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Modern methods and trends in mycotoxin analysis*

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Introduction

Mycotoxins are toxic secondary metabolites naturally produced by molds (fungi) that may contaminate agricultural commodities when environmental conditions are favourable. Because molds are present in soil and plant debris, and are spread by wind currents, insects, and rain, they are frequently found in/on foods together with their associated mycotoxins (1). The public health concerns resulting from the finding of mycotoxins and the observation of both acute and chronic effects in animals has prompted the research effort focusing on analytical methods development. Analysis for mycotoxins is essential to minimize the consumption of contaminated food and feed.

However, method development and evaluation for mycotoxins is not a simple task. Determining the concentrations of toxins in grains at the µg/kg or parts-per-billion levels required for the most important mycotoxins is difficult. The approach generally followed consists of obtaining a relatively large primary sample representing a lot, reducing it in bulk and particle size to a manageable quantity, and finally performing the analysis on a small representative portion (2).

Given the vast number of methods that have been developed for the determination of mycotoxins in a variety of foods and feeds, a great deal of judgment is required for the selection of the optimum protocol of analysis. Besides performance criteria such as precision and trueness (contributing jointly to the accuracy), analytical procedures are characterized by three very practical criteria: (a) the speed with which the analysis can be performed, (b) the level of technical skills required to perform the assay, and (c) whether the assay provides a qualitative or quantitative result (3). Clearly, the most desirable methods incorporate all three: they are rapid, easy to use, and quantitative. In reality, most methods are a compromise and it is left to the users to determine the relative importance of each criterion for their application. This decision is the basis for selection of an analytical method that, in turn, will determine the technical expertise required to run the assays and the overall cost.

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Regardless of the method chosen, mycotoxin analysis usually involves extraction, cleanup, and detection.

Extraction

The purpose of extraction is to remove as much of the mycotoxin from the food matrix as possible into a solvent suitable for subsequent cleanup and determination. The assumption inherent in all extraction procedures is that the mycotoxin will be distributed evenly among the liquid phase and excluded from the solid phase of the mixture. The extent to which this assumption is valid will be reflected in the efficiency of mycotoxin recovery (3). However, the important factor is not the recovery figure itself (which is usually expected to range between 70 and 110%), but its consistency. Foods are typically extracted with mixtures of water and relatively polar solvents (acetone, acetonitrile, ethyl acetate, methanol). Acetonitrile and methanol are by far the most common solvents used for extracting the major mycotoxins, with the notable exception of patulin for which ethyl acetate is preferred. The composition of the extraction solvent is determined empirically from physical and chemical characteristics of the mycotoxin, the commodity, safety considerations, and the analytical procedure (4). The extraction solvent is often a compromise between the solvent strength required to efficiently extract toxins from foods and the compatibility of solvents with the analytical test system. Chlorinated solvents, like methylene chloride, very efficiently extract aflatoxins from corn, but their immiscibility with water precludes their use in aqueous-based analytical tests such as enzyme-linked immunosorbent assays (ELISAs). Miscibility is not a factor, however, if aflatoxins will be isolated using solid-phase extraction columns. Safety considerations and costs for waste solvent disposal may also affect the selection of extraction solvent.

There are two widely used approaches for extraction, either high speed blending with a solvent for a few minutes or shaking with a solvent for 30 minutes to 2 hours. Protocols with blending are more rapid than protocols with shaking when a small number of samples are being analyzed. In blending, care must be taken to ensure that the entire sample is continuously washed with the extraction solvent. However, if large numbers of samples are analyzed, the shaking protocol may be preferable, because modern shakers are capable of holding up to 20 samples concurrently. Once the solid sample has been shaken or blended with the extraction solvent, the liquid is separated from the solids either by filtration or centrifugation. The extract is then either cleaned up further to isolate the toxins, or applied directly to the determinative step in the procedure.

Cleanup

Once the mycotoxin has been extracted from the solid matrix, the liquid extract is cleaned up to remove impurities before the determinative, or quantitation step. The cleanup involves isolating the toxin from the extract and is a requirement for

most (but not all) analytical methods. In addition to removing sample impurities, the cleanup may further concentrate the mycotoxin prior to the determinative step. However, many screening methods, e.g. ELISA, require no cleanup other than dilution of the extract and/or filtration before analysis.

Generally, cleanup of the extracts is accomplished using solid-phase extraction (SPE) columns. SPE columns are usually a porous silica, the surface of which has been modified to provide selective absorption of either the analyte or impurities. In some cases, the analyte is retained on the columns while impurities pass through and are washed off. The analyte is then selectively removed by changing the composition of the rinse solution. In other cases, the SPE columns are designed to trap impurities and permit the analyte to pass through (5). The advantage of the latter type of column (known as multifunctional column) is that only the extraction solvent is needed to purify the sample, eliminating the need for additional solvent for elution. Recently, the packing materials of traditional SPE columns have been incorporated into new formats, including disks and 96-well plates, to permit more rapid isolation (3).

