Chapter

Aflatoxin and Mycotoxin Analysis: An Overview Including Options for Resource-limited Settings

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Abstract

Aflatoxins are fungal toxins of serious human health concern, more so in some developing countries where significant contamination of staple foods occurs and the prevalence of aflatoxin-related health effects is high. A plethora of techniques for food mycotoxin testing has been developed. Modern chromatographic techniques allow quantitative determination with high accuracy and sensitivity, but are expensive and difficult to operate and maintain. Rapid tests provide a cheaper alternative for screening large numbers of samples, although they need validation on all food matrices that are tested. One important aspect of tackling aflatoxin contamination and exposure is to ensure the availability of suitable methods for detection and quantification that are rapid, sensitive, accurate, robust, and cost-effective for food surveillance in resource-limited settings.

Keywords: mycotoxins, analysis, food safety, rapid tests

1. Introduction

Food contamination with mycotoxins is a serious human health concern worldwide and of greatest significance in developing countries [1, 2]. Of all the mycotoxins, aflatoxins are more toxic, widespread in nature, and have been associated with significant health effects in humans and reduced productivity in farmed animals [3–5]. Recent estimates suggest that 60–80% 0f crops contain detectable concentrations of mycotoxins. In many instances, there is co-contamination with more than one toxin and this is geographically dependent on climate and farming practices [6, 7].

Aflatoxins affect approximately 4.5 billion people in developing countries, causing acute fatal hepatitis in individuals exposed to highly contaminated grains. Low level, chronic exposure to aflatoxins is associated with the development of liver cancer in adults, reduced immunity, and lowered growth and stunting in infants and children [8–10]. Monitoring food for contamination with aflatoxins is essential, although a number of challenges must be faced, including low concentrations and variable distribution of the toxin in contaminated grains within storage facilities. These factors

will contribute to variable test results as well as issues related to test sensitivity and specificity in varied food matrices [5, 11].

This chapter provides an overview of sample extraction and cleanup procedures, together with analytical techniques developed for mycotoxins, including aflatoxins. The advantages and disadvantages of the different approaches affecting suitability for use in aflatoxin food surveillance and quantitative confirmation are outlined. In developing countries, rapid tests make a significant contribution to aflatoxin control and a perspective on their application in resource-limited settings is given.

2. General mycotoxin analytical techniques

Mycotoxins present a great analytical challenge. Not only do they include a diversity of chemical compounds, but are heterogeneously distributed at varying concentrations in a wide range of agricultural commodities, foods, feeds, and biological samples that require specific extraction, cleanup, separation, and detection methods [11]. Some mycotoxins, especially deoxynivalenol and zearalenone, are conjugated as a result of plant metabolism, and these "masked" mycotoxins may contribute 20% of total of the parent mycotoxin but are not detected during conventional analysis [5, 11].

Quantification of mycotoxins requires expensive laboratory equipment that needs well-trained personnel to operate [12], as well as involving a series of steps and procedures that may be laborious and time-consuming [11]. The need for high sensitivity tests to detect the minimum levels of the mycotoxin possible for regulatory purposes, coupled with rapidity, high accuracy, simplicity, robustness, and selectivity have been the main driving forces behind the improvement and development of new mycotoxin analytical protocols [11, 13]. Mycotoxin analysis is essential to quantify the toxin for risk evaluation, diagnosis, and monitoring mitigation strategies [5].

3. Sampling

Sampling for aflatoxin determination in food commodities poses a particular challenge given uneven toxin distribution and the low levels at which mycotoxins occur [5]. As a result, some national and international food safety authorities and organizations have prescribed sampling methods for various food commodities for the purpose of achieving representative samples that may be used to determine concentrations of various mycotoxins in foodstuffs for official control purposes; sampling is potentially the biggest source of error in mycotoxin testing [14]. For many commodities, detailed sampling plans have been devised [15]. To obtain a representative sample from a grain storage facility, for example, incremental samples have to be taken from different places of the facility [11] with the entire primary sample ground, mixed, and subsampled to ensure that the analyzed portion has a similar toxin concentration as the original sample [11, 16].

4. Analytical procedures

Analytical procedures for mycotoxins entail extraction from the matrix with a suitable solvent, cleanup of co-extracted matrix components, and identification/

quantification of the toxin using suitable analytical facilities [12, 17]. Some exceptional techniques, such as infra-red spectroscopy, may detect mycotoxin contamination directly in ground samples without prior solvent extraction or cleanup but are limited to screening purposes because of high matrix interference and lack of suitable calibration materials [11]. Although additional cleanup is essential for chromatographic determination, the diluted extracts may be directly used with immunoanalytical methods [13].

5. Sample extraction

Extraction liberates the mycotoxin from the sample matrix with subsequent extract cleanup to reduce matrix interference, hence improving the sensitivity and robustness of the technique [11, 18]. Depending on the physicochemical properties of the mycotoxins and sample matrix, various combinations of extraction solvents may be used [11]. Relatively polar solvents, such as methanol, acetone, acetonitrile, ethyl acetate, diethyl ether, 1-octanol, toluene, dichloromethane, chloroform, or a mixture of them, may provide efficient extraction of mycotoxins, with minimal addition of water and acid solution helping to enhance extraction efficiency [19, 20]. A suitable extraction solvent should only remove the mycotoxins from the sample with high efficiency as well as being cheap, safe to use, and reduce matrix interference. For this purpose, mixtures of methanol-water and acetonitrile-water at appropriate ratios are the most frequently used extraction solvents for mycotoxin analysis [20].

