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SPECIAL ISSUE: *Aflatoxins in maize and other crops*

Developments in detection and determination of aflatoxins

H. Yao[1*], Z. Hruska[1] and J. Diana Di Mavungu[2]

[1]Mississippi State University, Geosystems Research Institute, 1021 Balch Blvd, Stennis Space Center, MS 39529, USA; [2]Ghent University, Laboratory of Food Analysis, Harelbekestraat 72, 9000 Ghent, Belgium; haibo@gri.msstate.edu

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REVIEW ARTICLE

Abstract

Since the discovery of aflatoxins in the 1960s, much research has focused on detecting the toxins in contaminated food and feedstuffs in the interest of public safety. Most traditional detection methods involved lengthy culturing and/or separation techniques or analytical instrumentation and complex, multistep procedures that required destruction of samples for accurate toxin determination. With more regulations for acceptable levels of aflatoxins in place, modern analytical methods have become quite sophisticated, capable of achieving results with very high precision and accuracy, suitable for regulatory laboratories and for post-harvest sample testing in developed countries. Unfortunately, many countries around the world that are affected by the aflatoxin problem do not have ready access to high performance liquid chromatography and mass spectrometry instrumentation and require alternate, readily available and simple detection methods that may be used by small holdings farmers in developing countries. This paper presents an overview of the existing detection and/or determination methods for aflatoxins. The traditional, quantitative, chemically-based analytical strategies for detecting aflatoxins in maize and their evolution to the modern instrumentation routinely used in developed countries are reviewed. Additionally, novel, more streamlined, user-friendly and in some instances, non-destructive, methods that may be useful for semi-quantitative or qualitative, quick-screening of contaminated maize samples appropriate also for use in developing countries, are discussed.

Keywords: aflatoxins, detection methods, quantitative analysis, non-invasive optical methods

1. Introduction

Under unfavourable field conditions stressful to the crop, some fungi produce mycotoxins, secondary metabolites that may contaminate various agricultural commodities in the field and in storage. Aflatoxins B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1, AFG_2) are mycotoxins produced primarily by the *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* fungal species (Ehrlich *et al.*, 2003). *A. flavus* produces the B aflatoxins and *A. parasiticus* and *A. nomius* produce both B and G aflatoxins. AFB_1 is the most prevalent and most toxic of the aflatoxins and is considered the most potent naturally occurring carcinogen with a Group 1 human carcinogen designation (IARC, 1993; Streit *et al.*, 2012) targeting the liver and lungs (Otim *et al.*, 2005; Turner *et al.*, 2009). Aflatoxins M_1 and M_2 (AFM_1 and AFM_2), the monohydroxylated derivatives of AFB_1 and AFB_2, respectively, occur in the milk of lactating mammals including humans, after ingestion of food or feed contaminated with AFB_1 (Streit *et al.*, 2012). Some of the more common crops susceptible to contamination with aflatoxins are cereals (e.g. maize, rice and wheat), tree nuts (e.g. pistachios, walnuts and Brazil nuts), cottonseed and groundnuts (Shephard, 2009). The most ambient climates for aflatoxin-production are high temperature and high humidity typically found in tropical and subtropical regions of the world including sub-Saharan Africa and Southern Asia. Drought stress and insect damage are additional factors that contribute to higher occurrence of aflatoxins in more temperate zones including Europe, South America and the United States. These conditions have the potential of significantly impeding the export markets from affected areas because of greater manifestation of aflatoxins and increasingly more stringent regulations imposed by the importing countries.

Public health concerns resulting from the discovery of aflatoxins in the 1960s with the outbreak of the Turkey-X

disease in the UK, directed more research toward finding appropriate analytical methods for detecting aflatoxins in commodities intended for food and feed. More emphasis was also directed toward appropriate regulations for acceptable levels of aflatoxins mainly in developed countries. More sensitive instrumentation was developed for quantifying aflatoxins in response to more stringent legislation particularly with respect to international trade including developed and developing countries. Currently close to 100 countries have passed legislation regulating level of aflatoxins in food and feed to some extent (FAO, 2004). Unfortunately there is no consensus among the regulating entities which presents problems for international trade (Cucci *et al.*, 2007). The European Union (EU), for example, has the most stringent regulations of 2 and 4 µg/kg AFB_1 and total aflatoxins in food, and 0.05 µg/kg of AFM_1 in milk. Most of the other countries are somewhere in the middle with 5 and 10 µg/kg AFB_1 and total aflatoxins in food, respectively. The US Food and Drug Administration (US-FDA) is among the most permissive, allowing 20 µg/kg total aflatoxins in food and up to 100 µg/kg aflatoxins in feed (Li *et al.*, 2013).

