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A review of rapid methods for the analysis of mycotoxins

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Abstract

An overview is presented of the analysis of mycotoxins by rapid methods such as: enzyme linked immunosorbent assay (ELISA); flow through membrane based immunoassay; immunochromatographic assay; fluorometric assay with immunoaffinity clean-up column or with a solid phase extraction clean-up column; and fluorescence polarization method. These methods are currently commercially available and are reliable, rapid methods. This review focuses on the basic principle of each rapid method as well as advantages and limitations of each method. Additionally, we address other emerging technologies of potential application in the analysis of mycotoxins.

Key words: analysis, antibody, biosensor, immuno-assay, mycotoxins, rapid methods

Introduction

Mycotoxins are secondary metabolites of fungi. The major fungal genera producing mycotoxins include *Aspergillus*, *Fusarium* and *Penicillium*. The most common mycotoxins are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, T-2 toxin and zearalenone. Many foods and feeds can become contaminated with mycotoxins since they can form in commodities before harvest, during the time between harvesting and drying, and in storage. Commodities and products frequently contaminated with mycotoxins include corn, wheat, barley, rice, oats, nuts, milk, cheese, peanuts and cottonseed. Mycotoxins produce a wide range of adverse and toxic effects in animals in addition to being foodborne hazards to humans [1]. Regulations for major mycotoxins in commodities and food exist in at least 100 countries, most of which are for aflatoxins; maximum tolerated levels differ greatly among countries [2]. The US Food and Drug Administration action levels and European Union regulations for aflatoxin are shown in Table 1 [1, 3–5].

To determine whether commodities are contaminated with mycotoxins, one must test for them. Proper sampling procedures are pre-requisite for obtaining reliable results because of the heterogeneous distribution of mycotoxins in grains and other commodities [6]. Conventional analytical methods for mycotoxins include thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC). Most of these methods employ solid phase column cleanup of extracts and immunoaffinity techniques to remove interferences to improve the measurement of mycotoxins. These analytical methods applied to food or feed samples yield results within hours or days. Competition within the food and feed industry forces them to reduce cost, employ cheaper labor and deliver goods rapidly. Additionally, increased analytical complexity in the food industry requires a rapid report for each individual contaminant. Thus,

Table 1. The US FDA action levels and European Union regulations on maximum levels for aflatoxins in foodstuffs and animal feedstuffs

United States		European Union[a]	
Product	Level (ppb)	Product	Level (ppb)
All foods	20	Groundnuts, nuts and dried fruits and processed products (direct human consumption)	4 (2)
Feedstuff (ingredient)s	20	Groundnuts (to undergo physical processing before human consumption)	15 (8)
Cottonseed meal intended for beef cattle/swine/poultry feedstuffs (regardless of age or breeding status)	300	Nuts and dried fruit (to undergo physical processing before human consumption)	10 (5)
Maize and peanut products intended for breeding beef cattles/swine or mature poultry	100	Cereals (for direct human consumption or to undergo physical processing before human consumption)	4 (2)
Maize and peanut products intended for finishing swine of 100 pounds or greater	200	Spices (*Capsicum* spp., *Piper* spp., *Myristica fragans, Zinziber officinale, Curcuma longa*)	10 (5)
Maize and peanut products intended for finishing beefcattle	300	Feed materials with exception of:	(50)
		– groundnut, copra, palm-kernel, cotton seed, babassu, maize and products derived from the processing thereof	(20)
		Complete feedingstuffs for cattle, sheep and goats with the exception of:	(50)
		– dairy cattle	(5)
		– calves and lambs	(10)
		Complete feedingstuffs for pigs and poultry (except young animals)	(20)
		Other complete feedingstuffs	(10)
		Complementary feedingstuffs for cattle, sheep and goats (except for diary animals, calves and lambs)	(50)
		Complementary feedingstuffs for pigs and poultry (except young animals)	(30)
		Other complementary feedingstuffs	(5)

[a] Numbers in parentheses refer to separate standard for aflatoxin B1 alone.

rapid methods for mycotoxin analysis have become increasingly important. Rapid methods are less expensive, easier to use and can be moved to an on-site environment. They can help to determine the effectiveness of food safety measures, to determine legal compliance, to achieve logistical and operational goals, to keep commodities and products moving rapidly through marketing channels, to save time and thus costs, to save investments in complex instruments and to employ staff with lesser technical training [7]. Most rapid methods provide qualitative or semi-quantitative results and are recommended for use in screening samples.

While developments in general technologies for detection of mycotoxins have been reviewed by Maragos [8, 9], this article provides a review of the current status of rapid, commercially available methods in mycotoxin analysis. Other emerging technologies that have potential for developing new format and real-time tests for mycotoxins are considered also.

Rapid methods for mycotoxin analysis

The term 'rapid method' usually refers to a method much faster than respective reference methods and also has a tendency of promoting the method. No such a definition for rapid methods in mycotoxin analysis exists but usually the time of analysis is in terms of minutes rather than hours. However, the rapid methods in mycotoxin analysis should have some common features: the method should be simple and easy-to-use and the method should be relatively fast and should be

capable of testing mycotoxins in the field. This article reviews rapid methods that are capable of detecting mycotoxins in a single, pre-ground sample in less than 30 min [10].

