100 × 2.1 mm, 1.8 μm) H\(_2\)O/MeOH with FA and HCOONH\(_4\) | Full MS and AIF (mass range m/z 50-1200) | [49] |
| AFB1 | Seeds, milk, flour, beer | UHPLC–Q-TOF | Eclipse Plus C\(_{8}\) RRHD (50 × 2.1 mm, 1.8 μm) H\(_2\)O/ACN with FA | No data | [28] |
| AFB1, AFB2, AFG1, AFG2 | Corn | UHPLC–Q-TOF | Hypersil Gold C\(_{18}\) (100 × 2.1 mm, 1.9 μm) H\(_2\)O/MeOH with FA and CH\(_3\)COONH\(_4\) | Full MS (m/z 100-1000) | [50] |

Table 1. Summary of LC-HRMS condition for AF determination.
Because occurrence of mycotoxins in cereals has been object of health concern worldwide for long time, numerous analytical methods for control of their presence have been developed. Vaclavik et al. proposed the use of direct analysis in real time (DART) with Orbitrap in positive mode for the control of different mycotoxins, including AFB1, AFB2, AFG1 and AFG2 in cereals, as wheat and maize [51]. Only ionization of some of tested AFs (AFB1, AFB2) was achieved by DART, being the sensitivity poor compared with triple quadrupole (QqQ). Lattanzio et al. [44] developed a LC-HRMS procedure for the simultaneous determination of AFs (B1, B2, G1, G2), and other mycotoxins in wheat flour, barley flour and crisp bread. Mycotoxin fragmentation patterns were obtained using Orbitrap. LODs in the 0.5–3.4 mg kg⁻¹ range were obtained for three cereal matrices and a critical comparison between the proposed method and a method based on QqQ showed similar performance. A comparison was also made by Renaud et al. [30] between a Q-Orbitrap and LC–MS/MS for AFB1, AFB2, AFG1 and AFG2 and other mycotoxins in maize. The linearity and LODs achieved were comparable by both methods. The technique TOF secondary ion mass spectrometry offers high sensitivity and identification of small molecules using the corresponding secondary molecular ions, which enabled a quantitative analysis of different aflatoxin analogues from corns with immunoaffinity columns (IACs) [52]. The detection sensitivity was estimated to be 10 μg L⁻¹ for the main four AFs. Tropical climate is a significantly favorable condition for fungus to develop as demonstrated by Giang et al. [43]. For this reason, mycotoxin contaminations are likely to occur on the cashew, especially aflatoxin. The toxins AFB1, AFB2, AFG1 and AFG2 were determined by UHPLC-HRMS in positive ion mode with LOQs ranged from 0.5 to 1.0 μg kg⁻¹. A method for the simultaneous determination of 9 mycotoxins, including the four main AFs in corn using UHPLC-Q-TOF was developed by Wang et al. [50]. LOQs ranged from 0.1 to 200 μg kg⁻¹. The developed method was applied to 130 corn samples, being AFB1 one of the most predominant mycotoxins, as it was found in 37 corn samples with concentrations between non-detected up to 593 μg kg⁻¹.

A simple and rapid multi-mycotoxin method for the determination of 17 mycotoxins simultaneously is described on durum and soft wheat pasta baby food samples [45] by UHPLC-Orbitrap in positive mode for AFB1. Twenty-nine samples were analyzed, 27 samples of durum wheat pasta, and two samples of baby food, and AFB1 was not detected in any sample (LOQ 0.11 μg kg⁻¹).

Beef is one of the most consumed cereal-based alcoholic beverages in the world, being usually obtained by fermenting certain starch-rich grains, such as malted barley and wheat, although other cereals like maize, oats, unmalted barley or rice, may be used. These raw materials can contain mycotoxins that are transferred to the final product. Bogdanova et al. [24] reports data on the occurrence of nine mycotoxins, including AFB1, in 100 beers using HPLC-TOF in positive mode. A LOQ of 0.150 μg kg⁻¹ was obtained for AFB1, being found contents between 0.1–0.19 μg kg⁻¹ for light beer samples. The low concentrations of AFB1 found in only a few samples could be related to its usually low prevalence in products manufactured and stored in the northern countries. AFB1 and other mycotoxins have also been determined in beer using LC-HRMS in positive mode by Rozentale et al. [25]. The LOD was 0.021 μg L⁻¹, and the toxin was not detected in any beer sample. Beer was also analyzed by Rubert et al. [23] using LC-ESI-IT-Orbitrap for the determination of 18 mycotoxins, including AFs (B1, B2, G1 and G2). The LOQs ranged from 9 to 30 μg L⁻¹. The method was applied to the analysis of 25 commercial beers, but no AFs were found.

Nutraceutical products as dietary supplements and functional foods contribute to a good nutrition, providing different alternatives for healthcare.
A multi-analytical strategy was proposed by Jia et al. [31] to screen mycotoxins and their transformation products in nutraceuticals from green tea. AFB1, AFB2, AFG1, AFG2 and AFM1 were determined using UHPLC-HRMS, obtaining entire spectrum of fragment ion peaks for each AF, which allows screening and quantitation of non-target mycotoxins. LOQs ranged between 0.02 and 0.40 μg kg\(^{-1}\) and AFB1 was found in two samples (0.95 and 0.97 μg kg\(^{-1}\)). Among the plants that can be used for the preparation of nutraceuticals, Ginkgo biloba is widely used, obtaining the extract from ginkgo leaves. Martínez-Domínguez et al. [35] determined more than 250 toxic substances, including pesticides and mycotoxins, in Ginkgo biloba nutraceutical products using UHPLC-Orbitrap. LOD and LOQ below 5 and 10 μg kg\(^{-1}\) were obtained, respectively. In the case of AFs, AFB1 (5–54 μg kg\(^{-1}\)) and AFB2 (4–300 μg kg\(^{-1}\)) were found in 6 samples. It should be noted that these levels are very high, especially the value obtained for AFB2 in one sample, considering the maximum contents allowed by the European legislation, and that AFB2 is one of the most toxic mycotoxins. The same authors [34] also applied the same approach for the analysis of isoflavone supplements obtained from soy, detecting AFB1 (8.2–171 μg kg\(^{-1}\)) and AFG2 (6.4 μg kg\(^{-1}\)), as well as to green tea and royal jelly supplements [36], quantifying AFB1 (5.4 μg kg\(^{-1}\)) in one of the green tea samples. Analysis of mycotoxins in functional and medicinal herbs is a challenge because herbs have complicated and diverse matrices from different parts of plants as well as different species. Thus, Cho et al. [46] developed a LC-tandem mass spectrometry (MS/MS) method for the determination of multi-class mycotoxins (including AFB1, AFB2, AFG1 and AFG2) in twenty different species of herbs that are used for both food and medicinal purposes. The results were further verified using Orbitrap in positive mode. AFB1 was found in six samples at levels around 5 ng g\(^{-1}\). Coix seed is an important food and traditional Chinese medicine which is currently being used for the treatment of COVID-19 in China. Wu et al. [37] developed a method by UHPLC-HRMS for the simultaneous detection of 24 mycotoxins in coix seeds. The LOQs for AFB1, AFB2, AFG1, AFG2 and AFM1 were 0.5 μg kg\(^{-1}\). More than 70 samples of coix seeds were collected from Chinese markets and were analyzed, being the results as follow: AFB1 positive ratio: 29.9%, range: 0.39–14.7 μg kg\(^{-1}\); AFB2 positive ratio: 5.2%, range: 0.15–0.97 μg kg\(^{-1}\); AFG1 positive ratio: 1.3%, range: 0.26–0.26 μg kg\(^{-1}\); AFG2 positive ratio: 1.3%, range: 0.12–0.12 μg kg\(^{-1}\); AFM1 positive ratio: 0%.