The stringent regulations place more emphasis on aflatoxins-determination in order to minimise exposure to aflatoxins in food and feed. Most of the current methods for quantitative aflatoxins determination include chromatographic methods such as thin layer chromatography (TLC), high performance liquid chromatography (HPLC), and more recently liquid chromatography tandem mass spectrometry (LC-MS/MS), suitable for use in regulatory laboratories (Shephard, 2009). Several immunology-based semi-quantitative and qualitative methods including enzyme-linked immunosorbent assays (ELISAs) and immuno-affinity column assays, were also developed for use at grain stations and silos (Pittet, 2005). Among the novel potential aflatoxins-detection systems are screening and detection methods for rapid in-field and laboratory applications including dip-stick kits (Pittet, 2005), optical-based sensing methods (e.g. hyperspectral imaging and electronic noses) (Harvey *et al.*, 2013; Stark, 2010; Yao *et al.*, 2010), and other emerging experimental methods including biosensors (Malhotra *et al.*, 2014; Pascale, 2009; Pittet, 2005; Tothill, 2011; Vidal *et al.*, 2013). Table 1 provides a summary of the above-mentioned methods.

2. Current analytical approaches for detecting aflatoxins in commodities

Most of the current analytical techniques used for detection and quantification of aflatoxins involve sampling, sample preparation including extraction and clean-up, followed by an appropriate detection method depending on the precision of the desired result.

Sampling and sample preparation

Because aflatoxin exposure risks are correlated with the amount of mycotoxin present in the diet consumed by people and domestic animals, it is important to test a truly representative sample from a given commodity. The sampling error in aflatoxins-determination can account for up to 90% of total variance in a particular sample (Whitaker, 2003). The large source of error is often associated with the skewed distribution of aflatoxins, where a few heavily contaminated grain particles can compromise an otherwise clean sample. Since it is not practical to individually screen tons of grain, several sampling and sub-sampling protocols were developed for sampling large-quantity receptacles including grain trucks and shipping containers, based on statistics (Harvey *et al.*, 2013). A sampling protocol, or plan, is specific to an analytical method and consists of a sampling phase, and an analytical phase, divided into sample preparation and instrumental analysis (Cheli *et al.*, 2012). Efforts for improving sampling and sample preparation for the detection of aflatoxins, particularly in food and feed, continue to be a priority for regulating agencies worldwide (Shephard *et al.*, 2011, 2012, 2013).

Removal of aflatoxins from a target commodity is accomplished by extracting the mycotoxins with an aqueous polar solvent, most commonly, methanol or acetonitrile (Pascale, 2009; Pittet, 2005). The choice of solvent depends on the chemical composition of the mycotoxin, the extraction matrix, safety concerns including the volume of waste generated, and the chosen analytical method (Pittet, 2005; Shephard, 2009). The extraction procedure involves blending or shaking ground samples in the preferred solvent, followed by filtration or centrifugation (Pascale, 2009; Pittet, 2005). The filtered liquid is further purified before the determination step or applied directly in methods that do not require clean-up including immune-based analytical methods such as ELISA (Krska and Molinelli, 2007) or LC-MS/MS procedures utilising 'dilute and shoot' protocols (Sulyok *et al.*, 2007).

The most frequently employed clean-up procedures for aflatoxins-determination are solid phase extraction (SPE) methods, recently reviewed by several authors, including multifunctional columns and immunoaffinity columns (IAC) (Pascale, 2009; Pittet, 2005; Shephard, 2009; Turner *et al.*, 2009). The SPE column contains a bonding phase such as porous silica, modified to allow selective absorption of impurities or the substance of interest (analyte). Typically, the silica traps the analyte, the impurities are washed off and a test-specific rinse solution releases the analyte from the column. In a multifunctional SPE column, the impurities are retained in the column and the analyte flows through. A more recent addition to the SPE clean-up methods is the widely adopted IAC which employs a specific monoclonal or polyclonal antibody binding to the analyte, in this case,

Table 1. Advantages and disadvantages of aflatoxin detection technology.