Enzyme linked immuno-sorbent assay (ELISA)

ELISA methods for mycotoxin assay have been available for more than a decade. The technology is based on the ability of a specific antibody to distinguish the three-dimensional structure of a specific mycotoxin. The direct competitive ELISA is commonly used in mycotoxin analysis [11]. A conventional microtiter plate ELISA requires equilibrium of the antibody–antigen reaction that would require an incubation time of approximately 1–2 h. Currently, most of the commercially available ELISA test kits for mycotoxins are working in the kinetics phase of antibody–antigen binding, which reduces the incubation time to minutes. Although reduction of incubation time may lead to some loss of assay sensitivity, the test kit can provide accurate and reproducible results. A typical principle of direct competitive ELISA is shown in Figure 1. After a mycotoxin is extracted from a ground sample with solvent, a portion of the sample extract and a conjugate of an enzyme-coupled mycotoxin are mixed and then added to the antibody-coated microtiter wells. Any mycotoxin in the sample extract or control standards is allowed to compete with the enzyme-conjugated mycotoxin for the antibody binding sites. After washing, an enzyme substrate is added and blue color develops. The intensity of the color is inversely proportional to the concentration of mycotoxin in the sample or standard. A solution is then added to stop the enzyme reaction. The intensity of the solution color in the microtiter wells is measured optically using an ELISA reader with an absorbance filter of 450 nm. The optical densities (OD) of the samples are compared to the ODs of the standards and an interpretative result is determined.

ELISA test kits are favored as high throughput assays with low sample volume requirements and often less sample extract clean-up procedures compared to conventional methods such as TLC and HPLC. The methods can be fully quantitative. They are rapid, simple, specific, sensitive and portable for use in the field for the detection of mycotoxins in foods and feeds [12]. An example of performance characteristics of ELISA method is given in the Table 2. Although the antibodies have the advantage of high specificity and sensitivity,

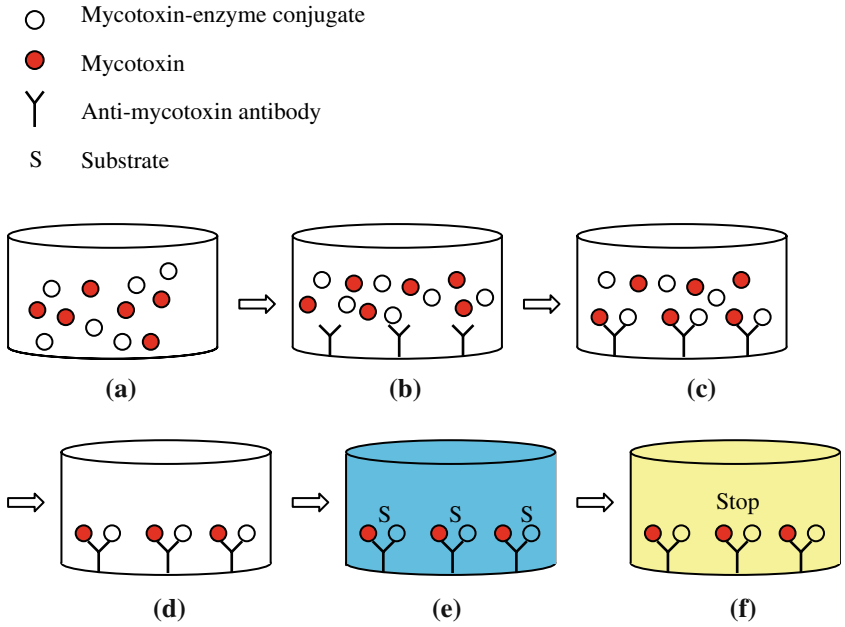

Figure 1. Principle of competitive ELISA for mycotoxin analysis. (a) Sample mixed with conjugate; (b) mixed content added to antibody coated well; (c) mycotoxin binds to antibody in the 1st incubation; (d) unbound materials are rinsed away in the washing step; (e) substrate is added to develop color; (f) stop solution is added to stop the reaction.

Table 2. Performance characteristics of different rapid methods for the detection of aflatoxin in corn

Performance characteristics	ELISA	Flow-through immunoassay	Lateral flow test	Fluorometric assay with IAC clean-up	Fluorometric assay with SPE clean-up
Quantitative or semi-quantitative	Quantitative	Semi-quantitative	Semi-quantitative	Quantitative	Quantitative
Detection limits	2.5 ppb	20 ppb	4, 10 or 20 ppb	1 ppb	5 ppb
Recovery (%)	93.7–122.6%	NA	NA	105–123%	92–102%
Relative Standard Deviation for Repeatability (%)	4.8–15.9%	NA	NA	11.75–16.57%	8.8–19.6%
Correct response for positive test samples spiked at the detection level	NA	97%	100%	NA	NA
Assay time[a]	< 25 min	< 5 min	5 min	< 15 min	< 5 min
Equipment	ELISA reader	NA	NA	Fluorometer	Fluorometer
Reference	[15]	[17]	[19]	[21]	[22]

[a] Assay time is the time needed to detect mycotoxins in a single, pre-ground sample after extraction.

because the target compounds are mycotoxins but not the antigens, compounds with similar chemical groups can also interact with the antibodies. This so-called matrix effect or matrix interference commonly occurs in ELISA methods resulting in underestimates or overestimates in mycotoxin concentrations in commodity samples [13]. Additionally, insufficient validation of ELISA methods causes the methods to be limited to those matrices for which they were validated [14]. Therefore, an extensive study on the accuracy and precision of an ELISA method over a wide range of commodities is needed and a full validation for an ELISA method is essential and critical [15].