Mycotoxins are frequently present in animal feed due to their misuse, carry-over or environmental contamination. The consequence is the incorporation of these substances into the food chain and monitoring the presence of these hazardous chemicals remains one of the main tasks for ensuring feed safety and human health [1]. León et al. [38] developed a procedure for the quantitative target analysis of mycotoxins and other substances in feed using LC–HRMS using Orbitrap. For post-target screening a customized theoretical database including the exact mass, the polarity of acquisition and the expected adducts was built. Castaldo et al. [32] used a strategy combining a quantitative method for 28 mycotoxins, including AFB1, AFB2, AFG1 and AFG2, and a post-target screening for other 245 fungal and bacterial metabolites in dry pet food samples using UHPLC-Q-Orbitrap in positive mode. Results showed mycotoxin contamination in 99% of pet food samples at concentrations of up to hundreds μg kg\(^{-1}\).

A wide range of foods such as peach seed, milk powder, corn flour and beer samples have been analyzed by Du et al. [28] for determining AFB1 and other fungi metabolites by UHPLC-Q-TOF with ESI in the positive mode. LODs were 0.0036–0.033 μg kg\(^{-1}\) for solid samples and 0.0022–0.017 μg L\(^{-1}\) for beer.

Modern MS detectors can be used not only as detectors, but also as a “separation” tool, due to significant advances in HRMS, achieving greater sensitivity and
selectivity. Thus, flow injection (FI) can be used to introduce the sample into the MS instrument, saving time and solvent compared to UHPLC analysis. Using this approach, Sapozhnikova et al. [53] proposed the simultaneous detection of twelve pesticides and seven mycotoxins in food and feed samples by FI-MS, using QqQ and ion mobility HR-TOF. LODs in standard solutions were below maximum permitted content, except for AFB1 with the lowest maximum permitted content of 0.002 mg kg\(^{-1}\).

Mycotoxins can be present in their parent forms and also in other forms, as “modified mycotoxins”, which are conjugated with glucoside, acetyl, sulphate, and/or glutathione or other substances. This term was first used for the hydroxylation product AFM1 of AFB1, which was present in mycotoxin-contaminated feed. Lu et al. [5] summarizes the target and non-target qualitative and quantitative analysis for modified mycotoxins using HRMS instruments, such as TOF and Orbitrap.

### 3.3 HRMS applications for biological sample analysis

The determination of mycotoxin exposure of human populations is difficult due to the heterogeneous distribution of mycotoxins in foods and the time lag between toxin intake and the development of chronic disease. Therefore, a more reliable and relevant indication of individual exposure could be provided by biomarkers measured in biological fluids.

Aflatoxins can bioaccumulate in the organs and tissues of animals and humans or be excreted by biological fluids or feces [39, 40]. Sensitive analytical procedures are required for the determination of AFs in biological samples due to the very low concentrations involved. Most of the analytical methods proposed are based on LC coupled to different detection systems such as spectrophotometry, fluorescence, MS or MS/MS. Recently, HRMS, including TOF and Orbitrap, resulted an excellent technique for target analysis of AFs as well as for identifying and screening of non-target compounds in metabolomic strategies for studies concerning bioaccumulation, toxicokinetics and excretion of AFs and their metabolites.

Most of the studies are related with urine and blood because sampling is non-invasive in the case of urine and minimally invasive in that of blood. Urine is an easily accessible sample which can be easily collected in a large quantity and contains numerous metabolites. Urine samples were analyzed for the presence of AFB1 and AFM1 and AFB1-N7-guanine adduct by Debegnach et al. [40]. LODs obtained using LC–HRMS were 0.8 and 1.5 ng L\(^{-1}\) urine for AFB1 and AFM1, respectively. No quantitative determination was possible for the adduct AFB1-N7-guanine. In the 120 urine samples analyzed, AFM1 was found in 14 samples in the range of 1.9–10.5 ng L\(^{-1}\) urine, while AFB1 and its adduct were not detected. These results indicated that no workplace exposure was originated.

In addition, blood is a biofluid which incorporate the functions of different parts of the body into a single sample, being very useful for metabolomic studies. Human plasma samples were used for monitoring 17 mycotoxins, including AFB1, AFB2, AFG1 and AFG2, using HRMS on hybrid IT-Orbitrap in ESI positive mode by Slobodchikova et al. [22]. The LOQs of all the AFs were 0.2 μg L\(^{-1}\). By using HRMS, the method can also be used for screening of the presence of different metabolites. Also, Slobodchikova et al. [54] performed human in vitro microsomal incubations of 17 mycotoxins and systematically characterize all resulting metabolites using LC–HRMS to build a library with screening of additional 188 metabolites, including 100 metabolites reported for the first time. NanoLC-Q-Orbitrap with isotope dilution MS was applied by McMillan et al. [26] to quantitate AFB1-lys in plasma samples from an extremely vulnerable population of Nigerian children suffering
from severe acute malnutrition. The plasma levels of AFB1-lys in the population were between 0.2 and 59.2 \( \mu \text{g kg}^{-1} \) albumin, with a median value of 2.6 \( \mu \text{g kg}^{-1} \) albumin. AFB1-lys concentrations were significantly higher in children suffering with severe acute malnutrition. Identification of 56 natural toxic substances by LC–Q-TOF has been proposed by Ogawa et al. to create a forensic toxicological library [29]. The product ion spectra include the four main AFs, with positive ionization. The method was applied to post-mortem blood samples from a death resulting from the intake of aconite. The developed library permits the screening of natural toxic substances in routine forensic toxicological analysis. Serum samples were collected for the determination of AFB1 and AFB1-lys adduct [39]. For AFB1, a high number of non-detected samples was found (LOQ 5 \( \mu \text{g kg}^{-1} \) serum) and none of the analyzed samples showed the presence of AFB1-lys adduct, thus the presence of AFB1 in serum cannot be attributable to occupational exposure. Serum AFB1-lys was also evaluated as an AFB1-specific biomarker for diagnostic purposes and for evaluating the efficacy of chemoprotective interventions in pigs [54]. The LOQ value was 10.3 \( \mu \text{g L}^{-1} \) and results indicated that AFB1-lys has potential as an AFB1 specific biomarker for diagnostic purposes.