Method	Pros	Cons	References
Thin layer chromatography	• reliable quantification method when combined with densitometry • accuracy and precision comparable to HPLC methods (HPTLC; OPLC) • official reference methodology for aflatoxins	• outdated equipment • destructive sample preparation • largely replaced with HPLC for quantitative analysis of aflatoxins	Rahmani *et al.*, 2009; Shephard, 2009
High performance liquid chromatography	• reliable, sensitive, selective and repeatable quantification methodology • may be automated • official reference method for aflatoxins	• expensive equipment requiring dedicated operator and specialist to interpret results • destructive sample preparation • -may require derivatization	Cho *et al.*, 2008; Shephard, 2009; Turner *et al.*, 2009;
Liquid chromatography/ mass spectrometry	• simultaneous analysis of mycotoxins • low limit of detection (LC-MS/MS) • confirmatory method • no derivatization required	• very expensive equipment requiring dedicated operator and specialist to interpret results • sensitivity relies on ionisation • matrix assisted calibration for quantitative analysis • lacks internal standards	Krska *et al.*, 2008; Li *et al.*, 2013; Pascale, 2009; Shephard, 2009
Enzyme-linked immunosorbent assay	• specific, rapid and relatively easy to use • inexpensive equipment • low limit of detection • simultaneous analysis of multiple samples • semi-quantitative (screening) or quantitative analysis possible • limited use of organic solvents	• possible cross reactivity with related mycotoxins • matrix interference • possible false positives/negatives • narrow detection range • confirmatory LC analysis may be required	Pascale, 2009; Pittet, 2005; Turner *et al.*, 2009
Immunoaffinity assay	• IAC in combination with liquid fluorometry is comparable to LC for determination of aflatoxins • official method	• sample destruction • limited to analysis of total aflatoxin	Pittet, 2005
Fluorescence polarisation immunoassay	• rapid, no cleanup required • mycotoxin-specific tracer for analysis and • very sensitive • portable	• limited validation with ELISA or HPLC • possible cross reactivity with related mycotoxins • matrix interference • limited to detecting one mycotoxin at a time	(Lattanzio *et al.*, 2011; Lippolis and Maragos, 2014; Pascale, 2009
Capillary electrophoresis	• useful for separating closely related mycotoxins • highly sensitive • capable of multi-constituent analysis when combined with immunoassays	• limited to lab use due to cumbersome instrumentation	Maragos, 2004
Biosensors	• rapid, no clean-up • high selectivity and low limit of detection • ease of use, low cost and portability • self-contained, simple design	• extraction sample prep for solid samples • extract clean-up needed to improve sensitivity • cross reactivity with related mycotoxins • variation in reproducibility and repeatability (improved with use on novel materials)	Malhotra *et al.*, 2014; Meneely and Elliott, 2014; Pascale, 2009; Rubert *et al.*, 2012a; Tothill, 2011
Near infrared spectroscopy	• rapid, non-destructive • no extraction or clean-up • user-friendly operation	• calibration model must be validated • knowledge of statistical methods • poor sensitivity (high limit of detection) • costly equipment	Berardo *et al.*, 2005; Dowell *et al.*, 2002; FAO, 2004; Gordon *et al.*, 1999; Hossain and Goto, 2014; Pearson and Wicklow, 2006; Pearson *et al.*, 2001; Tallada *et al.*, 2011
Hyperspectral imaging	• rapid, non-destructive • no extraction or clean-up • user-friendly operation • high spectral and spatial resolution • potential for in-line detecting applications	• calibration model must be validated • knowledge of statistical methods • poor sensitivity (high limit of detection) • low signal level (for fluorescence) • costly equipment	Del Fiore *et al.*, 2010; Hruska *et al.*, 2013; Yao *et al.*, 2008, 2010
Electronic nose	• rapid means for controlling and improving the microbiological quality of food	• need to improve selectivity and reduce interferences (e.g. to humidity) • compensate for drift effects • limited feasibility studies and poor validation	De Lucca *et al.*, 2012; Gardner and Bartlett, 1994

the mycotoxin. The impurities in this type of column are washed with distilled water and the mycotoxin is eluted with a solvent that dissolves the antibody-analyte bond. IACs have been widely used in mycotoxin, particularly aflatoxins, analysis. The eluted IAC extracts may be read directly in a fluorometer utilising commercially developed methods. Alternately, the eluted extracts may be combined with HPLC-based methods approved by the Association of Official Analytical Chemists (AOAC), for improved quantitative analyses.

Quantitative determination methods

Several chromatographic methods are available for quantification of aflatoxins. The traditional thin layer chromatography (TLC) method is considered a powerful screening tool for the presence of aflatoxins and a reliable quantification method when combined with densitometry. Sample extract is spotted alongside a standard control on to a glass plate covered with a coat of silica gel or a silica-covered alumina sheet, stationary phase. The plate is placed into a vertical vessel containing a solvent mobile phase that travels upwards and separates the samples into different constituents present in the extract depending on their individual solubility and adsorption to the plate matrix. The plate is dried and separation results are typically visualised under UV fluorescence followed by densitometry for quantitative determination of aflatoxins. Advancement in TLC methodology was realised by high performance TLC (HPTLC), two-dimensional TLC, and overpressured-layer chromatography (OPLC) where improvements to the stationary phase layer (HPTLC), and innovative mobile phase application, resulted in accuracies and precision comparable to HPLC methods (Rahmani *et al.*, 2009). When the aflatoxin concentrations on the TLC plates were quantified with fluorescence densitometry, the limits of detection were close to 0.5 µg/kg (Shephard, 2009). Although still accepted as an approved reference method for determination of aflatoxins, TLC has been largely replaced with HPLC for quantitative analysis of aflatoxins.