One invaluable form of SPE column is the immunoaffinity column, which currently represents the state-of-the-art of mycotoxin cleanup and analysis. In this format, antibodies attached to an inert support material are used to specifically bind the analyte while sample impurities pass through. The analyte is then removed from the column with a solvent that denatures the antibody. Several commercial immunoaffinity columns are available for aflatoxins, ochratoxin A, fumonisins, zearalenone, deoxynivalenol, and recently also for T-2 and HT-2 toxin (6). One advantage of immunoaffinity columns is the very specific nature of the interaction between the mycotoxin and the antibody. Although this may be offset somewhat by nonspecific interactions between matrix components and solid-phase support material, in most cases affinity columns are very efficient for removing sample impurities. Factors that influence antibody activity will influence the ability of the column to bind mycotoxins and therefore the ability to recover the toxin from foods. Factors such as the solvent strength applied to the column, the flow rate, and the volume of extract must be optimized to obtain accurate and reproducible results (3).

Despite potential pitfalls (like cross-reactivity of the antibody with closely-related compounds, or capacity problems leading to overloaded columns), it must be recognized that since the introduction of commercial immunoaffinity columns for mycotoxin analysis, there has been a steady and significant improvement in the precision achieved in collaborative studies, particularly at low ppb and sub-ppb concentrations (7–9). This has been due to ease of use of columns, high recoveries, and good selectivity irrespective of the type of matrices (10). The original basis for assessing performance characteristics of collaborative trial data was the HORRAT value (11), but since this was largely based on historical data, which fail to recognize recent improved performance, a modified assessment of this function has been introduced (12): there is now a general consensus on the fact that at mycotoxin con-

centrations less than 120 µg/kg, the relative standard deviation of reproducibility (RSD_R) is constant and equal to 22 % (13).

Detection

The final step in an analytical protocol involves determining whether the toxin is present, using at least one detection technology. Usually we make a distinction between *reference (confirmatory) methods,* which allow to detect, identify and quantify mycotoxins in various matrices, and *rapid screening methods* (also often classified as alternative methods) which are aimed essentially at detecting the presence of a mycotoxin or group of toxins.

Reference or confirmatory methods

Reference methods have several purposes: one is to confirm samples that have been determined to contain mycotoxins, based on rapid screening tests. The second is to more accurately quantitate the amount of toxin present. Reference methods for mycotoxins generally involve a chromatographic technique such as thin-layer chromatography (TLC), gas chromatography (GC), high-performance liquid chromatography (HPLC), or liquid chromatography with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) to further separate mycotoxins from extract impurities.

Thin-layer chromatography

Many of the pioneering studies on mycotoxins relied on thin-layer chromatographic (TLC) methods well before the general availability of HPLC and immunological methods. Although TLC is a reference method, it is often used as a mycotoxin-screening assay. TLC is a very powerful tool to determine the presence of one or more mycotoxins in a sample, but does not permit critical quantitation that may be required unless densitometry can be used (14). Typically, TLC involves the spotting of extracts, individually, near one end of a glass or aluminium plate on which a thin layer of silica gel or similar matrix has been placed. Suitable standards are also spotted on the plate for comparison after the plate has been developed. During development, the edge of the plate nearest the location of the spotted extracts and standards is placed in a specified solvent preparation covering the bottom of a tank that allows the plate to stand nearly vertical. The solvent is adsorbed by the silica or similar matrix and travels up the plate through the spotted extracts and standards. As this occurs, the various compounds in an extract spot are separated, depending on their adsorption to the matrix and solubility (3). Because these properties vary, the compounds are deposited at different heights on the plate. The plate can be removed from the tank when the solvent front nears the top of the plate, dried, and the spots can be visualized. Recently, a rapid, low-cost TLC secreening method was developed in our laboratory for the detection of ochratoxin A in green coffee at a control level of 10 µg/kg (15). This method, based on visual estimation of fluores-

cence intensity under a UV lamp, was shown to give results that were in good agreement with those obtained by a reference immunoaffinity/HPLC method on a series of naturally contaminated green coffees (15).

Densitometric quantitation conducted on a TLC plate is more accurate when the compounds are colored or fluorescent and the analyst does not have to spray or dip the plate to visualize the spots. TLC often can be used with little or no cleanup prior to spotting. In some cases, two-dimensional TLC can be utilized to find mycotoxins in extremely dirty samples. In such cases, a plate is developed with one solvent mixture in one direction, then rotated through 90° and run with a second solvent system. Often, unknown mycotoxins have been found on TLC plates that would not have been evident using other, more-quantitative methods. Moreover, confirming the identity of a specific mycotoxin can sometimes be conducted directly on a thin-layer chromatogram, e.g. by spraying a suitable derivatization reagent that causes a change in color.