In comparison to other biological fluids such as blood, plasma and urine, the database on multi-mycotoxin levels in human milk is rather small. Breast milk is a relevant source of mycotoxins for neonates. Rubert et al. [33] proposed the use of LC–HRMS as excellent tool in the screening, quantitation and confirmation of targeted mycotoxins and their metabolites, including AFB1, AFB2, AFG1, AFG2 and AFM1. None of the AFs were detected in the breast milk samples.

Several studies propose to analyze several biological samples for metabolic studies. Thus, the correlation between exposure to AFB1 and early-stage renal damage was evaluated by Díaz de León-Martínez et al. [48] by analyzing urine and blood samples. The exposure to AFB1 was measured through the biomarker AFB1-lys. Plasma, urine and feces of pigs and plasma and urine of broiler chickens were used in a LC–HRMS procedure by Lauwers et al. [49] to determine mycotoxins, including AFs, and their metabolites with LOQ values of 1 \( \mu \text{g L}^{-1} \). LC–HRMS was used on non-targeted qualitatively determination phase I and II metabolites, for which analytical standards are not always commercially available. The multi-method was successfully applied in a toxicokinetic study and a screening study to monitor the exposure of individual animals.

**Figure 3.**
*Type of samples more frequently analyzed. The number of published articles dealing with each matrix is indicated.*
Figure 3 shows a distribution of the type of food and biological samples for which LC-HRMS methods have been applied in AF determination.

4. Sample treatments for determination of aflatoxins

Method accuracy and precision are strongly conditioned by the effectiveness and robustness of the sample treatment stage. Both physicochemical properties of the AFs and the sample matrix composition need to be considered in the extraction procedure selection, which should ideally isolate and concentrate the analytes, eliminate interferences, and provide extracts compatible with the analytical technique to be used. For example, AFB1 was extracted in acidified ethyl acetate (EtAc) from serum previously submitted to enzymatic digestion (ED) and lipid removing by liquid–liquid extraction (LLE) with hexane. Nevertheless, this extraction medium was unable to isolate the hydrophilic metabolite AFB1-lys from the same sample and, a salting-out step with a quick, easy, cheap, effective, rugged and safe (QuEChERS) mixture was applied [39]. The objective conditions the adoption of a more or less selective sample treatment. Thus, non-selective extractions are applied for non-targeted strategies, allowing retrospective analysis of any potential compound, whereas for targeted analysis, clean and concentrated extracts are required.

Solid samples are generally homogenized by grinding [37, 38, 43, 44, 46], in order to obtain representative sample aliquots before being submitted to a solid–liquid extraction (SLE) stage. Freeze-drying of feces has also been proposed for eliminating any variations due to different moisture contents [49]. For SLE, aqueous mixtures of polar organic solvents such as MeOH, acetone or ACN have been used for AFs isolation from biological [49] and food [37, 43, 44, 46, 52] samples, being the mixtures mechanically shaken. The application of external energy in SLE procedures is sometimes proposed in order to enhance analyte recoveries in low times. Ultrasound assisted extraction (UAE) [30, 32, 50] and microwave assisted extraction (MAE) [28] have efficiently extract AFs from food matrices.

On the other hand, even though food and biological liquid samples could be directly analyzed HRMS or LC combined with HRMS, previous steps are generally applied to minimize matrix effects. Thus, the addition of organic solvents such as ACN or MeOH allowed deproteinization of plasma [26, 29, 49] and milk [42] samples. Enzymatic reactions have also proven to be effective for human serum deproteinization [39, 48].

The removing of non-polar sample components, such as phospholipids, has been proposed by LLE with hexane [39, 46], by solid-phase extraction (SPE) using SPE-phospholipid cartridges for pig feces [49] and well-plates for chicken plasma [49].

The isolation of AFB1, AFB2, AFG1, AFG2 and AFM1 by LLE using EtAc has been applied for urine [49] and serum [22, 39], being recommended though a three-step LLE by Slobodchikova et al. [22] which resulted in better recoveries than those provided by SPE or protein precipitation (PP) procedures in a multi-mycotoxin method.

The simultaneous sample matrix purification and AF isolation is accomplished by many of the applied treatments in a single step. Thus, an on-line SPE device allowed the simultaneous isolation of 12 mycotoxins, including AFB1, and matrix purification of beer samples. Although SPE is more commonly applied under offline mode, as used by Rubert et al. for isolation of AFB1, AFB2, AFG1, AFG2 in a multi-mycotoxin method proposed for beer [23]. SLE extracts obtained from solid food matrices have also been submitted to SPE [43, 44]. Polymeric sorbents are used in SPE isolation of AFs in their free forms. Whereas mixed-mode SPE-sorbents are selected for the retention of AFB1-lys adduct. Thus, a modified extraction procedure involving a PP step before enzymatic digestion (ED) with Pronase® and
SPE-clean-up using strong ion mixed-mode-SPE has allowed both metabolic profiling and AFB1-lys adduct quantification in serum samples [26, 48].

Specific antibody-analyte binding is exploited as clean-up procedure in IACs, reporting interesting applications in AF analysis. Thus, gel suspensions of monoclonal antibody specific for AFs have allowed the purification of AFB1, AFB2, AFG1 and AFG2 from urine [40]. IAC have also been proposed for multi-mycotoxin studies, including AFs, for the analysis of functional and medicinal herbs [46]. In both articles reviewed dealing with IAC, AFs were eluted with MeOH after a washing step for impurities elimination using water [40] of aqueous buffer solutions [46].

QuEChERS methodology has been applied for AFs determination in both food [24, 32, 38, 41, 45, 50, 51, 53, 56] and biological samples [33, 39], using ACN as extractant solvent and a dispersive SPE (DSPE) step with the appropriate sorbents. When QuEChERS clean-up is applied omitting the use of sorbents, so that only implying organic solvent and salts, the procedure is named as simplified QuEChERS, and it has been proposed for isolation of AFB1, AFB2, AFG1, AFG2 and AFM1 from human breast milk [33] and AFB1 from durum wheat pasta [45]. The possibilities of different mixtures of solid sorbents for multi-class determinations of more than 250 compounds, pesticides and mycotoxins, including AFB1, AFB2, AFG1 and AFG2, applied to nutraceutical products have been studied by Martínez-Domínguez et al. [34–36] and compared with SLE, called in this case as “dilute and shoot” procedure. Best results have been reported by the latter procedure followed by a clean-up step using a mixture of sorbents in a DSPE mode [35, 36] or cartridge packed [34], this clean-up stage applied in order to enhance analyte recoveries and/or maintain the equipment performance for longer periods of times. When “dilute and shoot” procedure was compared to IAC for AFs in urine sample, lower LODs were achieved by the latter, because cleaner extracts with higher AF concentrations were obtained, being therefore selected [40]. An on-line automated sample preparation procedure is developed by Jia et al. [31] for multiple mycotoxin screening in nutraceutical products involving SLE, using aqueous acid solution and ACN, and the obtained supernatant being transferred to a disposable pipette extraction containing salt previously to the application of a clean-up step based on DSPE.