HPLC is the most frequently used chromatographic technique for quantitative analysis of mycotoxins, particularly aflatoxins. Many HPLC-based methods were developed over the years and optimised for specific applications. Extracted samples are injected into the normal or reverse-phase HPLC chromatography column and individual compounds are separated based on their affinity for the column matrix and the mobile phase solvent. Pre- or post-column clean-up (e.g. IAC) and/or derivatization for enhancement of fluorescence, are usually required prior to detection. HPLC protocols used for the determination of aflatoxins in various commodities include reverse-phase HPLC (RP-HPLC), normal phase HPLC, and UV-HPLC with different combination of amperometric, spectrofluorometric and fluorescence detectors (Turner *et al.*, 2009). Most commonly, methods in aflatoxins-determination utilised reverse-phase HPLC systems where the optimum mobile phase was determined to be a ternary mixture of water, methanol and acetonitrile, and the application of fluorescence detection after post-column bromination with KOBRA cell (Shephard, 2009). Improved column technology with reduced particle size of the packing material from 5 µm in HPLC to 1-2 µm in ultra-HPLC (UHPLC or UPLC) achieved separation with unsurpassed resolution and sensitivity (Cho *et al.*, 2008).

More restrictive regulatory guidelines for aflatoxins in food and feed required methods that were more sensitive and selective, with improved detection limits. Great strides in aflatoxins determination were achieved by coupling liquid chromatography techniques to mass-spectrometry (e.g. LC-MS; LC-MS/MS). The combination resulted in a variety of novel methods for detecting and quantifying single mycotoxin, a group of the same type of mycotoxin, such as aflatoxins or fumonisins, and the analysis of many concurrent mycotoxins (Li *et al.*, 2013). The main advantages of the LC-MS or LC-MS/MS techniques are the improved detection limits, visual fragmentation of sample constituents and elimination of impurities based on mass (Krska *et al.*, 2008; Shephard, 2009). The down side is the costly and complicated instrumentation involved in these analyses. Considering that determination of aflatoxins is easily achieved via HPLC with fluorescence detection, these techniques may not be practical for most routine analyses.

High resolution mass spectrometry for detection of aflatoxins

As alluded above, liquid chromatography coupled to mass spectrometry (LC-MS) plays an important role in the determination of aflatoxins because of its ability to provide sensitive and simultaneous analysis of fungal metabolites in complex matrices. Analyses are typically carried out using triple quadrupole mass spectrometry (QqQ-MS) in selected reaction monitoring (SRM) acquisition mode. A drawback of this approach is its inability to detect compounds that have not been previously optimised for detection, or to perform retrospective data analysis. The use of high resolution MS (HRMS) allows comprehensive profiling of known as well as unknown metabolites in a sample. Indeed, a major advantage of HRMS instruments is the ability to record an unlimited number of compounds in full-scan mode. This characteristic allows the development of screening strategies based on accurate-mass databases for the identification of mycotoxins, including aflatoxins, and their masked derivatives, which are not yet integrated into current monitoring plans. Once confirmation of new mycotoxin conjugates have been established, food and feed commodities can be screened for these compounds with different MS techniques. If standards are available, analytical methods can be developed and known derivatives

can be quantified. Although very little attention has been paid to masked forms of aflatoxins, the possibility that these derivatives may be present in plants and increase the exposure levels to aflatoxins cannot be excluded. Besides its role in the detection of less studied or unexpected mycotoxin derivatives, HRMS is currently more frequently used to remove interferences in quantitation. The required resolving power for the MS instrument depends on the complexity of the sample matrices. The Fourier-transform Orbitrap mass spectrometer allows the achievement of a higher resolution (up to 450,000 full width at half maximum) and a higher mass accuracy (mass error lower than 2 mg/kg) as compared to the Time of Flight (ToF) instruments, thereby leading to a higher selectivity for the analysis of complex samples. Orbitrap-based high resolution mass spectrometry has proved to be a very reliable and robust alternative tool for AFB_1, AFB_2, AFG_1, and AFG_2 and other mycotoxins determination in food matrices compared to the well-established triple quadrupole technology (Lattanzio *et al.*, 2011; Rubert *et al.*, 2012b). A methodology for simultaneous determination of 32 mycotoxins, including aflatoxins, in food matrices was developed by Rubert *et al.* (2012a). Despite the great structural variability between 32 mycotoxins, the validated UHPLC-Orbitrap MS approach proved to be accurate, precise and sensitive. Albeit less popular and less robust compared to Orbitrap MS, ToF high resolution technology has also demonstrated its suitability for the screening of aflatoxins in foodstuffs rapidly and with high sensitivity (Tanaka *et al.*, 2006). As for the triple quadrupole type MS instruments, the drawback of HRMS remains the cost and the need for trained staff.

Semi-quantitative or qualitative determination methods

More user-friendly separation techniques that have been developed, use aflatoxin-specific antibodies in immunoassay analysis format. One of these immune-based methods is the well-established and widely used ELISA. Several ELISA kits are commercially available for quantification, semi-quantification and qualitative assessment of different mycotoxins. The principle of ELISA is based on competition between a primary antibody specific to the toxin in question or a labelled toxin-enzyme conjugate and the toxin from the sample, for a finite number of binding sites. The resulting complex reacts with a chromogen that can be measured with a reader (Pittet, 2005; Turner *et al.*, 2009). Most ELISAs are specific, rapid and relatively easy to use. However, these kits have drawbacks including potential cross-reactivity and dependence on a specific matrix. Therefore, it is important to adhere to the kit instructions and only use it for the test specified by the manufacturer (Pittet, 2005).