Other parameters, such as sample/extractive solvent ratio, temperature, and time of extraction, may affect the extraction process; therefore, need to be carefully controlled to achieve accurate quantification [16]. High temperature and pressure instruments, such as accelerated solvent extraction/pressurized liquid extraction and microwave-assisted extraction methods hasten the process by speeding up and automating the extraction, use less solvent and provide better extraction efficiencies (in terms of extraction yield and/or recovery) compared to classical solvent extraction techniques. However, they are limited by the high cost of the equipment and may not be suitable for thermally unstable analytes [11, 16]. Non-polar solvents, such as hexane and cyclohexane, may be used before or following the extraction procedure to remove lipids in certain sample types, for example, groundnuts and maize [16, 19]. The presence of pigments, essential oils, and fatty acids in some samples may make extraction difficult and necessitate the use of different extraction solvents, such as a mixture of ethyl acetate-formic acid [20]. Chlorinated solvents are considered to be toxic and ecologically harmful, hence should be avoided, where possible in the extraction process [12]. Deep eutectic solvent has been recently reported as an environmentally safe extraction solvent limiting the use of traditional solvents and derivatization reagents [20].

Extraction is usually enabled by the high-speed blending of ground sample-extraction solvent mixture or employing a mechanical shaker followed by filtration before subsequent purification step, where applicable [19]. Evaluation of extraction procedures based on methanol-water and acetone-water in maize found [21] that the acetone-water mixture (6 + 4 v/v) showed the best extraction efficiency for all aflatoxins (B_1, B_2, G_1, and G_2) compared to the commonly used mixtures of methanol-water (8 + 2 v/v) or acetone-water (85 + 15 v/v).

Purification of sample extracts is required to reduce matrix-induced signal suppression or enhancement in mycotoxin detection [11, 19, 22]. Immunoaffinity

columns (IAC), solid-phase extraction (SPE), column chromatography, multi-functional columns, and liquid-liquid partitioning (LLE) may be used for purification purposes with the purified sample reconstituted in a suitable solvent before chromatographic analysis [13].

5.1 Solvent extraction methods

5.1.1 Liquid extraction/partitioning

Liquid extraction or partitioning is a common and arguably the simplest method of sample purification relying on the solubility of the target compounds in a particular solvent, and the insolubility of competing or interfering compounds in the same solvent [18].

5.1.2 Liquid-liquid partitioning/extraction

Liquid-liquid extraction (LLE) is used repetitively to extract analytes quantitatively by concentrating those analytes that migrate between two partitioned immiscible solvents [19]. In LLE, traditional solvents with a low dielectric constant (those that tend to be immiscible with water) are poor at extracting polar compounds, including most mycotoxins. Suitable solvents, such as methanol or acetonitrile, should be mixed with water in the presence of salts to reduce the mutual miscibility, allowing the polar analytes to move selectively into the polar organic phase from the aqueous phase [18]. Solvents, such as hexane and cyclohexane, for example, may be used to remove non-polar contaminants, for example, lipids and cholesterol through liquid-liquid extraction [23]. However, the method is used infrequently because it is labor intensive, uses vast amounts of solvent, leads to losses, and is time-consuming [13, 19].

5.1.3 Dispersive liquid-liquid microextraction

Dispersive liquid-liquid microextraction (DLLME) is a recently introduced miniaturized extraction procedure. The technique is based on the formation of a cloudy solution consisting of fine droplets of the extractant solvent dispersed entirely in the aqueous (continuous) phase. This occurs following the rapid addition of a mixture of a water-immiscible extractant solvent, and a water-miscible dispersive solvent into an aqueous solution containing the analytes. As a result of a very large surface area formed by the dispersed extractant micro-droplets, the analytes are rapidly and efficiently enriched in the extractive solvent and, after centrifugation, can be separated in the sediment phase [16, 24].

This technique is cheap, environmentally safe, simple, fast, and efficient [16]. However, it is difficult to automate and necessitates using a third component (disperser solvent), which commonly decreases the partition coefficient of analytes into the extractant solvent [24].

5.1.4 Vortex-assisted liquid-liquid microextraction

Vortex-assisted liquid-liquid microextraction is a new equilibrium-based solvent microextraction technique. It is based on the dispersion of micro-droplets of the extraction solvent into the aqueous sample and is achieved by vortex agitation,

forming a mild emulsification process [24, 25]. Separation of the two phases occurs upon centrifugation, with the floating extractant phase restoring its original single micro drop shape; it is easily collected with the help of a microsyringe and used for HPLC analysis [24, 26].

Several experimental parameters, namely, organic solvent, agitation time, rotational speed of the vortex agitator, acceptor phase volume, aqueous sample volume, pH, and salt addition may affect the extraction process, and these need to be controlled and optimized for optimum performance of the procedure [24]. Surfactants, such as Triton X-114, Tween-20, Triton X-100, and cetyltrimethylammonium bromide (CTAB), may be used to enhance extraction efficiency [25].

This technique is rapid, as the fine droplets formed, extract target analytes toward equilibrium faster because of the shorter diffusion distance and larger specific surface area compared to the DLLME where the need for a disperser solvent is mandatory [24, 26].