Gas chromatography

Gas chromatography (GC) often is used in more technical laboratories for some of the mycotoxins and in particular for the analysis of type-A trichothecenes (T-2 toxin, HT-2 toxin, neosolaniol and diacetoxyscirpenol) that do not render themselves readily amenable to HPLC analysis. Components are separated using the relative affinity of the compounds for a stationary column and a mobile, inert gas. Analytes separated on the column and eluted with the inert gas are detected by chemical or physical means. Various detection systems may be utilized as coupled to GC, but in most cases electron-capture detection (ECD) and mass spectrometry (MS) have been employed. GC-ECD and GC-MS are highly sensitive methods that enable the simultaneous determination of several trichothecenes even in complex food matrices in the lower µg/kg range (16). However, major problems were identified within the framework of a recent EC-funded project (17, 18). These included higher trichothecene response for calibrants in presence of matrix than for pure calibrants, non-linear calibration curves, matrix interferences, and carry-over memory effects from previous samples (16–18). Although several recommendations could be made at the end of the study to reduce or eliminate these problems, analysis of trichothecenes by GC is still liable to unacceptable variations in repeatability and reproducibility (5, 16). Consequently, immunoaffinity and HPLC is now preferred to GC-ECD or GC-MS for the analysis of deoxynivalenol (19–21) and T-2 and HT-2 toxins (6), which are the most prevalent trichothecenes and the only ones currently subjected to national and/or international regulations.

High-performance liquid chromatography

High-performance liquid chromatography (HPLC) is the most frequently and widely used method of mycotoxin analysis (22). HPLC reference methods that are quite sensitive and have reasonably low levels of detection have been developed for

most of the major mycotoxins; thus, these are good quantitative methods. HPLC separates a mixture of compounds, usually present in an extract of a sample by relative affinity of the compounds for a stationary column and a mobile solvent. Compounds eluted from the column pass through a detector of some sort (usually fluorescence or ultraviolet (UV) depending on the physical and chemical attributes of the analyte of interest), and the detector helps quantitate the specific compounds in the original sample injected onto the column. It is sometimes necessary (or an advantage) to use precolumn or postcolumn derivatization to assist sensitive detection of the mycotoxin. For example, in the case of aflatoxins, on-line electrochemical bromination using a "Kobra" cell has become a well-established and robust procedure in order to enhance fluorescence before passing through the detector (7, 23). For many mycotoxins, the time for analysis following injection onto the column is less than 20 minutes, but the extract must be substantially cleaned up (e.g. through an immunoaffinity column) before injection. In contrast to TLC where plates are used once, the life of HPLC analytical columns depends on the operating conditions. Injection of relatively "dirty" samples will drastically shorten column life and may lead to broader peaks if residues build up in the injector or column (24). Indeed, final extracts may still contain compounds not removed during cleanup that may or may not be related to the target mycotoxin. These substances may have retention times similar to the target analytes, thereby leading to false positives or mis-identification. An example of such interference that has caused problems in the determination of patulin (which cannot be purified onto immunoaffinity columns) is 5-hydroxymethylfurfural (HMF) formed during the production and storage of apple juice. However, HPLC conditions can be easily modified to avoid such problems (25, 26). Similarly spices (and in particular nutmeg) have often caused problems during analysis for aflatoxins because of the large number of volatile compounds that are naturally present (24). Often the identity of the interfering compound is unknown, and in such cases liquid chromatography-mass spectrometry is most useful to avoid reporting false positive results.

Liquid chromatography-mass spectrometry

Liquid chromatography coupled with mass spectrometry (LC-MS) or tandem mass spectrometry (LC-MS/MS) is a powerful tool for mycotoxin detection and identification, particularly for those toxins for which there is little ultraviolet/visible (UV/VIS) absorbance or native fluorescence, like the fumonisins and *Alternaria* toxins. However, the majority of LC-MS and LC-MS/MS methods published so far have been developed for the determination of trichothecene mycotoxins in various cereals. Most of these methods employ either atmospheric pressure chemical ionization (APCI) or electrospray ionization (ESI) interfaces coupled with single or triple quadrupole mass spectrometers (27, 28). Ion-trap instruments have also been utilized for trace level quantification of mycotoxins, but compared to triple quadru-

pole instruments, they suffer from certain drawbacks like lower limits of detection, poor calibration linearity, and lower measurement repeatability (27).

In our laboratory, a method was developed for the simultaneous quantitative determination of the *Fusarium* mycotoxins deoxynivalenol, fumonisin B_1 and zearalenone in corn by liquid chromatography-atmospheric pressure chemical ionization tandem mass spectrometry (LC-APCI-MS/MS), using stable isotopically labelled and structural analogues internal standards (29). This procedure, which involves accelerated solvent extraction followed by two solid-phase cleanup steps on strong anion exchange resin and multifunctional column, allowed to reach quantitation limits of 50, 50, and 10 µg/kg for deoxynivalenol, fumonisin B_1 and zearalenone, respectively (29). Other totally different approaches have been developed that do not require any sample cleanup. For example, in his lecture given at the XIth International IUPAC Symposium on Mycotoxins and Phycotoxins (Bethesda, USA, 17–21 May 2004), *M.C. Spanjer* presented a multimycotoxin LC-ESI-MS/MS method allowing the determination of aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fumonisins B_1 and B_2, diacetoxyscirpenol, zearalenone, T-2 toxin, HT-2 toxin, roquefortine, and sterigmatocystin in single sample extracts (30). Validation data were shown for aflatoxins in peanuts and figs and for ochratoxin A and deoxynivalenol in wheat, but the method has still not been officially published. The author also reported strong matrix effects for peanuts and cornflakes, which were attributed to the absence of cleanup. The fact that in the electrospray ionization (ESI) process coextracted and coeluted matrix components can decrease the yield of analyte ion production by competition is now a well-recognized effect. The use of an internal standard that undergoes the same signal suppression as the analyte surely eliminates the inaccuracy problem, but in most cases the signal is still weakened (31). Similar problems were also reported by *Buttinger et al.* (32), who compared HPLC/fluorescence detection with triple quadrupole LC-MS/MS for analyzing ochratoxin A in corn samples. They observed significantly lower recoveries with LC-MS/MS, which could be traced down to ionization suppression of co-eluting matrix components, and concluded that LC-fluorescence was superior with respect to performance characteristics and for economic reasons (32).