IAC used for initial clean-up of aflatoxin-contaminated samples intended for HPLC analysis, have been developed into commercially available semi-quantitative tests for several mycotoxins. The IAC eluate is derivatised with a bromine developer solution to enhance fluorescence and the toxin in question is detected in a tabletop fluorometer (Pittet, 2005; Shephard, 2009). Unfortunately, only total aflatoxins, not specific sub-types (AFB_1, AFB_2, etc.), can be determined in a given sample. An AOAC/IUPAC collaborative study determined that the immunoaffinity column test in combination with solution fluorometry was comparable to liquid chromatography for determination of aflatoxins in maize, raw peanuts and peanut butter. The technique is now a widely used registered AOAC method (Pittet, 2005).

An increased demand for rapid immune-chromatographic tests for direct detection of mycotoxins in a preferably portable and disposable tool produced the dipsticks and lateral flow devices. These membrane-based tests can provide a qualitative yes/no determination of the presence of a target molecule or a threshold, semi-quantitative result in 5-10 min. Advantages of this type of test are their portability and ease of execution. Disadvantages include a subjective interpretation and a higher cost per test compared to the ELISAs. These types of rapid devices were developed in both, Europe and the USA for total aflatoxins in cereals with thresholds at 4, 10 and 20 µg/kg (Pittet, 2005).

Other methods worth mentioning that are promising, but had not yet reached the popularity of ELISAs, or the methods mentioned earlier, are capillary electrophoresis (CE), fluorescence polarisation immunoassay (FPI), and biosensors, e.g. surface plasmon resonance (SPR) (Cheli *et al.*, 2012; Goryacheva *et al.*, 2007; Lippolis and Maragos, 2014; Maragos, 2004; Meneely and Elliott, 2014). All three methods have been assessed in aflatoxins-determination. CE is useful for separating closely related mycotoxins, but is limited by cumbersome instrumentation to laboratory use. However, it is highly sensitive and when combined with immunoassays, is capable of multi-constituent analysis. The other two tests have the potential of portability that would allow them to be used in field applications. The solution-based FPI utilises a mycotoxin-specific tracer for analysis and is very sensitive (Lippolis and Maragos, 2014; Sheng *et al.*, 2014). The limiting factor of this assay lies in its inability to detect more than one mycotoxin at a time. Biosensors are discussed at length in the following section.

Biosensors

Compared to the classical mycotoxin determination methods, biosensors have emerged as attractive alternative tools offering high selectivity and sensitivity, ease of use, low cost and portability in a self-contained, simple design. The basic premise of biosensors is to convert biological responses resulting from the interaction between an analyte and a biological element (bioreceptor) into electrical signals via a transducer detector element. These signals are further amplified, measured and displayed (Figure 1). A biosensor

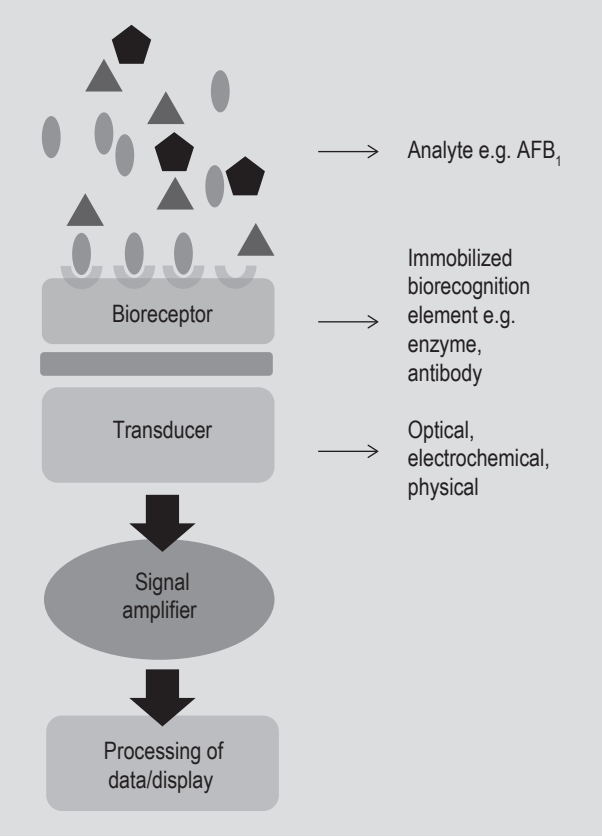

Figure 1. Schematic overview of a biosensor.

typically consists of three main parts: a bioreceptor, an immobilising matrix and a transducer. The bioreceptor used for recognition of the analyte of interest can be an enzyme, a cell receptor protein, antibody, nucleic acid, whole cell, or tissue, immobilised in a matrix consisting of various materials including conducting polymers, nanomaterials, self-assembled monolayers, or sol-gel films (Malhotra *et al.*, 2014). The third part is the transducer (optical, electrochemical, or physical) which converts the biochemical reaction product into an identifiable electronically processed output signal. Among the optical biosensors are included interferometric, fluorimetric, and refractometric transducers as well as those based on the surface plasmon resonance platform. Electrochemical biosensors can employ potentiometric, amperometric, capacitative and conductometric transducers. Transducers included in the physical biosensor are magnetic, piezoelectric, calorimetric and the surface acoustic wave (Malhotra *et al.*, 2014).