5.1.5 Dilute and shoot method

The dilute-and-shoot (DaS) method utilizes the improved sensitivity and robustness of modern equipment. It is based on dilution followed by direct injection of samples that are presumed to be inherently clean enough to not require full preparation, thus reducing cost. It has the benefits of rapidity, can work with multiple analytes, and limits the potential loss of analyte due to pretreatment, although it still has a risk of matrix interference that can overwhelm instrument sensitivity [18].

5.2 Solid-phase extraction methods

5.2.1 Solid-phase extraction

The solid-phase extraction (SPE) technique utilizes small disposable cartridges packed with silica gel or bonded phases that are in the stationary phase to bind impurities or target analytes. The impurities can be washed off, and the analyte recovered using a suitable rinse solution [19, 23, 27].

In SPE, the aqueous sample extract is applied to the conditioned column followed by rinsing to remove matrix compounds, with the analyte eluted from the column using an organic solvent. Evaporation of excessive solvent can be employed for further concentration [13, 19].

Compared to LLE, SPE has the advantage of rapidity, efficiency, reproducibility, uses considerably less solvent, and offers a wide range of selectivity, however, it is limited by the fact that there is no single fit-for-all cartridge [19, 23].

5.2.2 Ion-exchange columns

Ion-exchange columns use ionic materials, such as SAX (strong anion exchange) in SPE to extract mycotoxins that present as ions, such as moniliformin, in aqueous solutions. The target molecule is bound to charged groups on the silica material and removed by the addition of a strong ionic solution because of its higher affinity to the sorbent or by the altered pH [19, 23].

5.2.3 Matrix solid-phase dispersion

Matrix solid-phase dispersion (MSPD) utilizes some SPE sorbent materials (usually octadecyl silica, silica gel, or alumina) that is ground typically with 1 g of a homogenized sample using a pestle and mortar. The solid mixture is then transferred to a glass column or cartridge containing a lower layer of co-sorbent material, such as carbon black, with the adsorbed residues selectively eluted with an appropriate solvent [16, 28, 29]. This technique has the advantages of flexibility and versatility and can be used in a single step with small amounts of sorbent and solvent, thus reducing the cost and time of analysis. However, it is not easily automated, often requiring an additional cleanup step that could be time-consuming for a large number of samples [16, 29].

5.2.4 Solid-phase microextraction

Solid-phase microextraction (SPME) combines extraction and concentration of analytes in a single step and is based on the extraction of analytes by adsorption to a thin fiber coated with different stationary phases. This is followed by thermal desorption into a heated injector for gas chromatography or with a solvent when liquid chromatography is used [16]. It is simple, safe, and has a wide application on polar and non-polar compounds [30]. However, it has the disadvantage of high cost, fiber fragility, and is susceptible to experimental conditions that can affect reproducibility and sensitivity [16, 31].

5.2.5 Micro-solid phase extraction

The recently introduced micro-solid phase extraction (μ-SPE) uses a sorbent material trapped in a porous membrane sheet to extract the analyte diffusing through it, the μ-SPE device tumbling to stir the process facilitating the mass transfer. Following extraction, desorption is carried out by ultrasonification with the extraction device immersed in a suitable organic solvent. The technique is simple as extraction and cleanup steps are carried out simultaneously and it uses less solvent and sorbent materials [30].

5.2.6 Magnetic solid-phase extraction

Magnetic solid-phase extraction (MSPE) is a new SPE technique that is based on the use of magnetic nanoparticles that are dispersed into the sample solution with separation effected by applying an external magnetic field outside the sample solution [31]. The technique avoids time-consuming column or filtration operations encountered in SPE with the large contact area between the adsorbent and the analyte ensuring a fast mass transfer, which guarantees high extraction efficiency compared to the SPME technique [31, 32].

5.2.7 Immunoaffinity columns

Immunoaffinity columns (IACs) are increasingly used for the cleanup and enrichment of sample extracts [11]. The column containing mycotoxin-specific antibodies bound to solid phase support within the cartridge selectively binds the mycotoxin in the extract. Mycotoxin desorption is achieved using a miscible solvent or by antibody denaturation [16, 19].

Compared to traditional solid-phase cleanup techniques, IAC is more sensitive, specific, easy to use, rapid, safe (minimizes use of hazardous solvents), and robust in terms of applicability to different matrices. However, columns are single-use, more expensive, suffer from storage limitations and stability problems regarding organic solvents, and the possibility of nonspecific interactions due to cross-reactivity with other mycotoxins [11, 12, 16, 19]. However, there is now a commercially available immunoaffinity column ('Myco 6in1'; Vicam, Milford, MA, USA) that may be used in a cleanup procedure for simultaneous determination of multiple mycotoxins [33] that helps mitigate the single use of these IACs.

5.2.8 MycoSep®/Multisep® columns

Mycosep® /Multisep® columns contain selected adsorbents packed in a plastic tube to recover individual mycotoxins from a sample extract [23]. Despite the practicability of the method, the columns are designed per analyte, hence not suitable for multi-toxin determination and may not provide effective purification for some matrixes [16, 23].

5.2.9 Molecular imprinted polymers and aptamers

Synthetic systems, such as molecular imprinted polymers (MIPs), aptamers, and peptides, have been developed to counter shortcomings related to the use of antibodies in IACs [20].