The potential of LC-MS/MS for screening large amounts of samples for the presence of a number of mycotoxins has been demonstrated in many publications, but international collaborative studies should be conducted before this type of method gains a more widespread acceptance in quality assurance laboratories.

Nevertheless, it must be emphasized that LC-MS/MS offers unprecedented performance for studying the formation of artefacts, degradation and reaction products of mycotoxins, as well as the binding of mycotoxins to matrix components during food processing. Several interesting articles on this subject have been published during the last five years (33–35).

Rapid screening methods

The majority of rapid screening methods rely on antibodies to detect mycotoxins (immunological assays), and differ according to how this antibody is used in the assay. Currently we have essentially 3 basic techniques: the enzyme-linked immunosorbent assays (ELISAs), the dipsticks and lateral flow tests, and the solution fluorometry.

Enzyme-linked immunosorbent assays (ELISAs)

Sensitive microtiter plate immunoassays (ELISA format) are commercially available for a variety of mycotoxins including aflatoxins BG, aflatoxin M_1, ochratoxin A, the fumonisins, zearalenone, deoxynivalenol, citrinin, and T-2 toxin. Most of these kits rely on a competitive, heterogeneous ELISA format in which the toxin from the sample competes with a labelled toxin (such as toxin-enzyme conjugate) for a limited number of antibody-binding sites. The greater the amount of toxin present in the sample, the lower the binding of the labelled toxin and the lower the signal generated by the assay. In such assays, the presence of toxin is therefore measured by the absence of a response (i.e. color). This is the Achilles' heel of the ELISA tests because any factor that diminishes the binding between the labelled toxin and the antibody can be mistaken for the presence of toxin (3). Such factors may include structurally related mycotoxins as well as matrix constituents that are completely unrelated to mycotoxins but simply interfere with conjugate attachment to the antibody by absorbing the conjugate or antibody, by denaturing the antibody, or by inhibiting the enzyme. In other words, cross-reactivities and matrix dependence are the major drawbacks of ELISA methods, which can sometimes lead to a strong overestimation of mycotoxin contamination levels (36). For these reasons, ELISA kits should be used only with the foods for which they have been extensively tested and demonstrated to work. Also, sufficient standards must be employed for each test, to ensure the validity of the quantitation.

Dipsticks and lateral flow tests

Besides the common ELISA procedures there is an increasing demand for quick immunochromatographic tests in which the presence of the mycotoxin is directly detected in a disposable device. One of these diagnostic tools is the dipstick immunoassay, resembling ELISA constructs: instead of microtiter plates, carrier membranes (usually polyvinylidene difluoride, nylon or nitro-cellulose) are used to immobilize either the antibody or the antigen. Depending on the test format, one to three working steps have to be performed for obtaining the results (5, 37). The first dipstick assay was developed by *Schneider et al.* (38) for the detection of fumonisin B_1 in corn-based foods and was reported to have a visual limit of detection of 40–60 ng fumonisin B_1/g sample (38). Then a multi-analyte dipstick immunoassay for the detection of various mycotoxins in wheat was developed by the same researchers, however with limited sensitivity (39). In this case the response of the

test (toxin-exposed) dipstick was compared to the response of a control dipstick (not exposed to toxin) for estimation of toxin presence.

In parallel, lateral flow immunoassays have been developed to allow for single-step tests that combine the negative control reaction on the same strip as the sample and require only the addition of the sample solution. Each lateral flow device is a single unit (sometimes enclosed within a plastic housing) allowing for manual testing of individual samples. The device contains an antibody coupled to a colored particle (such as colloidal gold or latex) which is deposited in a reservoir pad, and a target mycotoxin immobilized onto the membrane (the strip itself). When the sample solution is poured onto the reservoir pad or when the strip is placed in a vial containing the sample solution, the labelled antibody is solubilized and binds to the target mycotoxin, if present. Then this mycotoxin-antibody complex flows with the liquid sample laterally along the surface of the strip. When the complex passes over the zone where the target mycotoxin has been immobilized, nothing happens because the complex has no binding sites available. As a consequence, only one band will appear in the results window, indicating that the liquid flowed properly up the strip. In the case of a blank sample, the labelled antibody remains free and can bind to the mycotoxin immobilized on the test zone of the membrane, producing the appearance of a colored band on the strip. Therefore, the presence of two bands in the results window indicates a negative result. The smallest mycotoxin concentration that results in no color intensity is considered to be the visual detection limit.