![Figure 4. Sample treatments for AF determination by LC-HRMS.](image-url)
| Matrix                        | Sample treatment                                                                                                                                                                                                 | Ref.  |
|-------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------|
| **Biological samples**        |                                                                                                                                                                                                                   |       |
| Human serum                   | ED of 0.5 mL sample with Pronase®, LLE degreasing with hexane and: LLE with acidified EtAc (for AFB1), QuEChERS (for FB1-lys)                                                                                       | [39]  |
| Human serum                   | ED of 0.25 mL sample with Pronase® and mixed-mode SPE. Elution with acidified MeOH                                                                                                                            | [48]  |
| Human plasma                  | PP of 0.23 mL sample with MeOH/water, supernatant ED with Pronase® and mixed-mode SPE. Elution with acidified MeOH                                                                                             | [26]  |
| Human plasma                  | PP of 0.1 mL sample with EtOH/ACN                                                                                                                                                                               | [29]  |
| Human plasma                  | 3-step LLE of 0.1 mL sample with EtAc                                                                                                                                                                            | [22]  |
| Human urine                   | IAC for 2 mL sample. Elution with MeOH                                                                                                                                                                          | [40]  |
| Human breast milk             | Simplified QuEChERS                                                                                                                                                                                              | [33]  |
| Pig plasma, urine, and feces. Broiler chicken plasma and excreta | Pig plasma: PP of 0.25 mL sample with ACN. Pig urine: LLE of 0.5 mL sample with EtAc. Pig feces: SLE of 0.25 g sample with acetone and SPE for phospholipid removal. Chicken plasma: PP of 0.15 mL sample with ACN and well-plates. Chicken excreta: SLE of 0.25 g sample with ACN | [49]  |
| **Foods**                     |                                                                                                                                                                                                                   |       |
| Beer                          | QuEChERS for 5 mL sample                                                                                                                                                                                          | [24]  |
| Beer                          | On-line SPE for 0.5 mL sample. Elution with acidified ACN/CH$_3$COONH$_4$                                                                                                                                                | [25]  |
| Beer                          | SPE for 10 mL sample. Elution with ACN/MeOH                                                                                                                                                                          | [23]  |
| Milk                          | PP of 4 g sample with ACN and MDSPE with 10 mg MNPs. Desorption with acidified EtAc                                                                                                                                 | [42]  |
| Milk                          | QuEChERS for 10 mL sample                                                                                                                                                                                          | [41]  |
| Peach seed, milk powder, corn flour and beer | MAE of solid samples (0.2 g) in MeOH/water and DSPE with 2 mg zirconia NPs. Desorption with MeOH                                                                                                                                 | [28]  |
| Maize                         | UAE of 0.7 g sample in acidified MeOH/dichloromethane/ EtAc                                                                                                                                                         | [55]  |
| Maize                         | UAE of 0.5 g sample with acidified ACN                                                                                                                                                                             | [30]  |
| Corn                          | UAE of 2 g sample in ACN/water and QuEChERS                                                                                                                                                                         | [50]  |
| Cereal foods (flours and bread) | SLE of 10 g sample with ACN/water and SPE. Elution with MeOH                                                                                                                                                       | [44]  |
| Durum wheat pasta and baby food pasta | Simplified QuEChERS for 4 g sample                                                                                                                                                                                      | [45]  |
| Cashew nut                    | SLE of 1 g sample in MeOH/water and SPE. Elution with MeOH                                                                                                                                                           | [43]  |
| Coix seed                     | SLE of 5 g sample with acidified ACN                                                                                                                                                                                | [37]  |
| Isoflavone supplements        | SLE of 2.5 g sample with acidified ACN and clean-up by SPE                                                                                                                                                           | [34]  |
| Ginkgo biloba nutraceuticals  | SLE of 2.5 g sample with acidified ACN and clean-up by DSPE                                                                                                                                                          | [35]  |
| Green tea and royal jelly supplements | SLE of 2.5 g sample with acidified ACN and clean-up by DSPE                                                                                                                                                       | [36]  |
| Green tea nutraceuticals      | SLE of 1 g sample with acidified ACN and clean-up by DSPE                                                                                                                                                           | [31]  |
| Functional and medicinal herbs | SLE of 2 g sample with PBS, LLE degreasing with hexane and IAC. Elution with MeOH                                                                                                                                     | [46]  |
In the last years, nanoparticles (NPs) have received great attention in analytical chemistry due to the high surface area to volume ratio if compared to particles of higher dimensions, thus leading to very efficient extractions in lower times. A mass of 2 mg of zirconia NPs dispersed in the aqueous extract obtained by MAE from food samples allowed the DSPE isolation of AFB1 in 2 min, being then submitted to a desorption step in acidified chloroform. When magnetized NPs are used, the collection of the enriched NPs is easily achieved by applying an external magnetic field, avoiding the centrifugation step. Under this named magnetic dispersive solid-phase extraction (MDSPE) methodology, AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2, in a multiclass mycotoxin analysis method, have been preconcentrated with multi-walled carbon nanotubes (MWCNTs) modified with polyethylene glycol. Although not implying HRMS detection for LC, it is noteworthy that AFB1 has also been preconcentrated with amino-modified magnetic MWCNTs.

With the aim to enhance sensitivity and clean-up purposes, the AF extracts finally obtained by applying the selected isolation procedure, are generally evaporated to dryness and reconstituted in low volumes of solvents compatible with the instrumental measurement step.

The data provided in this review demonstrate that most of the studies dealing with AF determination by LC-HRMS are focused on food, feed and biological samples analysis. In fact, only two manuscripts dealing with other matrices, waters and snus, were found. A triple-stage SPE, consisting of a hand-made cartridge, packed with porous graphitized carbon and modified styrene-divinylbenzene polymer, coupled to a commercial HLB plus cartridge, allowed the isolation of natural toxins of different polarities. Thus, a screening method is proposed for the tentative identification of mycotoxins, cyanotoxins and plant toxins in surface waters. The use of multiple Heart-cutting 2D-LC-Q-Orbitrap for AF separation and detection, respectively, has probably allowed to apply a very simple procedure in the treatment of snus samples. Thus, LLE in acidified EtAc provided similar analyte recoveries than QuEChERS method.

As can be appreciated in Figure 4, sample treatments for AF determination by LC-HRMS have been proposed both applying a unique methodology or through the combination of different procedures generally sequentially applied, for both food and biological samples.

A summary of the sample treatments used in the AF determination by LC-HRMS is provided as well in Table 2.