The molecular Imprinted Polymer (MIP) is a synthetic material providing an artificially generated three-dimensional network that is able to specifically rebind a target molecule. It is a cheaper alternative for mycotoxin cleanup and preconcentration as well as affording chemical and thermal stability and solvent compatibility, which is contrary to immunoaffinity columns [11, 34].

During molecular imprinting, cross-linked polymers are formed by free-radical copolymerization of functional monomers. The cross-linking occurs in the presence of an analyte serving as a template followed by template removal by liquid extraction (washing). This leaves highly selective three-dimensional binding pockets complementary in size, shape, and functionality to the imprinted molecule remaining in the polymer matrix [13]. Despite offering promise for future application, MIP may still be affected by the low specificity and robustness of the technique in terms of kinetics, reuse, ability to withstand unfavorable solvents, and potential sample contamination by template bleeding [23].

On the other hand, aptamers are small fragments of oligonucleotide sequences (single-stranded DNA or RNA), usually containing 10 to 100 bases that bind to their targets by folding into specific three-dimensional structures [35]. Compared to antibodies, they are cheap, stable, reversible, not limited by immunogenicity of targets, and do not require immunization of animals during production [35]. Although difficult to develop, they provide an important avenue for exploitation in mycotoxin cleanup procedures and in sensing instruments [20, 35].

5.2.10 QuEChERS extraction/cleanup

QuEChERS (quick, easy, cheap, effective, rugged, and safe) as a sample pretreatment technique entails solvent extraction, partitioning with magnesium sulfate and other salts, such as NaCl, and cleanup using a dispersive solid-phase extraction

(d-SPE) sorbent, especially the primary secondary amine (PSA) and extract centrifugation before analysis [36]. Magnesium sulfate along with NaCl is used to reduce water in the sample during extraction, while PSA retains co-extracted compounds during cleaning [16]. This procedure is simple, rapid, cost-effective, and enables multi-residue determination [16, 36]. The use of QuEChERS is becoming a popular alternative to the dilute-and-shoot approach for multi-mycotoxin determination using LC/MS-based techniques to reduce matrix interference [37]. However, it should be noted that the several QuEChERS commercial kits or QuEChERS-like protocols differ in extraction, partitioning, or dispersive solid-phase extraction (dSPE) steps. They, therefore, may show different cleanup efficiencies, and for optimization an additional cleanup step may be needed to improve the performance of QuEChERS protocols [38].

6. Toxin determination

6.1 Conventional analytical techniques

Conventional analytical methods employ chromatographic separation, particularly, liquid chromatography (LC), thin layer chromatography (TLC) and gas chromatography (GC) coupled to a detection system, with high-performance liquid chromatography (HPLC) after immunoaffinity cleanup combined with fluorescence detection (FLD) or mass spectrometry (MS) frequently employed for the quantitative determination of regulated mycotoxins in food [11, 18].

6.1.1 Thin layer chromatography

Thin layer chromatography (TLC) was traditionally the most widely used mycotoxin screening technique that did not require expensive equipment and enabled high sample throughput [23]. The TLC techniques lack separating power that limits discrimination of co-extracted interference from the analyte of interest. This may, however, be overcome through improved modern cleanup techniques that remove impurities [12].

6.1.2 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is now most commonly used for mycotoxin determination offering good sensitivity and precision, coupled with ease of automation [12, 19]. After extraction and cleanup, samples are injected into the HPLC column, individual compounds are separated based on their affinity for the column matrix and the mobile phase solvent [27]. To enhance fluorescence for better mycotoxin quantification using the HPLC-FLD technique, derivatization is important [12]. Pre-column derivatization with trifluoroacetic acid (TFA), or post-column derivatization, with bromine or iodine, can be used to identify aflatoxins [17]. Photochemical post-column derivatization may provide a cheaper alternative, whereas specific cyclodextrins may be incorporated in the mobile phase for non-chemical enhancement of fluorescence [28]. Despite offering good sensitivity and specificity, HPLC-FLD techniques are limited by expensive equipment requiring operation by experienced staff and may require laborious sample preparation procedures [12, 19].

Recent utilization of ultra-HPLC (UHPLC) methods that are based upon increasing the mobile phase pumping pressure up to 1000 bar and above and reduction of particle size from 5 μm (HPLC) to 1–2 μm (UHPLC) improves resolution, sensitivity, and achieves rapid chromatographic separation as a result of increased speed and resolution between analytes [39, 40]. However, to avoid the high cost of the UHPLC system, columns packed with materials having solid core particles, coated with an outer layer of porous material can achieve more efficient separations at a much faster rate than with standard columns eliminating the need for expensive high-pressure facility because they are able to work at standard pressures (up to 600 bar) and can be used on all HPLC systems [41].

6.1.3 Liquid chromatography/mass spectrometry

Liquid chromatography with mass spectrometry (LC/MS) is a technique that allows more sensitive and selective determination of multiple mycotoxins in complex matrices with improved limits of detection and quantification [27]. Atmospheric pressure chemical ionization (APCI), atmospheric pressure photo ionization (APPI), and electrospray ionization (ESI) interfaces are currently employed in modern LC/MS instrumentation owing to their robustness, easy handling, high sensitivity, accuracy, and analyte selectivity and compatibility to a wide range of compound polarities [22, 42].

Liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) equipment has been developed that can significantly increase the sensitivity and specificity of multi-mycotoxin assays [37].

Liquid chromatography with tandem mass spectrometry (LC-MS/MS) has been increasingly used to provide a simultaneous determination of different classes of mycotoxins, including the regulated mycotoxins, thus affording to increase sample throughput and decreasing the cost per analysis [11]. However, given the complexity of matrixes to be analyzed and the wide-ranging physical and chemical mycotoxin properties, such methods require great skill to develop [11, 22, 42].

Mass spectrometry detectors linked to HPLC, UHPLC, and GC systems can increase their separation and identification power. However, the MS facilities are expensive with complex laboratory requirements, require skilled operators, and may suffer solvent limitations [23].

6.1.4 Gas chromatography

Gas chromatography (GC) may be used to determine mycotoxins that are volatile within the column [19]. For example, GC coupled with electron capture detection (ECD), flame ionization detection (FID) or mass spectrometric detection (MS) may be used for trichothecene or patulin determination. However, when compared to alternative methods, GC requires prior cleanup of extracts and pre-column derivatization to increase the volatility and sensitivity of the toxins [11, 17]. Although having successful applications, GC has several disadvantages that include the analyzed sample to be volatile or converted into a volatile sample, problems with the thermal stability of the sample leading to losses, and the high cost of the equipment [23].

6.2 Rapid screening methods

Rapid screening methods, that include immunochemical techniques, varying from simple lateral flow and enzyme-linked immunosorbent assays (ELISA) to highly

sophisticated immunosensors, are based upon binding of an antigen, for example a mycotoxin to a specific antibody, and often do not require any cleanup or analyte enrichment steps [11, 18, 43].

6.2.1 ELISA techniques

The enzyme-linked immunosorbent assay (ELISA) technique utilizes a specific antibody to bind the target molecule directly or conjugated with the enzyme and interaction with a chromogenic substrate to give a measurable result [18, 23]. However, due to the low molecular weight of mycotoxins, they are not immunogenic and must be conjugated to a carrier molecule to achieve immunogenicity. The ELISA technique can be highly sensitive and specific, portable, rapid, and simple to use with high sample turnover. However, ELISA has a number of disadvantages that include single-use kits that can increase the cost of bulk screening, high matrix dependence, cross-reactivity, and limited detection range due to the narrow sensitivity of the antibodies [13, 23].

6.2.2 Immunosensor/biosensor techniques

Biosensors are based upon the interaction of a mycotoxin with a recognition system fabricated as a layer onto the surface of a matrix substance that induces a change that is converted into a measurable electronic signal by a transducer. This provides great sensitivity and selectivity, easy application, low cost, and portability [27, 44]. Biosensors are often classified by the type of toxin-binding element (e.g., antibody, aptamer, imprinted polymers, etc.) as well as by the technology used for signal transduction and detection (e.g., optical, electrochemical, piezoelectric, etc.) [33].

A number of biosensor/immunosensor assays and techniques have been developed for mycotoxin determination, including fiber optic devices, surface plasmon resonance (SPR), dip-stick and lateral flow devices, fluorescence polarization, time-resolved fluorescence, microbead, capillary electrophoresis (CE), and electrochemical and piezoelectric immunoassays [45, 46]. These techniques are outlined in the following subsections.

6.2.2.1 Optical biosensors and fiber optic devices

Optical sensors, based on a variation of optical signals generated by a transducer from molecular recognition events on a sensing element are divided into many subclasses depending on the type of signal generated, including calorimetric, fluorescent, chemiluminescent, and surface plasmon resonance [35]. Photoelectrochemical optical biosensors use light as an excitation source and photocurrent as the recognition signal, whereas another subset of optical biosensors uses total internal reflection ellipsometry with localized surface plasmon resonance for detection with an optical planar waveguide polarization interferometer [33]. For example, fluorescent-based fiber optic devices can capture fluorescence emission from the fluorescently labeled mycotoxin or the naturally fluorescent mycotoxin, for example, aflatoxin when they bind to the fiber optic surface and transmit it to a sensitive detector [45]. A commercial device "Octet" based on biolayer interferometry to detect changes in the interference pattern of light reflected from the surface of optical fiber when materials bind to the tip of the fiber has been developed and available from ForteBio (Menlo Park, Calif., USA) [47, 48].

6.2.2.2 Surface plasmon resonance

The SPR technique is based upon the property that the binding of materials to a surface, for example, the binding of antibodies to the mycotoxin, can alter the refractive index near that surface. The SPR device measures the small changes in the angle, or intensity, of internally reflected light that results from the binding event, and the magnitude of the response is influenced by the amount of material adhering to the surface. Alternatively, surface plasmons may be used to excite fluorophores captured on a surface, a technique is known as surface plasmon-enhanced fluorescence spectroscopy (SPFS). With this technique, light is used to excite plasmons (electron charge density waves) in a thin film of gold foil attached to the surface of a glass prism, the resonance of which enhances the fluorescence of the captured fluorophores, for example, the labeled antibody [45, 46, 49]. Using imaging, SPR (iSPR) allows multiple binding events on different regions of the sensor surface to be monitored simultaneously (multiplexing), hence capable of measuring multiple antigen-antibody interactions simultaneously in a single injection [49, 50].