A given biosensor is only as sensitive as the bioreceptor employed. For this reason, a range of mycotoxin-specific recognition molecules have been developed. Although most widely used recognition molecules are antibodies (polyclonal and monoclonal), biomimetic, synthetic materials have more recently been developed to serve as molecular recognition elements, providing the required sensitivity and specificity for low level toxin detection achieved with antibodies, with the advantage of greater stability (Tothill, 2011).

To date, most common biosensors developed for detecting aflatoxins have been optical or electrochemical, however, piezoelectric sensors (e.g. quartz crystal microbalance (QCM)) based on change in mass on the sensor surface have been used in mycotoxin detection applications. For example, a QCM based sensor was developed for detecting AFB_1in liquids. With the aid of a biocatalysed deposition amplification system the sensor achieved detection in milk samples within the range of 0.01-10.0 µg/l (Jin *et al.*, 2009).

Optical sensors including a fibre-optics device based on evanescent waves for detecting AFB_1 (Maragos and Thompson, 1999) and an aflatoxins sensor based on fluorescence polarisation (Nasir and Jolley, 2002) were among the earliest biosensors developed for mycotoxins. SPR is another promising optical technology for the rapid detection of aflatoxins based on detecting change in the refractive index of a medium after the binding of an analyte to an immobilised recognition molecule (Mosiello and Lamberti, 2011). When the analyte in liquid form binds to the biorecognition element of the SPR sensor, an optically measurable increase in the refractive index is produced at the sensor surface. The SPR assay developed for detecting aflatoxins in maize extracts using elastase-functionalised surface plasmon (Cuccioloni *et al.*, 2008) exhibited limits of detection for AFB_1 lower than ELISA's and analysis results of naturally contaminated samples concurred with those measured with, the more highly regarded LC-MS, instruments (Cheli *et al.*, 2012; Mosiello and Lamberti, 2011; Tüdös *et al.*, 2003). Additional SPR immunoassays for rapid determination of mycotoxins in cereals and cereal-based foods were recently reviewed by Meneely and Elliott (2014). The downside of optical biosensors is their high cost and lack of portability (Tothill and Turner, 2003). This may be alleviated with the introduction of biomaterials. Xu *et al.* (2013) developed a simple and rapid one step and label-free optical biosensor for the determination of AFB_1 based on competitive dispersion of gold nano-rods that demonstrated high degree of sensitivity and selectivity for the mycotoxin with a linear detection range from 0.5-20 ng/ml with a limit of detection of 0.16 ng/ml. The authors did not indicate the corresponding detection limit in µg/kg matrix. However, based on the details provided on the extraction procedure from peanuts, this detection limit could be estimated to be approximately 1.25 µg/kg.

Electrochemical biosensors have the greatest potential for mycotoxin determination in the form of simple, highly sensitive and cost effective portable devices, analogous to the glucose testers widely used for monitoring blood sugar in diabetics. The principle of electrochemical sensors in mycotoxin analysis lies in two directions depending whether

the total toxin load of a sample (based on enzyme inhibition), or a specific toxin (based on affinity sensors), is sought (Tothill, 2011). Most biosensors for detecting mycotoxins are affinity based enzymatic catalysis of antibody-antigen immunoreactions that produce or consume electrons. The resulting signal can be measured as current or potential from which the corresponding concentration of the analyte can be extrapolated. More current immunosensors, based on electrochemical transducers, have been reported to be highly sensitive and selective at targeting a specific mycotoxin (Piermarini *et al.*, 2007; Tothill, 2011), particularly when used with gold electrodes which are less prone to non-specific binding than conventional carbon electrodes (Heurich *et al.*, 2011; Tothill, 2011). The label-free immunosensors based on electrochemical impedance spectroscopy (EIS) were recently developed as simple, cost-effective, and highly sensitive devices for the determination of AFB_1 (Li *et al.*, 2010; Malhotra *et al.*, 2014; Vidal *et al.*, 2013). Additionally, an electrochemical immunosensor array with a 96-well screen-printed microplate for detecting AFB_1 at a level of 30 pg/ml corresponding to a detection limit of 0.75 µg/kg maize with working range between 0.05 and 2 ng/ml (1.25-50 µg/kg maize) was developed by Piermarini *et al.* (2007). The incorporation of carbon nanomaterials including graphene and graphene derivatives in biosensors developed for toxin detection in foods have greatly enhanced the overall stability and performance of these sensors, achieving sensitivity and accuracy within the detection range of conventional quantification methods (Malhotra *et al.*, 2014).

Most biosensors require minimal sample preparation which may be incorporated as part of the sensor system process, particularly for liquid samples. Solid samples (e.g. maize kernels) may require extraction and clean-up procedures similar to those used for HPLC and MS techniques (Tothill, 2011).