Membrane based immunoassay

Flow-through assay

Membrane-based, flow-through assay for mycotoxins has been available since the late 1980s. This assay typically is based on a principle of direct competitive ELISA [16–18]. Anti-mycotoxin antibody is coated on a membrane surface (Figure 2). The mycotoxin is extracted from a ground sample and a portion of the extract is then added to the membrane, followed by an addition of mycotoxin-enzyme conjugate. Mycotoxin and mycotoxin-enzyme conjugate compete for the limited antibody binding sites. After a washing step, the enzyme substrate is added and reacts with the mycotoxin-coupled enzyme and color develops. For a negative sample, i.e., mycotoxin level less than the assay cut-off level, there will be a visible color spot in the center of the membrane. For a positive sample, i.e., mycotoxin level greater than/equal to the cut-off level, there will be no color spot on the membrane. The mycotoxin concentrations in positive samples can be confirmed by a quantitative method such as HPLC.

The flow-through assay is rapid, easy-to-use and is suitable for testing mycotoxins in the field. The method does not require any equipment and most any individual can perform this assay. An example of performance characteristics of a flow-through test is given in the Table 2. However, since the method is semi-quantitative, interpretation of results may be difficult when the mycotoxin concentration of the test sample is close to the method cut-off level.

Lateral flow test

The technology of the immunochromatographic test, also called lateral flow test or strip test, has been used for many years. However, its application in food safety, especially mycotoxin testing, is quite recent [19]. A typical immunochromatography test strip is composed of a sample pad, a conjugate pad, a membrane, an absorbent pad and an adhesive backing. The competitive reaction scheme is used most often when testing for small molecules with single antigenic determinants such as mycotoxins (Figure 3). A sample extract is added onto the sample pad. Any mycotoxin present binds to the anti-mycotoxin antibody gold particle complex in the conjugate pad and they migrate together with the anti-2^{nd} antibody gold particle complex along the membrane. The membrane contains a test zone and a control zone, onto

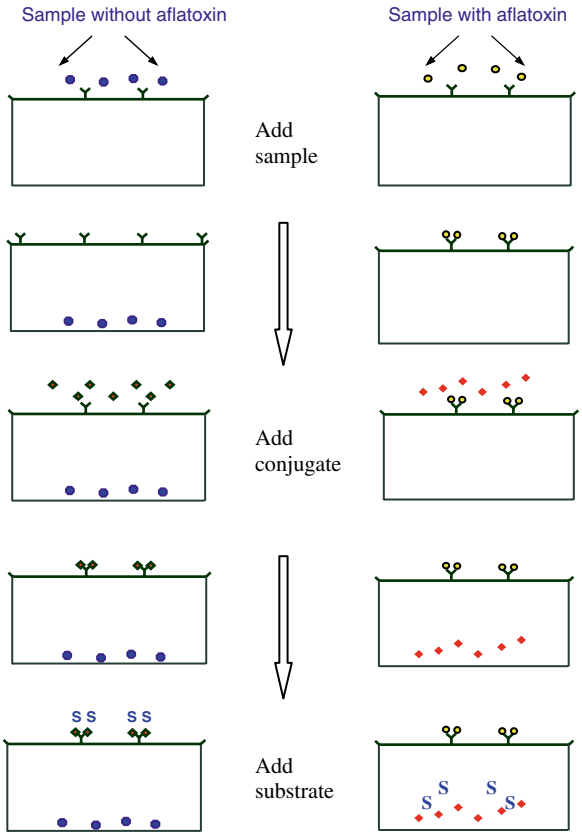

Figure 2. Principle of membrane-based flow through test (in the absence of toxin (left), color is developed; in the presence of toxin (right), color development is suppressed).

which a mycotoxin-protein conjugate and a 2nd antibody are dried, respectively. The mycotoxin-protein conjugate in the test zone can capture any free anti-mycotoxin antibody gold particle complex, allowing color particles to concentrate and form a visible line. Hence, a positive sample with a mycotoxin concentration greater than or equal to the assay cut-off level will result in no visible line in the test zone. Conversely, a negative sample with a mycotoxin concentration less than the cut-off level will form a visible line in the test zone. The control zone will always be visible regardless of the presence or absence of mycotoxin because the 2nd antibody always captures the anti-2nd antibody gold particle complex indicating the validity of the performed test.

The benefits of the immunochromatographic test are that they are user-friendly, very rapid, have long-term stability over a wide range of climates, and are particularly suitable for testing for mycotoxins in the field. An example of performance characteristics of a lateral flow test for the detection of total aflatoxins in corn is given in the Table 2. However, the technology can only provide semi-quantitative results; for any positive samples, the exact mycotoxin concentration would require confirmation by a reference method such as HPLC.