The advantages of SPR include rapid and simple cleanup procedures, short analysis times, reusable sensor chips, and not necessarily requiring competition or labeled reagents for detection. It has great potential for multiplexing, with a wide variety of commercially available devices [46, 49]. However, like most immunoassays, SPR can be influenced by matrix effects that can be dealt with by increasing the dilution of the sample extract or by cleanup of the extract before the detection step [46].

6.2.2.3 Lateral flow devices

Lateral flow strip and dipstick devices (immunochromatographic test devices) use rapid disposable devices that may be attached with the toxin or the antibody that can bear enzymatic, liposome associated, or colloidal gold labels to detect the presence of mycotoxins [45]. Colloidal gold is frequently used as a label in test strips developed for mycotoxins due to availability, ease of production, and ease of conjugate formation with antibodies [51]. "Mycotoxin in the sample extract interacts with colloidal gold conjugated anti-mycotoxin antibodies at the base of the stick, with both bound and unbound antibodies moving along the stick membrane, passing a test line composed of immobilized mycotoxin, which will bind free antibody to form a visible line indicating a level of aflatoxin contamination below the test cut-off value. The control line further along the stick is composed of anti-antibodies to ensure complete extract migration along the strip" [28].

The related, membrane-based flow-through device, also known as enzyme-linked immunofiltration assay (ELIFA) differs from lateral flow devices, in that the applied liquid flows perpendicularly through the membrane rather than laterally, where it is collected on an absorbent pad on the opposite side of the membrane. It uses an enzymatic label that requires a substrate-incubation step, with the test and control lines being generated by an enzyme-substrate color reaction [28, 45].

Because of their easy application, efforts to develop dipstick and lateral flow assays for mycotoxins are likely to continue, particularly using stable, nonenzymatic labels [45], with a number of devices already being commercially available [17]. Also, innovative labels based on nanoparticle applications, such as quantum dots (QDs), gold nanoparticles (AuNPs), magnetic nanoparticles (Fe_3O_4), carbon nanoparticles (CNPs), time-resolved fluorescent microspheres (TRFM), have been developed for signal amplification in LFD, which can improve detection. Moreover, the advent of a

fluorescence quenching principle in lateral flow immunoassays (LFIA) in contrast to traditional competitive LFIA increases the sensitivity of the LFIA [35, 52].

6.2.2.4 Fluorescence polarization and time-resolved fluorescence

Fluorescence polarization (FP) immunoassays are solution-phase assays that rely on the measurement of change in the rate of rotation of a fluorescent-labeled mycotoxin (tracer) when it forms an immune complex with the added antitoxin antibody after competing with unlabeled mycotoxin in the sample extract [28, 45, 46]. FP can be used to measure the rate of association of the toxin with the antibody (kinetic assays) or the equilibrium point in a competition reaction (equilibrium assays). Critically, FP relies on the proper selection of antibody and tracer pairs [45, 46].

Unlike FP immunoassays, time-resolved fluorescent immunoassays (TR-FIA) use the property of fluorescence lifetime to measure the rate of decay of a fluorophore that is associated with a mycotoxin [45]. The newer fluorescent materials known as lanthanides, such as Eu (III) and Tb (III), have much longer fluorescence lifetimes that can eliminate the background fluorescence interference from the matrix, thus improving the sensitivity of methods based on TR-FIA [35].

The fact that FP is a homogeneous assay that does not require the separation of the free and bound tracer, may eliminate additional steps, such as washing, in competitive ELISA, thus increasing method rapidity [53]. However, like most immunoassays, it can be affected by the presence of a matrix, which can be controlled through dilution, cleanup, matrix-matched calibration curves, or data normalization [46, 53]. Although the available FP immunoassay readers are not capable of multi-mycotoxin detection, the potential speed of FP assays combined with the portability of the devices, suggests this technology has a promising future [46].

6.2.2.5 Microbead assays

Microbead assays use antibodies or antigens attached to the microbeads in miniaturized IAC assays, often with the cleanup and detection steps performed on a single instrument. It can be affected by poor re-usability of the columns due to fouling and reduced functional capacity of antibodies [45].

6.2.2.6 Capillary electrophoretic immunoassays

Capillary electrophoresis (CE) employs capillaries that are injected with the cleaned sample extracts in aqueous buffer solutions where they are separated in an electrical field before detection, typically using fluorescence or UV absorbance [23, 45]. The CE methods have comparable sensitivity, precision, and accuracy to HPLC methods, use less expensive capillaries, eliminate the use of organic solvents and take shorter analysis times, thus making them viable alternatives to HPLC [17].

6.2.2.7 Electrochemical immunosensor assays

Electrochemical immunosensors for mycotoxin determination are based on the high-affinity interaction between antigen and specific antibodies that can be transformed into a measured electrochemical signal based on a variety of electrochemical techniques [54]. They can be categorized into amperometric,

potentiometric, conductometric, impedimetric, and voltammetric sensors according to the types of detectable electrical signals [35]. In their simplest format, the immobilized antibody is bound to the surface of a screen-printed electrode, and the final enzymatic stage develops a reaction product that can be measured by its electrical properties [28].

These electrochemical assays can be affected by factors that influence the interface between antigen and antibody, including solvent-matrix interactions and the reduction/oxidation potential of the diluent. The extent of testing using this technology, the accessibility of components, and the capacity for miniaturization, suggest future utility of these devices in the detection of aflatoxins [46].