A lateral flow test can provide either a yes/no determination of the presence of the target mycotoxin or a threshold (semi-quantitative) result, typically in 5–10 min. Advantages of this format are that it is field portable, with all reagents immobilized onto the lateral flow dipstick, and that it requires no specialized equipment. Disadvantages include a subjective interpretation, and a much higher cost per test when compared with ELISA. Several companies in Europe and the United States have recently launched a variety of dipsticks and lateral flow tests, for example for the detection of total aflatoxins in cereals at cut-off levels of 4, 10 and 20 µg/kg, or for the detection of deoxynivalenol in wheat at a control level of 1000 µg/kg.

An interesting variant of lateral flow format is the flow-through immunoassay, which is different in that the flow is directed through the membrane rather than across it. In this format the applied liquids flow through the membrane and are collected on an absorbent pad on the opposite side of the membrane. The label is enzymatic, which requires a substrate-incubation step. This type of assay is also known as an enzyme-linked immunofiltration assay (ELIFA). Flow-through immunoassays have recently been described in the literature for the screening of ochratoxin A in green coffee beans (40) and roasted coffee beans (41), aflatoxin B_1 in nuts (42), and zearalenone in corn, wheat and feed samples (43). Moreover, a collaborative study of kits to detect ochratoxin A and T-2 toxin in wheat, rye, corn, and barley has been conducted by *De Saeger et al.* (44). The limits of detection for ochratoxin A and T-2 toxin were reported at 4 and 50 µg/kg, respectively.

Solution fluorometry

In addition to being a widely used tool for HPLC or GC techniques, immunoaffinity columns can also be used as the basis of a semi-quantitative test for some mycotoxins. In this case, the toxin is eluted in a cuvette, derivatized (usually by adding a bromine developer solution) to enhance fluorescence, and then detected in a portable fluorometer. Recent applications include the determination of fumonisins in corn (45), aflatoxin M_1 in cheese (46), ochratoxin A in ham (47), aflatoxins in sesame butter (48), and aflatoxins in grains and raw peanuts (49). In the latter three papers, a very good correlation was demonstrated between solution fluorometry data and quantitative results obtained by HPLC with fluorescence detection. However, in the case of aflatoxins, it must be emphasized that solution fluorometry does not allow to measure the aflatoxins B_1, B_2, G_1, and G_2 individually, but only gives an estimate of the total aflatoxin concentration. In-house data also showed that if the instrument is properly calibrated, the solution fluorometry technique works particularly well for the detection of aflatoxin M_1 in fresh milk, and ochratoxin A in wheat and green coffee beans (unpublished results). Finally, it is worth mentioning that almost 15 years ago, an AOAC/IUPAC collaborative study conducted to evaluate the effectiveness of an immunoaffinity column coupled with either solution fluorometry or liquid chromatography for determination of aflatoxins in corn, raw peanuts, and peanut butter led to the endorsement of both techniques by the two organizations (50). This method, now registered as AOAC Official Method 991.31, is still widely used today.

New Trends – Research Methods

Studies of the biosynthesis and mode of action of mycotoxins, measurements to determine their fate during food processing, the discovery of new mycotoxins, the development of new technologies, and the need to decrease analytical costs are factors that have helped to drive the development of so-called "research" methods. These are methods with limited application, or methods that are not yet widely used due to their novelty (3). Typical examples are near- and mid-infrared spectroscopy (NIR, MIR), molecularly imprinted polymers (MIPs), capillary electrophoresis (CE), fluorescence polarization (FP), fluorescence labelled optical-read dipstick assays (FLORIDAs), and immunological biosensors based on surface plasmon resonance or fiber optic probes.

Near- and mid-infrared spectroscopy (NIR, MIR)

The use of near infrared transmittance instrumentation for determination of the mycotoxin deoxynivalenol in wheat kernel samples was investigated in a Nordic pilot project (51). Dilution series of *Fusarium*-infected wheat were used for calibrating a Foss Infratec 1241 Grain Analyzer with an extended wavelength range of 570–1100 nm, and the results obtained on wheat materials were compared with HPLC and GC data. This work showed that it is possible to predict DON concen-

tration in wheat kernels by near infrared transmittance spectroscopy at levels higher than ca. 400 µg/kg (51). In 2003, another method based on mid (MIR)-infrared/attenuated total reflection (ATR) was developed for the rapid determination of *Fusarium graminearum* infection on corn (52). The sample was pressed onto the ATR crystal and reproducible pressure was applied. The recorded spectra were then subjected to principal component analysis and classified using cluster analysis. The method was shown to enable the separation of samples with a deoxynivalenol content as low as 310 µg/kg from non-contaminated (blank) samples. The investigated DON concentration range was 310–2596 µg/kg, and the percentage of correctly classified samples was up to 100 % for individual samples compared with a number of blank samples (52). However, for both methods, further work is needed to reveal the true potential of infrared spectroscopy for predicting mycotoxin levels in cereal samples.