Fluorometric assay

The fluorometric assay is an effective, quantitative method for mycotoxin analysis that has been available for over a decade. To obtain accurate mycotoxin results by fluorometric assay, it is very important to remove interferences before the fluorometric measurement because other compounds in the mycotoxin sample extract may have fluorescence that could alter the readings. Available fluorometric assays use two sample clean-up methods which have been proven to be effective to remove assay interferences: (1) immunoaffinity

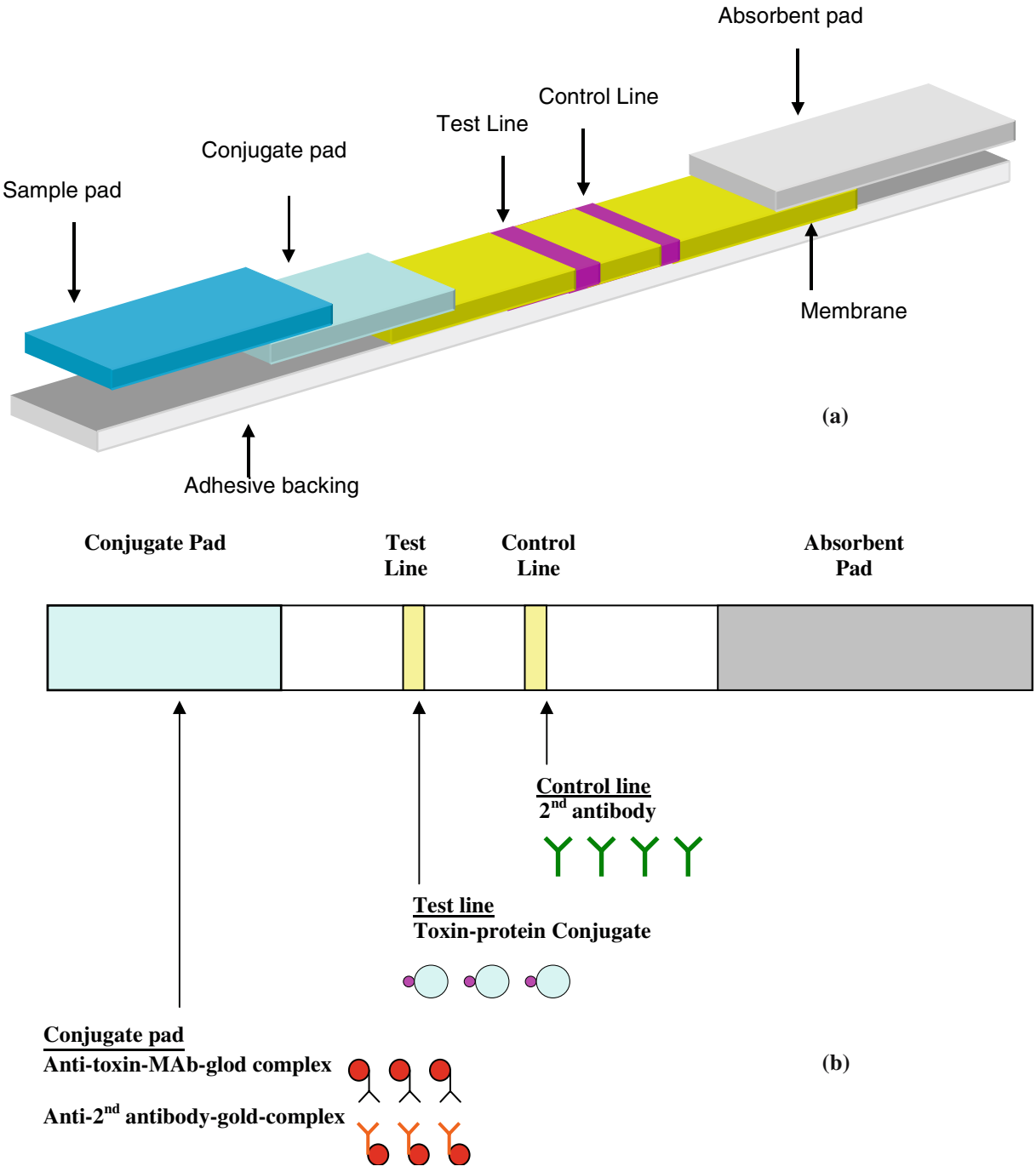

Figure 3. Schematic illustration of a lateral flow test. (a) Configuration; (b) reagents placement.

column clean-up; and (2) solid phase extraction column clean-up. Additionally, to enhance the fluorescence signal, most mycotoxins are derivatized prior to the fluorometric measurement.

Immunoaffinity column clean-up

The immunoaffinity column (IAC) has been used widely for sample clean-up in the mycotoxin analysis [20]. The IAC contains anti-mycotoxin

antibody that is immobilized onto a solid support such as agarose gel in phosphate buffer, all of which is contained in a small plastic cartridge (Figure 4). The sample extract is applied to an IAC containing specific antibodies to a certain mycotoxin. The mycotoxin binds to the antibody and water is passed through the column to remove any impurities. Then by passing a solvent such as methanol through the column, the captured mycotoxin is removed from the antibody and thus eluted from the column. The mycotoxin in the methanol elute is then further developed by addition of a chemical substance to either enhance the fluorescence or render the mycotoxin fluorescent before measuring in a fluorometer. Prior to adding a fluorescent enhancing chemical, the methanol solution can be used for HPLC analysis as well. With IAC clean-up, the mycotoxin can be concentrated in the column, thereby increasing the fluorometric assay sensitivity or decrease its limit of detection. However, IACs have a limited loading capacity and the sample clean-up procedures are more complicated compared to others in the rapid methods for mycotoxins. An example of the performance characteristics of the fluorometric assay with IAC clean-up for the detection of aflatoxins in corn is given in Table 2 [21].