3. Novel rapid and non-invasive detection with spectroscopy, hyperspectral imaging and electronic nose

Spectroscopy provides a potential approach for rapid and non-invasive detection of fungal infection and aflatoxins-contamination. The spectral data can be obtained from a spectrometer using a fibre optic-based method. The spectral data can also be obtained from an imaging spectrometer such as a hyperspectral imager. Depending on the application, the spectral data can be collected as reflectance, transmittance, and fluorescence.

When spectral data are collected with a spectrometer, the fibre optic of the spectrometer is pointed at the target (Pearson *et al.*, 2001). This method produces narrowband reflectance measurements. The narrowband reflectance has the capability of revealing subtle differences in maize reflectance and transmittance, which could be useful information for the detection of diseases and contamination. Many applications used spectrometer data for single maize kernel analysis including moisture and protein content prediction through near infrared (NIR) measurements (Armstrong, 2006), aflatoxins-detection with reflectance and transmittance (Pearson *et al.*, 2001), and *A. flavus* infection assessment (Gordon *et al.*, 1999; Hossain and Goto, 2014; Pearson and Wicklow, 2006) as well as detection of mycotoxin contamination in agricultural commodities (Hossain and Goto, 2014). Recently, Tallada *et al.* (2011) used both NIR spectra (904-1,685 nm) and colour images to discriminate fungal-infected maize kernels. A total of eight fungal species including *A. flavus* were investigated in the study. The results indicated that the NIR spectrometry had better accuracy than the colour imaging method. NIR spectroscopy (850-1,650 nm) was also used for maize kernel heat damage assessment (Esteve Agelet *et al.*, 2012) with an accuracy of 99%. The instrumentation used in the spectrometer approach is simple and enables quick data acquisition. On the other hand, the fibre optic-based reflectance measurement does not provide spatial information in the data. It is a single point measurement of the maize kernel. The spectral data are mixed signals of all the spectra within the fibre's field of view (FOV). If the FOV is larger than the kernel, unwanted background signal would also be acquired. Thus, the FOV of the fibre optic should be maintained within the kernel surface. However, this approach limits the viewing area and the coverage of the grain kernel could be partial or incomplete.

While most research using spectroscopy focused on fungal detection, one study concentrated on detecting aflatoxins in single maize kernels (Pearson *et al.*, 2001). In this work, both transmittance (500-950 nm) and reflectance (550-1,700 nm) spectra were used. For kernels with either high (>100 µg/kg) or low (<10 µg/kg) levels of aflatoxins, the classification accuracy was found to be 95%. The accuracy for kernels with medium levels (10-100 µg/kg) of contamination was 25%. Similar results were later applied (Pearson *et al.*, 2004) to a high speed sorting machine to remove maize kernels contaminated with aflatoxins and fumonisins. Two wavelengths, 750 and 1,200 nm, were used by the sorter. A discriminant analysis process was used to select the two wavelengths, which were filtered out through a dual-peak filter mounted in front of the silicon and indium-gallium-arsenide detectors. It was found that aflatoxin level was reduced by 81% from an initial average of 53 µg/kg.

To better differentiate a maize kernel from the background, it is preferred to use spectral data with both high spatial and spectral resolutions. In this case, an imaging spectrometer or hyperspectral imaging system (Yao *et al.*, 2008) can be used. The spectral range of a hyperspectral image can be from 400-2,500 nm (visible to shortwave near infrared) in the electromagnetic spectrum. The use of

hyperspectral imaging makes it possible to do pixel based data analysis. Each pixel has the same wavelength range and spectral resolution as the entire image. Multivariate statistical analysis and pattern recognition methods are typically used to process hyperspectral imagery. Recently, hyperspectral reflectance imaging has been used in mycotoxin-producing fungal research. For example, Yao *et al.* (2008) used hyperspectral imaging (400-900 nm) to differentiate five fungal species including *A. parasiticus,* and *A. flavus*. Among the five species some were toxigenic (toxin producing) and some were atoxigenic (non-toxin producing). The overall classification accuracy was 97.7%. Del Fiore *et al.* (2010) demonstrated the potential of using hyperspectral reflectance (400-900 nm) for rapid and non-destructive detection of maize kernels infected with toxigenic *Aspergillus niger* or *A. flavus*. The above research focused mainly on using hyperspectral reflectance imagery for fungal identification or fungal infection on maize kernels.