6.2.2.8 Piezoelectric sensors

Piezoelectric sensors often called quartz crystal microbalance (QCM) are based upon piezoelectric quartz crystals and they work through the application of an alternating current to a quartz crystal, which induces oscillations of the crystal, the frequency of which depends in part on the thickness of the crystal, for example, after mycotoxin binding on immobilized antibodies [46]. Mass change on the sensory layer of the surface of the gold-plated crystal quartz transducer causes specific measurable vibrations of the crystal in response to an electrical signal [20]. The advantage of QCM is that they do not require the use of labeled reagents [46].

In general terms, immunochemical techniques are affected by high matrix dependence, cross-reactivity, and loss of antibody stability under the extreme environment, such as pH, organic solvents, and high temperature. Moreover, the cost of their development may be high and requires a stable source of antibodies to ensure continuity of analytical performance and stability. Therefore, the development of synthetic receptors can solve some of these challenges, particularly, problems associated with antibody stability in an extreme environment [18, 44, 54]. As an example, [55] developed an aptamer-based assay for the detection of AFB_1 in corn samples that exhibited a wide dynamic range from 0.1 to 10 ng/mL, limit of detection of 0.11 ng/mL, and recovery values between 60.4 and 105.5% that were described as promising results.

It is worthy to note that, chemical and biochemical sensor devices are increasingly developed based on advanced microchip technology, including microfluidic chips and microarrays for portability, easy on-site field application, robustness, reliability, reduced cost, rapidity, high throughput, and increased sensitivity. Also, the advent of innovative labels based on nanoparticle application has led to a significant improvement in their detection capability. Examples of these include the microfluidic devices based on flow-through (capillary electromigration) and lateral flow formats and the emerging microchip-based sensing methods, such as surface plasmon resonance (SPR) and magnet nonotag-based detection [35, 56, 57].

6.3 Noninvasive techniques

Noninvasive techniques, such as spectroscopic and imaging techniques [27], DNA microarrays, electronic chemical sensors (electronic nose and tongue), and polymerase chain reaction-based methods [27, 44] provide a potential approach for rapid nondestructive detection of fungal infection and mycotoxin contamination on grains. However, many of these techniques may either be expensive and/or may need further validation studies.

7. Conclusions and analysis in resource-limited settings

All components of the food supply chain can become contaminated with aflatoxins and other mycotoxins. For food to meet safety standards, and for the development of mitigation strategies, determination of the degree of contamination is required. Analytical procedures for aflatoxin detection and quantification remain central to resolving this important food and feed safety issues. The many issues surrounding robust mycotoxin analysis have been addressed in a number of books [58–61] that the interested reader may wish to consult.

Since the discovery of aflatoxins, there has been a huge international effort to develop appropriate analytical procedures. However, all techniques have had to overcome a number of significant problems, including:

1. Diverse chemical structures that require individual methods for different mycotoxins;

2. Separation of structurally similar compounds;

3. Mycotoxins occur in very low concentrations in different commodities, thus removal or cleanup of the food/feed matrix is required. Each commodity may require a different cleanup procedure;

4. Due to the uneven distribution of mycotoxins in a commodity, it is important to analyze many samples that have been collected using a validated sampling plan.

As is apparent in this chapter, there are a plethora of approaches, both quantitative and qualitative, for aflatoxin analysis that overcame these problems. The advantages and disadvantages of the different analytical approaches are listed in Appendix 1 and examples of biosensor platforms for mycotoxin detection and their performance in terms of limit of detection is given in Appendix 2. Although, the conventional analytical techniques, particularly HPLC linked to the mass spectrometer or fluorescent detectors are indispensable to confirm the quantities of contamination and for determining the chemical identity of the various groups of mycotoxins, equipment is very expensive and there are ongoing instrument maintenance and solvent costs, and specially trained analysts are required. However, these techniques do not apply to resource-limited settings. The initial technique used for aflatoxin analysis was TLC, and it is still used in many laboratories, especially in developing countries, as it does not require expensive laboratory equipment.

The development of screening methods that provide rapid, low-cost analysis of large number of samples is required for food surveillance, particularly in low-income countries. For the most part, screening methods are specific, sensitive, and relatively simple to operate. There is also a need in low-income rural communities for rapid screening methods, where an electrical supply is often unavailable [62]. ELISAs and dipstick/lateral flow devices are simple to operate and are used widely in developing countries. However, before use, operators need to be confident that the assay kit is "fit for purpose," and is appropriate for the commodity matrix to be tested [62]. If the assay kit is not valid for the commodity tested, cross-reactivity may occur and the number of the false positive sample will increase. There are increasing efforts to develop multi-toxin screening assays, as aflatoxin is often found in association with other mycotoxins, including fumonisins and deoxynivalenol [6]. This information is

important, as it is essential to know the extent of toxin exposure so the appropriate public health and mitigation steps can be undertaken. Finally, it is very important that the results obtained in the field with rapid screening tests give comparable results to quantitative analysis in regulatory laboratories.

Appendix

Appendix 1.

Advantages and disadvantages of conventional mycotoxin analytical techniques.