Solid-phase extraction (SPE) column clean-up

Using solid phase extraction columns for purifications is rapid and economical. The most commonly used packing materials in the SPE columns or cartridges are silica gel, C_{18} bonded to silica gel, florisil or ion exchange resins. Conventional SPE column retain the analytes on the adsorbent, the non-mycotoxin materials are eluted and then the mycotoxins are eluted. A one-step SPE cleanup column has been developed for rapid clean-up of mycotoxin for the application in a fluorometric method [22, 23]. The packing material of the one-step SPE column is packed with a porous frit at the top of the column packing, in a funnel-shaped, durable plastic tube with plastic caps at both ends (Figure 5). A sample extract is added to the sample reservoir and a rubber syringe plunger, or a similar device, is used to push the sample extract through the one-step SPE column. The purified extract collected at the lower end of the tube contains the mycotoxin, which can immediately be derivatized and placed in a fluorometer for analysis. The

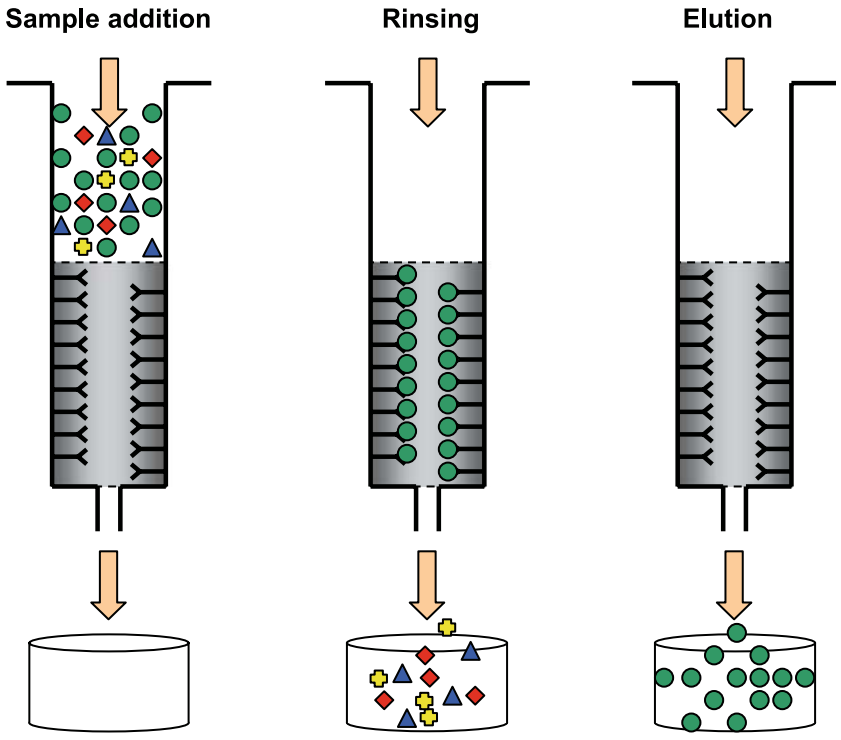

Figure 4. Principle of immunoaffinity columns (mycotoxin: ●; imprities: ◆ ▲ ✚).