### 5. LC-HRMS for AF biosynthesis and degradation studies

AFs are synthesized via multiple intermediates by a complex pathway in several species of the *Aspergillus* genus, including *Aspergillus flavus*. LC-HRMS
combination has allowed the study of the biosynthesis pathway by monitoring the changes in metabolite profiles, as proposed Xie et al. [58] under different growth and environmental conditions and using Orbitrap. On the other hand, the role of quercetin as inhibitor of the AF biosynthesis has been studied by Tiwari and Shankar [59], using nano-LC-Q-TOF for protein identification and HPLC-UV for AFB1 levels monitorization. The results obtained showed the power of quercetin as anti-aflatoxic agent in *A. flavus*. A strategy based on UHPLC-Orbitrap has been proposed by Arroyo et al. [60] to study the function of certain genes of *A. flavus*, allowing to verify some steps of the biosynthesis pathway of AFs.

As regards mycotoxin degradation, decontamination techniques for AFs in food and feed attract continuous interest due to their adverse health effects and large economic losses for producers. In this sense, physical, chemical and biological strategies have been proposed. Thus, AFB1 degradation products by electron beam irradiation have been identified, as well as the possible pathway, using UHPLC-Q-TOF-MS [61, 62]. High-voltage atmospheric cold plasma (HVACP) is other physical strategy applied for AFB1 decontamination, providing a 76% efficiency when the non-thermal treatment was applied for 5 min in air containing 40% relative humidity. Thus, molecular formulas of six degradation products were elucidated by HPLC-TOF and their structures were further studied by Orbitrap MS. Two of the detected degradation compounds were ozonolysis products of AFB1, and the other four indicated the action of other reactive species besides ozone, generated during HVACP treatment [63]. The proven degradation power of ultrasounds for AFB1 aqueous solutions allows to perceive this physical detoxification technology as promising for food industry. An ultrasound exposure of 80 min degraded AFB1 by 85.1%, being eight main reaction products identified by UHPLC-Q-Orbitrap [64]. The study of degradation pathways and structural identification of photodegradation products of AFB1 in aqueous medium [65], ACN [66] and on peanut surface [67], has been carried out using UHPLC-Q-TOF after ultraviolet irradiation of different intensities.

Biological degradation, mainly caused by bacterial and fungal enzymes, appears as a strategy for AFB1 removal, with inherent advantages over physical and chemical strategies such as being friendly to the environment. LC-Q-TOF has been applied in the monitorization of AFB1 degradation products, and the obtained results lead the authors to propose bacterial strain *Bacillus licheniformis* BL010 for detoxification of AFB1 [68]. The degrading properties of *P. aeruginosa* towards AFB1 have been studied by Sangare et al. [69], and as no degradation products were identified by LC-Q-TOF, the decontamination route was proposed through the formation of products of chemical properties different from that of the parent AF. On the other hand, the enzymatic reaction based on the recombinant Rh_DypB peroxidase for the *in vitro* biotransformation of AFB1 has been studied by Loi et al. [70], proving an efficient detoxification at low enzyme and hydrogen peroxide concentrations. A 96% AFB1 hydroxylation yield to a less toxic AF, AFQ1, was observed after 96 h of reaction. The authors indicated the convenience to explore the translation of this strategy towards contaminated matrices. On the other hand, the salt tolerant yeast *Candida versatilis* CGMCC has also demonstrated its capacity to degrade AFB1, through the formation of four non-toxic products which have been identified by LC-Q-TOF [71].

6. Conclusion

AFs are secondary toxic metabolites which may be present mainly in contaminated food and biological samples at very low levels. Among them, AFB1 is
considered the most toxic, being classified as a human carcinogen. The analysis of food and biological samples is very complex and includes different steps as extraction, clean-up, separation, and detection approaches. This chapter reports the main analytical procedures developed for the AF determination by LC-HRMS. Different sample preparation techniques have been proposed, being QuEChERS, SPE and SLE the more frequently used. New nanomaterials including magnetic nanoparticles have been recently applied as adsorbents, increasing extraction efficiency and specificity. Separation of AFs is usually performed using HPLC, which performance was improved when using UHPLC. Different detectors are proposed, being MS or MS/MS widely applied, ensuring a specific confirmation for targeted analysis. However, the toxicological pathway of AFs in biological samples leads to the appearance of modified or masked mycotoxins, whose structures must be accurately established, making their detection difficult using routine analytical methods. On the other hand, the lack of commercial analytical standards results a great challenge for accurate identification and quantitation of modified AFs. In this field, HRMS has proven to be a very effective tool to enable the rapid determination of both parent and modified AFs. The use of metabolomic platforms combined with HRMS is nowadays considered the most appropriate way to study the toxicokinetic behavior of AFs in order to establish, when possible, maximum tolerable intakes and to investigate whether they have any relationship with certain clinical pathologies and cancer processes.

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Abbreviations

AA acetic acid
ACN acetonitrile
AFAR aflatoxin aldehyde reductase
AFL Aflatoxicol
AFs aflatoxins
AFB1 aflatoxin B1
AFB2 aflatoxin B2
AFBO aflatoxin-8,9-epoxide
AFG1 aflatoxin G1
AFG1 aflatoxin G2
AFM1 aflatoxin M1
AFM2 aflatoxin M2
AFP1 aflatoxin P1
AFQ1 aflatoxin Q1
AIF all-ion fragmentation
AP aputinic
DART direct analysis in real time
2D Two-dimensional
dd-MS² data dependent
DIA data independent
DMSPE dispersive magnetic solid-phase extraction
DPEP dipeptidase
DSPE dispersive solid-phase extraction
ED enzymatic digestion
ESI electrospray ionization
EtAc ethyl acetate
EtOH ethanol
FA formic acid
FAPy formamidopyramidine
FI flow injection
GGT \( \gamma \)-glutamyltransferase
GSH glutathione
GST glutathione S-transferase
HCD high energy collision dissociation
HESI heated electrospray ionization
HRMS high resolution mass spectrometry
HVACP high-voltage atmospheric cold plasma
IACs immunoaffinity columns
IARC International Agency for Research on Cancer
IDMS isotope dilution mass spectrometry
IT ion trap
LC liquid chromatography
LLE liquid–liquid extraction
LOD limit of detection
LOQ limit of quantification
lys lysine
MAE microwave assisted extraction
MDSPE magnetic dispersed solid-phase extraction
MeOH methanol
MS mass spectrometry
MS/MS tandem mass spectrometry
MWCNT multi-walled carbon nanotubes
NAT N-acetyltransferase
NPs nanoparticles
PBS phosphate-buffered solution
PFP pentafluorophenyl
PP protein precipitation
PRM parallel reaction monitoring mode
Q quadrupole
QqQ triple quadrupole
QuEChERS quick easy cheap effective rugged and safe
SIM selected ion monitoring
SLE solid–liquid extraction
SPE solid-phase extraction
TOF time-of-flight
TOF-SIMS time-of-flight secondary ion mass spectrometry
UAE ultrasound assisted extraction
UHPLC ultra-high performance liquid chromatography
WHO World Health Organization.
Author details

Natalia Arroyo-Manzanares, Natalia Campillo, Ignacio López-García and Pilar Viñas*
Department of Analytical Chemistry, Faculty of Chemistry, Regional Campus of International Excellence “Campus Mare Nostrum”, University of Murcia, Murcia, Spain

*Address all correspondence to: pilarvi@um.es
References

[1] Marin S, Ramos AJ, Cano-Sancho G, Sanchis V. Mycotoxins: Occurrence, toxicology, and exposure assessment. Food and Chemical Toxicology. 2013;60:218-237. DOI: 10.1016/j.fct.2013.07.047.