Molecularly imprinted polymers (MIPs)

While the focus of this article has been on antibody-based technologies, an interesting alternative is the development of non-biologically based binding elements like molecularly imprinted polymers (MIPs). Molecular imprinting is a process where functional and cross-linking monomers are co-polymerized in the presence of a target analyte (the imprint molecule), which acts as a molecular template (53). The functional monomers initially form a complex with the imprint molecule, and following polymerization, their functional groups are held in position by the highly crosslinked polymeric structure. Subsequent removal (by liquid extraction) of the imprint molecule reveals binding sites that are complementary in size and shape to the analyte. In that way, a molecular memory is introduced into the polymer, which is now capable of selectively rebinding the analyte. The association between the imprint molecule and the monomers can be based on covalent, non-covalent or metal coordination interactions (5). The stability, ease of preparation and low cost of these materials make them particularly attractive (53). High affinity MIPs could essentially perform the same functions as antibodies in immunoassays and might benefit from greater solvent tolerance or tolerance to extremes of pH or ionic strength. Recently, MIPs have been reported for ochratoxin A (54–57), deoxynivalenol, and zearalenone (58). For example a MIP able to recognize ochratoxin A was prepared by using the mimic N-(4-chloro-1-hydroxy-2-naphtoyl-amido)-(L)-phenylalanine as a template (54). Experimental results showed that the MIP recognizes both the template and ochratoxin A. The specific molecular recognition effect was because of hydrogen-bonding interactions, but to ensure the full recognition effect, adjunctive steric factors seem to be necessary (54). While the affinities are not yet competitive with those of antibodies, this technique offers an excellent potential for further developments, even if applicability to real matrices has not yet been demonstrated (59).

Capillary electrophoresis

Capillary electrophoresis (CE) is a chromatographic technique in which mycotoxins are separated from one another and from matrix components using electrical potential (60). In brief, a fused silica capillary (typical length 50 cm) is filled with an aqueous run buffer and an electric field is applied to the capillary. Separation is achieved by migration of charged particles in the run buffer. Cations migrate to the cathode and anions migrate to the anode under the influence of an electroosmotic flow. The analytes are then detected using fluorescence or UV absorbance. The advantage of this technique is the potential decrease of solvent usage during the determinative step of the analysis, due to the relatively small volumes of sample injected (nanoliters) and the small volume of waste generated. Because the buffers used in the separation are aqueous, use of solvents beyond the extraction and cleanup steps can be virtually eliminated (3).

Capillary electrophoresis with laser-induced fluorescence detection has been used to analyze the fumonisins, aflatoxins, and ochratoxin A at sensitivities comparable to those achieved by more-traditional chromatographic techniques. For example *Corneli & Maragos* (61) developed a method for the determination of OTA in roasted coffee, corn and sorghum. The extraction and isolation procedures combined a silica column and an immunoaffinity cleanup column, as in other chromatographic methods. Due to the strong native fluorescence of OTA, a limit of detection of 0.2 ng/g has been achieved with this method (61).

Recent advances in mycotoxin detection with CE include the use of β-cyclodextrins combined with multiphoton-excited fluorescence for aflatoxin detection (62), and the use of micellar electrokinetic capillary chromatography (MECC) for the detection of neutral compounds or mixtures of neutral and charged compounds (63, 64). MECC is a variant of capillary zone electrophoresis which is performed by addition of micelle-forming compounds (surfactants) such as sodium dodecyl sulfate in the run buffer at a concentration above their critical micelle concentration (65).

The combination of CE with immunoassay has also been proposed for the analysis of fumonisin B_1 in corn (66). In this format an antibody is combined with sample extract and a fluorescein-tagged fumonisin (tracer). Then bound and unbound tracer are separated in an electrical field. With increasing fumonisin in the sample, the level of bound tracer decreased and the level of unbound tracer increased, signaling the presence of the toxin. However, the level of sensitivity was poor compared with alternative methods of analysis including derivatization of fumonisin B_1 for direct analysis by CE (37).

Fluorescence polarization

Fluorescence polarization (FP) is a technique widely used in the clinical area, which has recently been extended to mycotoxin analysis as well. A number of applications have already been described for measuring the aflatoxins (67), ochratoxin A (68), deoxynivalenol (69, 70), fumonisins (71) and zearalenone (72, 73).

Unlike most of the other immunoassays described in this review, the FP immunoassays are solution-phase assays: they can be conducted without the attachment of antibody to a solid surface. Fluorescence polarization detectors are indirectly measuring the rate of rotation of a fluorophore in solution (37). The rate of rotation is directly related to the size of molecules, with larger molecules rotating slower at a given temperature. With FP immunoassay a mycotoxin-fluorophore conjugate (tracer) is used. The tracer has a low molecular weight and rotates rapidly in solution. The addition of anti-toxin antibody results in the formation of an immune complex of the tracer with the antibody, effectively slowing the rate of rotation of the fluorophore and increasing the polarization (37).

The FP immunoassay therefore allows detection of low molecular weight materials in solution without requiring a step to separate the "free" and "antibody-bound" toxin, which is a significant advantage over traditional ELISA techniques (68). However, the technique has limitations as well. Indeed, when compared with HPLC data, most of the FP experiments undertaken with various mycotoxins have shown a positive bias of 20 to 30 %, which is resulting partly from known cross-reactivities of the antibodies towards other fungal metabolites, and partly from matrix effects (69). Such bias is not a limiting constraint if screening data are interpreted appropriately, which means that samples containing an excessive amount of mycotoxin as measured by the FP screening immunoassay must be re-tested with a reference method for confirmation.