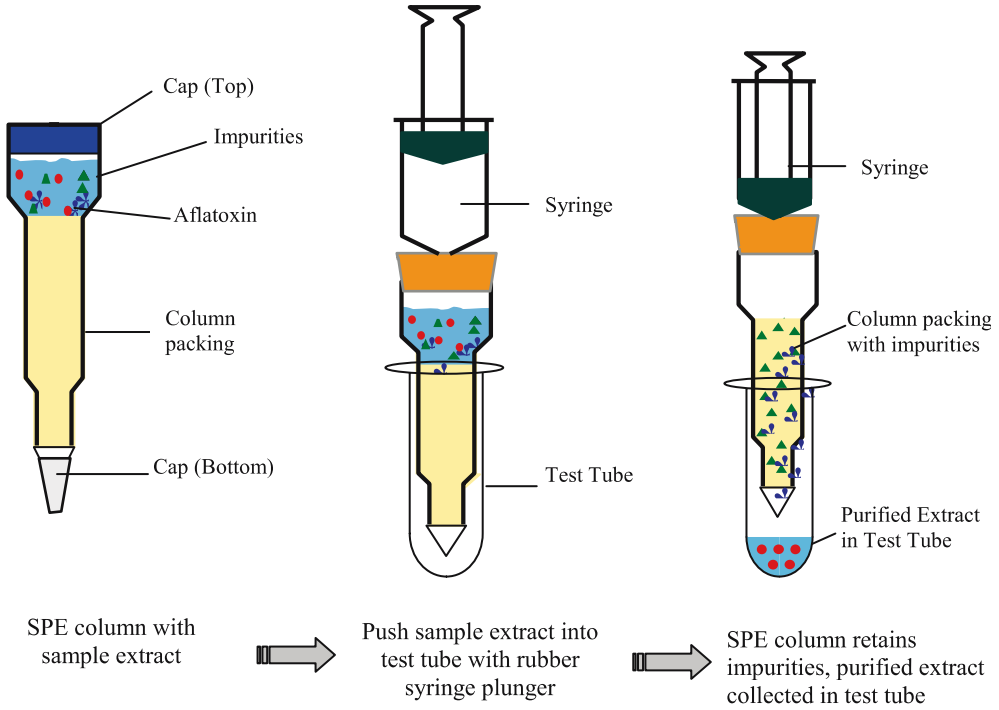

Figure 5. Principle of a one-step solid phase extraction column (aflatoxin: ●; impurities: ♣ ▲).

method is very rapid and simple due to the fact that the elution of the purified mycotoxin from the column is a single pass procedure using the extract solvent as the eluting solvent. An example of performance characteristics of the fluorometric assay with SPE column cleanup for the detection of aflatoxins is given in the Table 2. The extracts from the clean-up column can also be used for multi-toxin analysis by other methods. The column has a long shelf-life because it contains no biological reagents, and can be stored at room temperature. However, unlike immunoaffinity columns, the one-step SPE columns cannot concentrate the analyte during the clean-up procedure, and also the recovery may vary dependent upon the complexity of the food samples.

Fluorescent polarization

Fluorescence polarization immunoassay (FP) is based on the competition between mycotoxin and a mycotoxin-fluorescein tracer for a mycotoxin-specific antibody. The polarization is a measure of the orientation of the fluorescence emission from both horizontal and vertical directions but not a direct measure of fluorophore concentration. The observed orientation of the fluorescence is related to the rate of rotation of the fluorophore in solution, which, in turn, is related to the size of the fluorophore in solution. Small molecules have higher rates of rotation and lower polarization than larger molecules. The FP utilizes the interaction of a mycotoxin-specific antibody with a mycotoxin–fluorophore conjugate (tracer) to effectively decrease the rate of rotation of the tracer. Binding of the antibody to the tracer increases polarization; in the presence of free mycotoxin, lesser antibody is bound to the tracer, reducing polarization [24, 25]. Thus, the polarization value is inversely proportional to mycotoxin concentration (Figure 6).

Fluorescence polarization immunoassay has two important differences from ELISA: the detection does not involve an enzyme reaction, and separation of the bound and free compounds is not required. As a result, FP assays do not require a wash step and do not require waiting for an enzyme reaction for color development. In one study using a rapid (3 min) extraction and fluorescence polarization with a 2-min detection step and gave a recovery of 71.2% for spiked deoxynivalenol in wheat [26]. The method is simple to

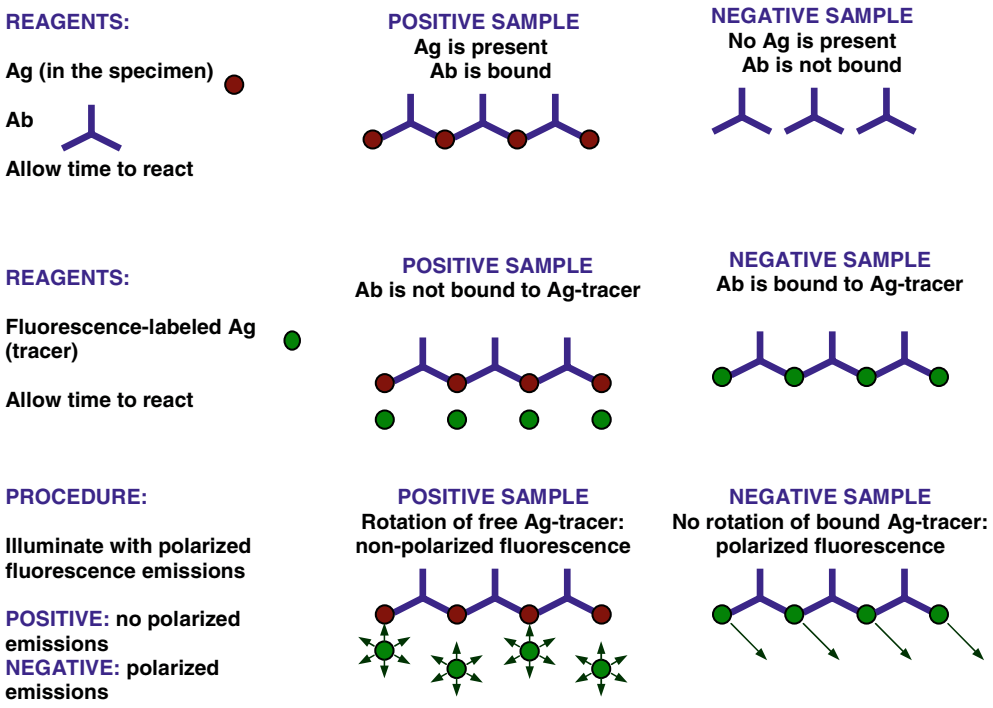

Figure 6. Principle of fluorescent polarization.

use and can be field portable. However, like ELISA methods, matrix effects may exist in FP and an extensive validation study is needed for the method application to different commodities. Additionally, the FP is not a high throughput method. Samples need to be analyzed serially, not as a batch.