[2] EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific opinion on ergot alkaloids in food and feed. EFSA Journal. 2012;10(7):2798. DOI: 10.2903/j.efsa.2012.2798.

[3] Singh C, Prakash C, Mishra P, Tiwari KN, Mishra SK, More RS, et al. Hepatoprotective efficacy of Premna integrifolia L. leaves against aflatoxin B1-induced toxicity in mice. Toxicon. 2019;166:88-100. DOI: 10.1016/j.toxicon.2019.05.014.

[4] Freire L, Sant’Ana AS. Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. Food and Chemical Toxicology. 2018;111:189-205. DOI: 10.1016/j.fct.2017.11.021.

[5] Lu Q, Qin JA, Fu YW, Luo JY, Lu JH, Logrieco AF, et al. Modified mycotoxins in foodstuffs, animal feed, and herbal medicine: A systematic review on global occurrence, transformation mechanism and analysis methods. TrAC-Trends in Analytical Chemistry. 2020;133:28. DOI: 10.1016/j.trac.2020.116088.

[6] Benkerroum N. Chronic and acute toxicities of aflatoxins: Mechanisms of action. International Journal on Environmental Research and Public Health. 2020;17(2):423. DOI:10.3390/ijerph17020423

[7] Kelly JD, Eaton DL, Guengerich FP, Coulombe RA. Aflatoxin B1 activation in human lung. Toxicology and Applied Pharmacology. 1997;144(1):88-95. DOI: 10.1006/taap.1997.8117.

[8] Marchese S, Polo A, Ariano A, Velotto S, Costantini S, Severino L. Aflatoxin B1 and M1: Biological properties and their involvement in cancer development. Toxins. 2018;10(6):214. DOI:10.3390/toxins10060214.

[9] Fouad AM, Ruan D, El-Senousey HK, Chen W, Jiang S, Zheng C. Harmful effects and control strategies of aflatoxin B1 produced by Aspergillus flavus and Aspergillus parasiticus strains on poultry: Review. Toxins. 2019;11(3):176. DOI:10.3390/toxins11030176.

[10] Scientific Committee on Food. “Opinion on aflatoxins, ochratoxin A and patulin” expressed on 23 September 1994. Reports of the Scientific Committee on Food-35° Series (European Commission, DG Industry). 1996.

[11] Claeys L, Romano C, De Ruyck K, Wilson H, Fervers B, Korenjak M, et al. Mycotoxin exposure and human cancer risk: A systematic review of epidemiological studies. Comprehensive Reviews in Food Science and Food Safety. 2020;19(4):1449-1464. DOI: 10.1111/1541-4337.12567.

[12] IARC, Monographs on the evaluation of carcinogenic risks to humans. Lyon, 2012; Vol. 100F, Chemical agents and related occupations.

[13] IARC, Monographs on the evaluation of carcinogenic risks to humans. Lyon, 1993; Vol. 56, Some naturally occurring substances: Food items and constituents, heterocyclic aromatic amines and mycotoxins.

[14] IARC, Monographs on the evaluation of carcinogenic risks to humans. Lyon, 2002; Vol. 82, Some traditional herbal medicines, some mycotoxins, naphthalene and styrene.

[15] Marin DE, Taranu I. Overview on aflatoxins and oxidative stress. Toxin
Aflatoxins - Occurrence, Detoxification, Determination and Health Risks

Reviews. 2012;31(3-4):32-43. DOI: 10.3109/15569543.2012.730092.

[16] Rushing BR, Selim MI. Aflatoxin B1: A review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. Food and Chemical Toxicology. 2019;124:81-100. DOI: 10.1016/j.fct.2018.11.047.

[17] Gross-Steinmeyer K, Eaton DL. Dietary modulation of the biotransformation and genotoxicity of aflatoxin B1. Toxicology. 2012;299(2):69-79. DOI: 10.1016/j.tox.2012.05.016.

[18] Bammler TK, Slone DH, Eaton DL. Effects of dietary oltipraz and ethoxyquin on aflatoxin B1 biotransformation in non-human primates. Toxicological Sciences. 2000;54(1):30-41. DOI: 10.1093/toxsci/54.1.30.

[19] Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union 2006;L364:5-24.

[20] Directive 2002/32/EC of the European Parliament and of the council of 7 May 2002 on undesirable substances in animal feed. Official Journal of the European Communities 2002;L140:10-21.

[21] Sekera ER, Wood TD. Sequencing proteins from bottom to top: Combining techniques for full sequence analysis of glucokinase. In: Woods AG, Darie CC, editors. Advancements of mass spectrometry in biomedical research. Cham: Springer International Publishing; 2019, p.111-119. DOI: 10.1007/978-3-030-15950-4_6.

[22] Slobodchikova I, Vuckovic D. Liquid chromatography – high resolution mass spectrometry method for monitoring of 17 mycotoxins in human plasma for exposure studies. Journal of Chromatography A. 2018;1548:51-63. DOI: 10.1016/j.chroma.2018.03.030.

[23] Rubert J, Manes J, James KJ, Soler C. Application of hybrid linear ion trap-high resolution mass spectrometry to the analysis of mycotoxins in beer. Food Additives & Contaminants Part A. 2011;28(10):1438-1446. DOI: 10.1080/19440049.2011.595015.

[24] Bogdanova E, Rozentale I, Pugajeva I, Emecheta EE, Bartkevics V. Occurrence and risk assessment of mycotoxins, acrylamide, and furan in Latvian beer. Food Additives & Contaminants Part B. 2018;11(2):126-137. DOI: 10.1080/19393210.2018.1440636.

[25] Rozentale I, Bogdanova E, Bartkevics V. A rapid and sensitive method for the control of selected regulated and emerging mycotoxins in beer. World Mycotoxin Journal. 2018;11(4):503-517. DOI: 10.3920/wmj2017.2298.

[26] McMillanA, RenaudJB, BurgessKMN, Orimadegun AE, Akinyinka OO, Allen SJ, et al. Aflatoxin exposure in Nigerian children with severe acute malnutrition. Food and Chemical Toxicology. 2018;111:356-362. DOI: 10.1016/j.fct.2017.11.030.