In addition to reflectance, hyperspectral technology can be implemented for fluorescence imaging of naturally fluorescing materials. When these substances are excited with shortwave radiation such as an UV light source or laser, they exhibit fluorescence emissions. These emissions can be recorded with a fluorescence hyperspectral imaging system. Since fluorescence hyperspectral imaging provides an alternate approach to non-invasive inspection and contamination detection (Kim *et al.*, 2001), this technology has been utilised in many food safety and quality related applications in the past decade including aflatoxins detection in commodities. One study, for example, used fluorescence hyperspectral imaging to detect aflatoxin contamination in ground red chili pepper flakes (Ataş *et al.*, 2012). Both reflectance and fluorescence hyperspectral data were collected in the spectral range from 400 to 720 nm in the study. The classification accuracy was 85-90%. Other work focused on using this method for aflatoxin contamination detection in maize (Hruska *et al.*, 2013; Yao *et al.*, 2010). Yao *et al.* (2010) used the imaging system for detecting single maize kernels contaminated with aflatoxins. Contaminated maize kernels were produced through artificial inoculation of toxigenic strain of *A. flavus*. The individual maize kernels were then imaged and chemically analysed to determine the actual concentration of aflatoxins in each kernel. The study reported a fluorescence peak shift phenomenon among different groups of kernels with different aflatoxin contamination levels. The fluorescence peak shift was more toward the longer wavelength in the blue region for the highly contaminated kernels and toward the shorter wavelength for the clean kernels. A multiple linear regression model was generated between the image bands and the measured aflatoxins levels. The correlation coefficient of determination equalled to 0.72. A later study (Yao *et al.*, 2013b) focused on imaging classification, yielded classification accuracies of 0.87 and 0.88, respectively, when 20 and 100 µg/g was used as detection thresholds. Most recently, Yao *et al.* (2013a) used fluorescence hyperspectral imaging to detect single maize kernels inoculated with toxigenic (AF13) and atoxigenic (AF38) strains of *A. flavus* field maize. It was found that the fluorescence data from the embryo side of the maize kernels provided better outcomes in discriminating the two infections than data from the endosperm side, especially when the infection was mild.

A major limitation of fluorescence emission imaging is the low signal level. One way to overcome this is to use a prolonged data acquisition time. However, this approach is not desirable for rapid detection of aflatoxins-contamination. Another approach is to use several key wavelengths. This approach can significantly reduce the amount of time for data acquisition. Yao *et al.* (2013b) calculated all possible two-band pairs and found that a normalised difference fluorescence index (NDFI) had the highest correlation (-0.81) with the measured aflatoxins. NDFI was calculated as (537 nm – 437 nm)/(537 nm + 437 nm). In a different experiment, Hruska *et al.* (2013) imaged all kernels shelled from individual maize ears. Two wavelengths, 437 and 537 nm, were used to form a ratio-based detector to identify all the potentially contaminated 'hot' kernels. The extracted 'hot' kernels demonstrated similar peak fluorescence shift feature seen in (Yao *et al.*, 2010).

An additional example of a rapid mycotoxin detection method is based on the electronic sense of smell. The odours and aromas of food contain many volatiles which may be used as sensory indicators of food quality (Cheli *et al.*, 2007). Volatile by-products of fungal spoilage are often associated with the formation of mycotoxins. The production of volatile metabolites was characterised by gas chromatography/ mass spectrometry (GC-MS) and found to correlate with fungal activity (Magan and Evans, 2000). Furthermore, patterns of fungal volatiles and their accumulation can be used as indicators of fungal activity as well as taxonomic markers for differentiating fungal species, and between fungal strains that produce toxins and those that do not. Because volatile headspace may be evaluated by the use of electronic nose (EN), the technique has found wider use for evaluating mould spoilage and assessing quality and safety of food stuffs. An EN is an instrument which attempts to mimic human senses using sensor arrays and pattern recognition systems, capable of recognising simple or complex odours (Gardner and Bartlett, 1994). The array made up of non-specific chemical detectors interacts with different volatile compounds and provides a fingerprint of the volatile molecules emanating from a target sample. The acquired fingerprint of the sample odour(s) may be subsequently identified and/or quantified by means of a pattern recognition system. It has been reported that aflatoxigenic fungi produce volatile compounds that differ from non-aflatoxigenic fungi (De Lucca *et al.*, 2012; Zeringue Jr. *et al.*, 1993). And in the instances where they

produce the same compounds, the release time point may differ between the two types of fungi. Furans, for example, are released by both the aflatoxigenic and non-aflatoxigenic *A. flavus* fungi, however, in the non-aflatoxigenic strains they are released several days earlier than in aflatoxin producing strains (De Lucca *et al.*, 2012). However, limited information is available regarding quantification capability of EN for predicting mycotoxins concentration in cereals (Cheli *et al.*, 2007).

4. Conclusions

Detection of aflatoxins in food and feed remains an important issue in food safety research and its applications. This review is a summary of current analytical approaches to aflatoxin detection and the latest research in rapid and non-invasive detection methods. The widely used chemically-based analytical methods can generally provide quantitative results with a high level of accuracy for extracted aflatoxin samples. However, the drawback of these methods is the sample variability during the sampling process. A major benefit of the rapid, non-invasive hyperspectral imaging-based methods is the demonstrated potential for screening large samples contaminated with aflatoxins, unfortunately a real drawback lies in the inability of achieving quantifiable results. Perhaps an integrated approach which incorporates rapid sample screening techniques with analytical methods may be most suitable for detecting aflatoxins in consumables.

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