Other emerging technologies

Other emerging technologies that are not yet commercially available for mycotoxin analysis include: (1) evanescent wave technology, (2) molecular imprinted polymers, (3) microarray technology, and (4) luminex xMAP® technology.

Evanescent wave technology

Surface plasmon resonance biosensor

At an interface between two transparent media of different refractive indices such as glass and water, light coming from the side of higher refractive index is partly reflected and partly refracted. Above a certain critical angle of incidence no light is refracted across the interface and total internal reflexion occurs at the metal film-liquid interface. Although the incident light is totally reflected, the evanescent wave penetrates a distance on the order of one wavelength into the lesser optical medium. If the interface between the medium of higher and lower refractive indices is coated with a thin metal film, then the propagation of the evanescent wave will interact with the electrons on the metal layer. These electrons are also known as plasmons, therefore, when surface plasmon resonance occurs, energy from the incident light is lost to the metal film resulting in a decrease in the reflected light intensity. The resonance phenomenon only occurs at an accurately defined angle of the incident light. This angle is dependent on the refractive index of the medium close to the metal-film surface. Changes in the refractive index of the buffer solution, to a distance of about 300 nm from the metal film surface will therefore alter the resonance angle. Continuous monitoring of this resonance angle allows the quantitation of changes in the refractive index of the buffer solution close to the metal-film surface (Figure 7). Since the change in the refractive index on the surface is a linear relationship to the amount of molecules bound, the content of molecules in buffer solution can be quantified [27].

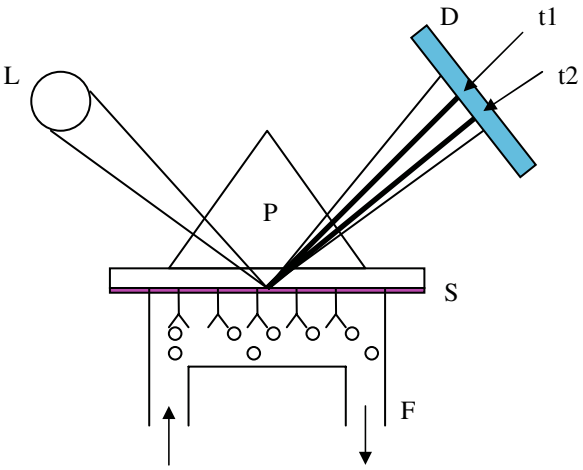

Figure 7. Principle of Surface Plasmon Resonance (SPR) L: light source, D: photodiode array detector, P: prism, S: sensor surface, F: flow cell. (The two thick lines in the reflected beam projected on to the D symbolize the dropping of light intensity following the resonance phenomenon at time = t_1 and t_2. The line projected at t_1 corresponds to the situation before binding of antigens to the antibodies on the surface and t_2 is the position of resonance after binding).

Substantial research in using SPR technology for mycotoxin analysis has been conducted and some results are quite promising [28–31]. SPR was developed for deoxynivalenol demonstrated an analytical range of 2.5–30 ng/mL with most results in agreement with LC-MS measurements on naturally contaminated wheat samples [30]. The SPR method also was developed for multiple mycotoxin analysis. Different mycotoxins were detected simultaneously within a time frame of 25 min, with detection limits for aflatoxin B1, zearalenone, ochratoxin A, fumonisin B1 and deoxynivalenol being 0.2, 0.01, 0.1, 50, 0.5 ppb, respectively [31]. The SPR method has several potential advantages such as: (1) a very small volume of sample is needed (in μL unit); (2) the metal chip can be re-used; (3) it can detect kinetics of antibody–antigen reaction; (4) it can detect a range of analytes; and (5) the method is user-friendly. However, sensitivity may be an obstacle for some SPR systems. Additionally, the monetary investment for SPR equipment is quite high.

Fiber optic immunosensor

An evanescent wave is generated at the interface between an optical fiber and an outside lower refractive index material (e.g., liquid or cladding) (Figure 8). Fluorescent molecules in this region can absorb energy from the evanescent wave and fluoresce. A portion of the fluorescence will be coupled back into the fiber and can be detected. By immobilizing antibodies to the surface of an optical fiber, fluorescent interference from the bulk solution is almost completely eliminated. The signal generated in the assay corresponds to the toxin concentration but varies depending on the assay format. Studies of the detection of fumonisin B1 by using a fiber-optic immunosensor showed that the sensor could detect fumonisin B1 in a quantitation range of 10–1000 ng/mL with a limit of detection of 10 ng/mL [32]. The advantages of the method are: (1) high specificity; (2) ease of miniaturization; (3) real-time monitoring and (4) adaptability for remote sensing. However, the method may have limitations in sensitivity. The sensitivity can be enhanced by using immunoaffinity column clean-up. Additionally, solvents may affect the accuracy of the method because they can change the refractive index of a medium.