[27] Qi DW, Fei T, Liu H, Yao HM, Wu D, Liu BZ. Development of multiple heart-cutting two-dimensional liquid chromatography coupled to quadrupole-Orbitrap high resolution mass spectrometry for simultaneous determination of aflatoxin B1, B2, G1, G2, and ochratoxin A in snus, a smokeless tobacco product. Journal of Agricultural and Food Chemistry. 2017;65(45):9923-9929. DOI: 10.1021/acs.jafc.7b04329.
extraction of mycotoxins in food using zirconia nanoparticles. Journal of Chromatography A. 2018;1561:1-12. DOI: 10.1016/j.chroma.2018.05.031.

[29] Ogawa T, Zaitsu K, Kokaji T, Suga K, Kondo F, Iwai M, et al. Development and application of a forensic toxicological library for identification of 56 natural toxic substances by liquid chromatography-quadrupole time-of-flight mass spectrometry. Forensic Toxicology. 2020;38(1):232-242. DOI: 10.1007/s11419-019-00506-w.

[30] Renaud JB, Sumarah MW. Data independent acquisition-digital archiving mass spectrometry: application to single kernel mycotoxin analysis of Fusarium graminearum infected maize. Analytical and Bioanalytical Chemistry. 2016;408(12):3083-3091. DOI: 10.1007/s00216-016-9391-5.

[31] Jia W, Shi L, Zhang F, Fan C, Chang J, Chu X. Multiplexing data independent untargeted workflows for mycotoxins screening on a quadrupole-Orbitrap high resolution mass spectrometry platform. Food Chemistry. 2019;278:67-76. DOI: 10.1016/j.foodchem.2018.11.056.

[32] Castaldo L, Graziani G, Gaspari A, Izzo L, Tolosa J, Rodríguez-Carrasco Y, et al. Target analysis and retrospective screening of multiple mycotoxins in pet food using UHPLC-Q-Orbitrap HRMS. Toxins. 2019;11(8):434. DOI: 10.3390/toxins11080434.

[33] Rubert J, León N, Sáez C, Martins CPB, Godula M, Yusà V, et al. Evaluation of mycotoxins and their metabolites in human breast milk using liquid chromatography coupled to high resolution mass spectrometry. Analytica Chimica Acta. 2014;820:39-46. DOI: 10.1016/j.aca.2014.02.009.

[34] Martínez-Domínguez G, Romero-González R, Arrebola FJ, Garrido Frenich A. Multi-class determination of pesticides and mycotoxins in isoflavones supplements obtained from soy by liquid chromatography coupled to Orbitrap high resolution mass spectrometry. Food Control. 2016;59:218-224. DOI: 10.1016/j.foodcont.2015.05.033.

[35] Martínez-Domínguez G, Romero-González R, Garrido Frenich A. Determination of toxic substances, pesticides and mycotoxins, in Ginkgo biloba nutraceutical products by liquid chromatography Orbitrap-mass spectrometry. Microchemical Journal. 2015;118:124-130. DOI: 10.1016/j.microc.2014.09.002.

[36] Martínez-Domínguez G, Romero-González R, Garrido Frenich A. Multi-class methodology to determine pesticides and mycotoxins in green tea and royal jelly supplements by liquid chromatography coupled to Orbitrap high resolution mass spectrometry. Food Chemistry. 2016;197:907-915. DOI: 10.1016/j.foodchem.2015.11.070.

[37] Wu Y, Ye J, Xuan Z, Li L, Wang H, Wang S, et al. Development and validation of a rapid and efficient method for simultaneous determination of mycotoxins in coix seed using one-step extraction and UHPLC-HRMS. Food Additives & Contaminants: Part A. 2021;38(1):148-159. DOI: 10.1080/19440049.2020.1833089.

[38] León N, Pastor A, Yusà V. Target analysis and retrospective screening of veterinary drugs, ergot alkaloids, plant toxins and other undesirable substances in feed using liquid chromatography-high resolution mass spectrometry. Talanta. 2016;149:43-52. DOI: 10.1016/j.talanta.2015.11.032.

[39] De Santis B, Debegnach F, Sonego E, Mazzilli G, Buiarelli F, Ferri F, et al. Biomonitoring data for assessing aflatoxins and ochratoxin A exposure by Italian feedstuffs workers. Toxins.
[40] Debegnach F, Brera C, Mazzilli G, Sonego E, Buiarelli F, Ferri F, et al. Optimization and validation of a LC-HRMS method for aflatoxins determination in urine samples. Mycotoxin Research. 2020;36(2):257-266. DOI: 10.1007/s12550-020-00389-6.

[41] Rodríguez-Carrasco Y, Izzo L, Gaspari A, Graziani G, Manes J, Ritieni A. Simultaneous determination of AFB1 and AFM1 in milk samples by ultra high performance liquid chromatography coupled to quadrupole Orbitrap mass spectrometry. Beverages. 2018;4(2):43. DOI: 10.3390/beverages4020043.

[42] Zhao Y, Yuan YC, Bai XL, Liu YM, Wu GF, Yang FS, et al. Multi-mycotoxins analysis in liquid milk by UHPLC-Q-Exactive HRMS after magnetic solid-phase extraction based on PEGylated multi-walled carbon nanotubes. Food Chemistry. 2020;305:125429. DOI: 10.1016/j.foodchem.2019.125429.

[43] Giang L, Thien TLT. Determination of aflatoxin B1, B2, G1, G2 in cashew nut by UHPLC-HRMS. Vietnam Journal of Chemistry 2020;58(4):540-547. DOI: 10.1002/vjch.202000027.

[44] Lattanzio VM, Gatta SD, Godula M, Visconti A. Quantitative analysis of mycotoxins in cereal foods by collision cell fragmentation-high-resolution mass spectrometry: performance and comparison with triple-stage quadrupole detection. Food Additives & Contaminants: Part A. 2011;28(10):1424-1437. DOI: 10.1080/19440049.2011.593192.

[45] Tolosa J, Graziani G, Gaspari A, Chianese D, Ferrer E, Manes J, et al. Multi-mycotoxin analysis in durum wheat pasta by liquid chromatography coupled to quadrupole Orbitrap mass spectrometry. Toxins. 2017;9(2):59. DOI: 10.3390/toxins9020059.

[46] Cho HD, Suh JH, Feng S, Eom T, Kim J, Hyun SM, et al. Comprehensive analysis of multi-class mycotoxins in twenty different species of functional and medicinal herbs using liquid chromatography-tandem mass spectrometry. Food Control. 2019;96:517-526. DOI: 10.1016/j.foodcont.2018.10.007.

[47] Picardo M, Sanchis J, Núñez O, Farré M. Suspect screening of natural toxins in surface and drinking water by high performance liquid chromatography and high-resolution mass spectrometry. Chemosphere. 2020;261:127888. DOI: 10.1016/j.chemosphere.2020.127888.