Method	Advantages	Disadvantages
TLC	Simple, inexpensive, and rapid Can be used for screening Simultaneous analysis of multiple mycotoxins Sensitive for aflatoxins and ochratoxin A	Poor sensitivity (for some mycotoxins) Poor precision Separation may require two-dimensional analysis Quantitative when used with a densitometer
GC	Simultaneous analysis of multiple mycotoxins Good sensitivity May be automated (autosampler) Provides confirmation (MS detector)	Expensive equipment Specialist expertise required Derivatization required Matrix interference problems Nonlinear calibration curve Drifting response Carry-over effects from previous sample Variation in reproducibility & repeatability
HPLC	Good sensitivity Good selectivity Good repeatability May be automated (autosampler) Short analysis times Official methods available	Expensive equipment Specialist expertise required May require derivatization
LC/MS	Simultaneous analysis of multiple mycotoxins Good sensitivity (LC/MS/MS) Provides confirmation No derivatization required	Very expensive equipment Specialist expertise requested Sensitivity relies on ionization technique Matrix-assisted calibration curve (for quantitative analysis)
ELISA	Simple sample preparation Inexpensive equipment High sensitivity Simultaneous analysis of multiple samples Suitable for screening Limited use of organic solvents Visual assessment	Cross-reactivity with related mycotoxins Matrix interference problems Possible false positive/negative results Confirmatory LC analysis required Critical quantitation near regulatory limits Semi-quantitative (visual assessment)
Rapid tests	Simple and fast (5–10 min) No expensive equipment required Limited use of organic solvents Suitable for screening purposes Can be used *in situ*	Qualitative or semi-quantitative (cut-off level) Possible false positive/negative results Cross-reactivity with related mycotoxins Matrix interference problems Lack of sensitivity near regulatory limits

TLC - Thin Layer Chromatography, GC - Gas Chromatography, HPLC - High-Performance Liquid Chromatography, LC/MS - Liquid Chromatography with Mass Spectrometry, LC/MS/MS - Liquid Chromatography with tandem Mass Spectrometry, MS - Mass Spectrometer, ELISA - Enzyme-linked Immunosorbent Assay.
Adapted from reference [17].

Appendix 2.

Examples of biosensor platforms for mycotoxin detection.

Target	Principle	Signal material	Sample	LOD
FB1/ DON	Competitive LFIA	gold nanospheres/gold nanoflowers	Grain	20/5 ng/mL
DON/ AFB1	Competitive fluorescent LFIA	α-Fe_2O_3 nanocubes	Food	0.18/0.01 ng/mL
ZEN/ OTA/ FB1	Competitive fluorescent LFIA	Quantum dot nanobeads	Wheat	5/20/10 ng/mL
AFB1/ ZEN	Competitive fluorescent LFIA	Time-resolved fluorescence microspheres	Maize	0.05/0.07 ng/mL
DON/T-2/ZEN	Competitive fluorescent LFIA	Amorphous carbon nanoparticles	Maize	20/13/1 μg/kg
OTA/ AFB1	Optical (calorimetric)	Aptamer, magnetic nanoparticles/graphene oxide, and magnetic nanoparticles@gold	Agricultural products	0.5/5 ng/mL
AFB1/ AFG1	Optical (calorimetric)	Gold and silver nanoparticles	Pistachio, wheat, coffee, milk	2.7/7.3 ng/mL
AFB1/ OTA/ FB1	Optical (fluorescent protein microarray)	Antibody, TiO2-modified porous silicon	Rice, maize, wheat	0.093 ng/mL
AFB1/ FB1	Optical (fluorescent)	Aptamer, graphene oxide/magnetic nanoparticles, and CdTe quantum dots	Peanut	6.2/16.2 pg/mL
FB1/ OTA	Optical (fluorescent)	Aptamer, time-resolved nanoparticles, and magnetic nanoparticles	Maize	0.015 pg/mL
AFB1	Optical (fluorescent quenching)	Aptamer, CdZnTe quantum dots, and gold nanoparticles	Peanut	20 pg/mL
AFB1/ OTA	Optical (Chemiluminescence)	Antibody and silver nanoparticles	Red yeast rice	0.44/0.83 pg/ mL
AFB1	Optical (SPR)	Antibody, gold chips	Grains	2.51 ppb
AFB1	Optical (SPR)	Antibody, gold nanoparticles, and self-assembled monolayer gold chips	Wheat	0.003 nmol/L
AFB1	Electrochemical (impedimetric)	Cysteine/carbon nanotubes-modified gold electrode immunosensor	Maize flour	0.79 pg/g
ZEN	Electrochemical (differential pulse voltammetry)	Screen-printed electrode immunosensor	Beer and wine	0.25 ng/mL

Target	Principle	Signal material	Sample	LOD
AFB1	Electrochemical (cyclic voltammetry)	Graphene quantum dots and gold nanoparticles-modified indium tin oxide electrode immunosensor	Maize	0.1 ng/mL
AFB1	Electrochemical (square wave voltammetry)	Gold electrode aptasensor	Beer	2nmol/L

FB1 - Fumonisin B1, DON - Deoxynivalenol, ZEN - Zearalenone, AFB1 - Aflatoxin B1, AFG1 - Aflatoxin G1, T-2 - T-2 toxin, OTA - Ochratoxin A, LFIA - Lateral Flow Immunoassay, SPR - Surface Plasmon Resonance, LOD - Limit of Detection.
Adapted from reference [35].

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