Molecular imprinted polymers (MIPS)

Molecular imprinting is a process of template-induced formation of specific recognition sites in materials where the template directs the positioning and orientation of the material's structural components by a self-assembling mechanism [33]. A polymer is formed around a molecule acting as a template. After the template is removed the imprints contain functional groups complementary to those of the template (Figure 9). Potential applications of MIPs in mycotoxin analysis are: (1) solid phase extraction; (2) biosensor devices; and (3) chromatographic matrices for separations [34, 35]. Theoretically, the advantages of MIPs are: (1) MIPs are arguably the most generic and cost effective technique for preparing synthetic receptors; (2) MIPs can be prepared for practically any compound; (3) MIPs have similar affinity to natural biomolecules but often have better specificity; (4) MIPs can work in organic solvents; (5) MIPs are stable at low/high pHs, pressure and temperature; (6) polymers are compatible with microfabrication; (7) polymers are inexpensive. However, MIPs also have some limitations: (1) template costs may vary considerably from one compound of interest to another; (2) the exact stereochemical structure of the imprint is not known; (3) the unfavorable adsorption isotherm

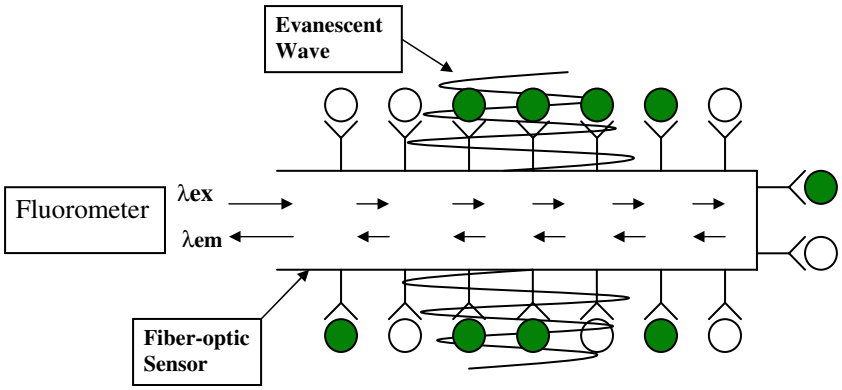

Figure 8. Principle of Fibre Optic Immunosensor (◯ Mycotoxin; ● Fluorescence labeled mycotoxin; Y Antibody).

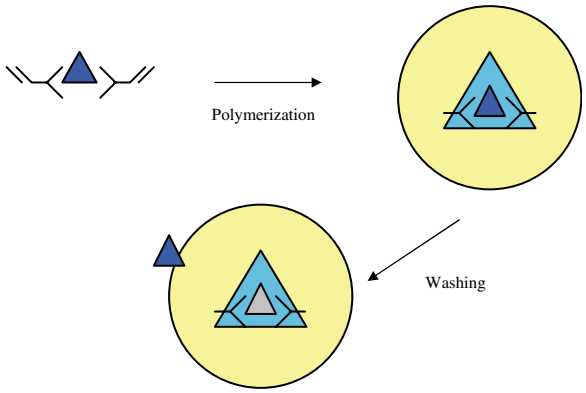

Figure 9. Principle of MIPs.

and slow mass transfer in the polymer matrix are among the major limitations for MIPs as separation media; (4) MIPs also tend to swell when solvent composition is altered leading to irreversible deformation of the imprinted cavity and loss of selectivity.

Microarray technology

Microarray technology, essentially a reverse dot blotting technique, is a new technology for the identification of DNA fragments such as PCR products, and may be a useful technique for identification and differentiation of high numbers of microorganisms in parallel [36]. An array of genus-, species- or sub-species specific capture oligonucleotides, representing the microorganisms to be identified, are immobilized on a solid support such as a glass slide. This glass slide may then be probed with labeled DNA from the sample of interest. Studies have been carried out for the identification and differentiation of a range of *Fusarium* species by designing and testing an oligonucleotide array [37]. The microarray technique has several advantages such as: high throughput analysis; small volume of sample needed and a wide range of microorganisms can be detected. However, it has several limitations in the application for mycotoxin analysis. Many researchers still do not trust data obtained from microarrays because of their high variability (low reproducibility). RNA extraction, amplification, and hybridization are all procedures involved in microarray research, and all have inherent problems leading to statistical errors. The method is designed to detect mycotoxin-producing fungal species, but not mycotoxins.

Luminex xMAP® technology

Luminex's xMAP® technology is comprised of existing technologies – flow cytometry, microspheres, lasers, digital signal processing and traditional chemistry. The range of applications

are considerable throughout the drug-discovery and diagnostics fields, as well as in basic research [38]. Microspheres are dyed to create 100 distinct colors. Each microsphere has a 'spectral address' based on red/infrared content. The suspendable microspheres are coated with capture reagents such as antibody or oligonucleotides. Sample is then added to microspheres and the analyte is captured by the microspheres. A fluorescent reporter tag is then added and results are read using a compact microsphere analyzer. The advantages of the technology are: high speed, high throughput, multi-analyte detection, versatility and reproducibility. There is no current method for mycotoxin analysis based on this technology.

Future developments in the analysis of mycotoxins should be for multi-mycotoxin analyses, further miniaturization, and portability for on-site mycotoxin testing. Also, the general trend is that methods must be user-friendly and cost-effective.

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