Fluorescence labelled optical-read immuno dipstick assay

In contrast to conventional rapid immunoassays which are labelled with gold or latex beads, the Fluorescence Labelled Optical-Read Immuno Dipstick Assay (FLORIDA) uses special fluorescent complexes, raising signals which are detected visually with a sensitivity of only few ppt (parts per trillion). The test signal is generated by a competitive immunoassay on a lateral flow test strip (74). Fluorescence labelled antibodies will be bound to the antigen immobilized at a capture line if the antigen (mycotoxin) is absent in the sample or below the limit of detection. With samples containing sufficient mycotoxin, an antibody-antigen complex is formed which passes the capture line. To check the test, secondary antibodies immobilized at a control line have to capture the labelled antibodies in any case. The assay is carried out by transferring an aliquot of liquid sample or sample extract into a test cup, followed by insertion of the test strip directly into the liquid. After a reaction time of approximately 2 minutes, the test strip is evaluated by excitation of the fluorophores into a handheld lamp, where the capture and control lines can be detected visually. The advantage of this technique lies in a special (patented) way of conjugating the antibodies with fluorophores, which allows to achieve an extremely high sensitivity (cut-off values are typically in the range of 10 ppt). Consequently, the FLORIDA technique can be regarded as one of the most promising approach

towards developing rapid and highly sensitive immunoassays, for example for the detection of aflatoxin M_1 in fresh milk.

Biosensors

The development of biosensors for the rapid, reliable and low-cost determination of mycotoxins in foodstuffs has received considerable attention in recent years, and various types of assays have already been devised for several of the major groups of mycotoxins (75). One format uses the phenomenon of *surface plasmon resonance* (SPR) to detect the change in mass that occurs when mycotoxin-specific antibodies attach to a mycotoxin that has been covalently bonded to the surface of a sensor chip (76–78). A recent application developed and optimized for measuring deoxynivalenol in wheat extracts gave results that were in good agreement with LC/MS data (78). Moreover, SPR sensor chips with immobilized deoxynivalenol could be re-used more than 500 times without significant loss of activity (78). Because the instrumentation is now commercially available, this format could find widespread application to future mycotoxin analysis. A second format using *fiber-optic probes* can be adapted for continuous monitoring of mycotoxin levels. This sensor uses the evanescent wave of light that can form around the surface of an optical fiber. Antibodies attached to the surface of the fiber trap fluorescent mycotoxins (e.g. aflatoxins) or fluorescent analogs of mycotoxins (e.g. derivatized fumonisins) with the evanescent zone, permitting their detection. Two different benchtop devices have been designed for the fumonisins and aflatoxins (79). Unfortunately, most of the SPR and fiber optic biosensor procedures for mycotoxin analysis still require some form of sample cleanup/preconcentration in order to be truly effective in the analysis of real samples and to achieve adequate sensitivity. Moreover, the majority of these devices lack the ability to perform simultaneous analyses of multiple samples.

Recently, *array biosensors* have been developed and demonstrated for a variety of applications. The ability of array biosensors to analyze multiple samples simultaneously for multiple analytes offers a significant advantage over other types of biosensors. In particular, a rapid, multianalyte array biosensor developed by *Ngundi et al.* (80) at the Naval Research Laboratory of Washington D.C., USA, has demonstrated the potential to be used as a screening and monitoring device for clinical, food, and environmental samples. The device, which is portable and fully automated, can be used with different immunoassay formats. One interesting application is the development of a competitive immunoassay for the detection and quantification of ochratoxin A in a variety of spiked food and beverage samples. A simple extraction procedure was employed with no need for cleanup or preconcentration of the sample extract. This is the first demonstration that a rapid biosensor can be used in a competitive assay format to detect a mycotoxin in extracts of relevant foods (80). However, further work aimed at developing a dual-analyte assay for deoxynivalenol and ochratoxin A showed that improvements are still necessary to reduce the analysis time and increase the sensitivity.

Multi-mycotoxin methods – the ultimate challenge

The ultimate goal in mycotoxin analysis could be to analyze all the common mycotoxins to the sensitivities required in a method with a single extraction, cleanup and detection procedure. However, the diverse chemical structures and properties of mycotoxins make this an extremely challenging task, yet to be achieved (24).

As discussed above, multi-mycotoxin determination of specific mycotoxin groups of related compounds, such as trichothecenes, is possible by using GC-ECD or GC-MS, while the determination of the four naturally occurring aflatoxins using HPLC has been long established. However, combining these two structurally inhomogeneous groups into a single method is extremely difficult. Even within the trichothecene group the individual metabolites exhibit a wide range of polarity from the non-polar T-2 toxin to the highly hydroxylated nivalenol. Problems are compounded when other unrelated mycotoxins are included so that the method may be unsuccessful even at the initial extraction stage where, at the best, a comprise solvent mixture must be found (24). If this hurdle is overcome, the cleanup necessary is likely to be very complex and the end detection of all toxins in one system is in itself a major problem. Here, the diode array UV detector is able to detect most mycotoxins, as demonstrated by the pioneer work of *Frisvad & Thrane* (81), who developed an HPLC-UV method for the analysis of 182 structurally different mycotoxins. This method is appropriate for a qualitative identification of mycotoxins produced in fungal cultures, but certainly not for the detection of trace levels of toxins in food and feed extracts. LC-MS may ultimately be the technique that comes closest to achieving the aim of a multi-analyte method, but would not be a cheap or simple solution available to all.