[48] Díaz de León-Martínez L, Díaz-Barriga F, Barbier O, Ortíz DLG, Ortega-Romero M, Pérez-Vázquez F, et al. Evaluation of emerging biomarkers of renal damage and exposure to aflatoxin-B1 in Mexican indigenous women: a pilot study. Environmental Science and Pollution Research. 2019;26:12205-12216. DOI: 10.1007/s11356-019-04634-z.

[49] Lauwers M, De Baere S, Letor B, Rycklik M, Croubels S, Devreese M. Multi LC-MS/MS and LC-HRMS methods for determination of 24 mycotoxins including major phase I and II biomarker metabolites in biological matrices from pigs and broiler chickens. Toxins. 2019;11(6):351. DOI: 10.3390/toxins11060351.
[51] Vaclavik L, Zachariasova M, Hrbeek V, Hajslova J. Analysis of multiple mycotoxins in cereals under ambient conditions using direct analysis in real time (DART) ionization coupled to high resolution mass spectrometry. Talanta. 2010;82(5):1950-7. DOI: 10.1016/j.talanta.2010.08.029.

[52] Ahn JH, Jeong YS, Lee TG, Kim YP, Kim HS. Sensitive and multiplexed analysis of aflatoxins using time-of-flight secondary ion mass spectrometry. BioChip Journal. 2012;6(1):34-40. DOI: 10.1007/s13206-012-6105-8.

[53] Sapozhnikova Y, Zomer P, Gerssen A, Nuñez A, Mol HGJ. Evaluation of flow injection mass spectrometry approach for rapid screening of selected pesticides and mycotoxins in grain and animal feed samples. Food Control. 2020;116:107323. DOI: 10.1016/j.foodcont.2020.107323.

[54] Di Gregorio MC, Jager AV, Souto PCMC, Costa AA, Rottinghaus GE, Passarelli D, et al. Determination of serum aflatoxin B1-lysine to evaluate the efficacy of an aflatoxin-adsorbing feed additive in pigs fed an aflatoxin B1-contaminated diet. Mycotoxin Research. 2017;33(2):93-102. DOI: 10.1007/s12550-016-0267-5.

[55] Mitema A, Feto NA, Rafudeen MS. Development and validation of TOF/Q-TOF MS/MS, HPLC method and in vitro bio-strategy for aflatoxin mitigation. Food Additives & Contaminants: Part A. 2020;37(12):2149-64. DOI: 10.1080/19440049.2020.1815861.

[56] Sirhan AY, Tan GH, Al-Shunnaq A, Abdulra’uf L, Wong RCS. QuEChERS-HPLC method for aflatoxin detection of domestic and imported food in JORDAN. Journal of Liquid Chromatography & Related Technologies. 2014;37(3):321-42. DOI: 10.1080/10826076.2012.745138.

[57] Li W-k, Zhang H-x, Shi Y-p. Simultaneous determination of aflatoxin B1 and zearalenone by magnetic nanoparticle filled amino-modified multi-walled carbon nanotubes. Analytical Methods. 2018;10(27):3353-63. DOI: 10.1039/C8AY00815A.

[58] Xie HL, Wang XP, Zhang LX, Wang T, Zhang W, Jiang J, et al. Monitoring metabolite production of aflatoxin biosynthesis by Orbitrap Fusion mass spectrometry and a D-optimal mixture design method. Analytical Chemistry. 2018;90(24):14331-8. DOI: 10.1021/acs.analchem.8b03703.

[59] Tiwari S, Shankar J. Integrated proteome and HPLC analysis revealed quercetin-mediated inhibition of aflatoxin B1 biosynthesis in Aspergillus flavus. 3 Biotech. 2018;8:47. DOI: 10.1007/s13205-017-1067-0.

[60] Arroyo-Manzanares N, Diana Di Mavungu J, Uka V, Malysheva SV, Cary JW, Ehrlich KC, et al. Use of UHPLC high-resolution Orbitrap mass spectrometry to investigate the genes involved in the production of secondary metabolites in Aspergillus flavus. Food Additives & Contaminants: Part A. 2015;32(10):1656-73. DOI: 10.1080/19440049.2015.1071499.

[61] Wang RQ, Liu RJ, Chang M, Jin QZ, Huang JH, Liu YF, et al. Ultra-performance liquid chromatography quadrupole time-of-flight MS for identification of electron beam from accelerator degradation products of aflatoxin B1. Applied Biochemistry and Biotechnology. 2015;175(3):1548-56. DOI: 10.1007/s12100-014-1377-1.

[62] Liu RJ, Wang RQ, Lu J, Chang M, Jin QZ, Du ZB, et al. Degradation of AFB1 in aqueous medium by electron beam irradiation: Kinetics, pathway and toxicology. Food Control. 2016;66:151-7. DOI: 10.1016/j.foodcont.2016.02.002.

[63] Shi H, Cooper B, Stroshine RL, Ileleji KE, Keener KM. Structures of
degradation products and degradation pathways of aflatoxin B$_1$ by high-voltage atmospheric cold plasma (HV ACP) treatment. Journal of Agricultural and Food Chemistry. 2017;65(30):6222-6230. DOI: 10.1021/acs.jafc.7b01604.

[64] Liu YF, Li MM, Liu YX, Bian K. Structures of reaction products and degradation pathways of aflatoxin B$_1$ by ultrasound treatment. Toxins. 2019;11(9):526. DOI: 10.3390/toxins11090526.

[65] Liu RJ, Jin QZ, Tao GJ, Shan L, Huang JH, Liu YF, et al. Photodegradation kinetics and byproducts identification of the aflatoxin B$_1$ in aqueous medium by ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry. Journal of Mass Spectrometry. 2010;45(5):553-559. DOI: 10.1002/jms.1741.

[66] Liu RJ, Jin QZ, Tao GJ, Shan L, Liu YF, Wang XG. LC-MS and UPLC-quadrupole time-of-flight MS for identification of photodegradation products of aflatoxin B$_1$. Chromatographia. 2010;71(1-2):107-112. DOI: 10.1365/s10337-009-1354-y.

[67] Chang M, Jin QZ, Liu YF, Liu RJ, Wang XG. Efficiency and safety evaluation of photodegradation of aflatoxin B$_1$ on peanut surface. International Journal of Food Science & Technology. 2013;48(12):2474-2479. DOI: 10.1111/ijfs.12238.

[68] Wang Y, Zhang HY, Yan H, Yin CH, Liu Y, Xu QQ, et al. Effective biodegradation of aflatoxin B$_1$ using the Bacillus licheniformis (BL010) strain. Toxins. 2018;10(12):497. DOI: 10.3390/toxins10120497.

[69] Sangare L, Zhao YJ, Folly YME, Chang JH, Li JH, Selvaraj JN, et al. Aflatoxin B$_1$ degradation by a Pseudomonas strain. Toxins.