There have been a number of successful attempts to integrate several mycotoxin groups into a single method. Most include complex cleanup, separation and detection schemes that cater to all the mycotoxins required so that the method soon loses its intrinsic advantage over simpler "single toxin" methodology (24). Perhaps the realistic approach with current technology is to limit multi-methods to groups of mycotoxins that commonly occur together. While most immunoaffinity columns have been designed for the analysis of single mycotoxins, new types of columns containing antibodies specific to both aflatoxins and ochratoxin A (82), or to aflatoxins, ochratoxin A and zearalenone (83), have recently become commercially available. This type of cleanup, which can be fully automated and combined with HPLC-fluorescence or LC-MS/MS, could find widespread use in the near future.

Conclusion

Besides well-established immunoaffinity/HPLC reference methods, emerging technologies for mycotoxin detection are at various stages in the progression to useful analytical tools. While some are advanced enough for field use, many others still face the challenge of making the transition from proof-of-concept assays using tox-

ins in buffer solutions to analysis of real food samples (37). However, despite these obstacles, detection technologies continue to advance, and the prospects for further improvements in mycotoxin analysis are excellent, even if universal multi-mycotoxin analysis appears unlikely in the near future.

Summary

The introduction of demanding mycotoxin regulations is increasing over the years and requires the development of ever more sensitive and reliable analytical methods that can provide the appropriate tests at an acceptable cost. In this review a distinction is made between reference (confirmatory) methods which allow to detect, identify and quantify mycotoxins in various matrices (TLC, GC, HPLC, LC-MS, LC-MS/MS), and rapid screening immunoassay methods which are aimed essentially at detecting the presence of a mycotoxin or group of toxins (ELISAs, dipstick assays, lateral flow tests, and solution fluorometry). This article also focuses on recent developments in technologies for detection of mycotoxins, with a particular emphasis on infrared spectroscopy (NIR, MIR), molecularly imprinted polymers (MIPs), capillary electrophoresis, fluorescence polarization, fluorescence labelled optical-read dipstick assays, biosensors based on surface plasmon resonance (SPR) or fiber-optic probes, and immunological array biosensors.

Zusammenfassung

Die zunehmende Tendenz zur Verschärfung der gesetzlichen Grenzwerte im Bereich der Mykotoxine erfordert gleichzeitig die Entwicklung immer sensitiverer verlässlicher analytischer Methoden, die die erforderlichen Kontrollen zu einem annehmbaren Preis erlauben. Der vorliegende Review vergleicht Referenzmethoden (Bestätigungsmethoden), die eine qualitative und quantitative Bestimmung von Mykotoxinen in verschiedenen Matrices ermöglichen (TLC, GC, HPLC, LC-MS, LC-MS/MS), mit immunologischen Schnelltests, die ausschliesslich die Präsenz eines individuellen Mykotoxins oder der einer Gruppe eines Toxins nachweisen können (ELISA, Dipstick, Lateral Flow Test, Solution Fluorometry). Der Artikel befasst sich weiterhin mit den neuesten Entwicklungstechnologien zum Nachweis von Mykotoxinen insbesondere der Infrarot-Spektroskopie (NIR, MIR), den molekular geprägten Polymeren (MIPs), der Kapillarzonenelektrophorese, der Fluoreszenz-polarisation, den fluoreszenz-markierten Dipsticks vom Typ FLORIDA, den Biosensoren basierend auf Oberflächenplasmonresonanz (SPR) oder faseroptischen Sonden, sowie mit immunologischen Array Biosensoren.

Résumé

La tendance à fixer des normes de plus en plus strictes pour les mycotoxines s'accentue au fil des années et nécessite le développement de méthodes d'analyse toujours plus sensibles et fiables afin de pouvoir réaliser les contrôles nécessaires à un coût acceptable. Dans cette revue, on fait une distinction entre les méthodes de

référence (ou de confirmation) qui permettent de détecter, identifier et quantifier les mycotoxines dans différentes matrices (CCM, GC, HPLC, LC-MS, LC-MS/MS), et les méthodes rapides de screening servant essentiellement à détecter la présence d'une mycotoxine ou d'un groupe de toxines (tests ELISA, tests de type bandelette, tests à débit latéral, et fluorométrie en solution). Cet article traite également des récents développements concernant les technologies utilisées pour la détection des mycotoxines, et plus particulièrement la spectroscopie infrarouge (NIR, MIR), les polymères à empreinte moléculaire (MIPs), l'électrophorèse capillaire, la polarisation de fluorescence, les bandelettes fluorescentes de type FLORIDA, les biocapteurs à résonance du plasmon de surface (SPR) ou à fibre optique, et finalement les capteurs immunologiques de type matriciel.

Key words

Mycotoxins, analysis, reference methods, rapid screening methods, research methods

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