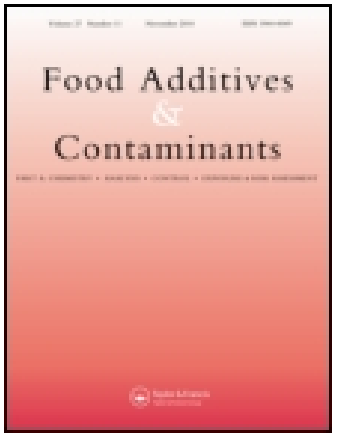

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Mycotoxin analysis: An update

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Mycotoxin analysis: An update

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Abstract
Mycotoxin contamination of cereals and related products used for feed can cause intoxication, especially in farm animals. Therefore, efficient analytical tools for the qualitative and quantitative analysis of toxic fungal metabolites in feed are required. Current methods usually include an extraction step, a clean-up step to reduce or eliminate unwanted co-extracted matrix components and a separation step with suitably specific detection ability. Quantitative methods of analysis for most mycotoxins use immunoaffinity clean-up with high-performance liquid chromatography (HPLC) separation in combination with UV and/or fluorescence detection. Screening of samples contaminated with mycotoxins is frequently performed by thin layer chromatography (TLC), which yields qualitative or semi-quantitative results. Nowadays, enzyme-linked immunosorbent assays (ELISA) are often used for rapid screening. A number of promising methods, such as fluorescence polarization immunoassays, dipsticks, and even newer methods such as biosensors and non-invasive techniques based on infrared spectroscopy, have shown great potential for mycotoxin analysis. Currently, there is a strong trend towards the use of multi-mycotoxin methods for the simultaneous analysis of several of the important *Fusarium* mycotoxins, which is best achieved by LC–MS/MS (liquid chromatography with tandem mass spectrometry). This review focuses on recent developments in the determination of mycotoxins with a special emphasis on LC–MS/MS and emerging rapid methods.

Keywords: *Mycotoxin, analysis, LC–MS/MS, sample preparation, chromatographic methods*

Introduction

Mycotoxins are natural, secondary metabolites produced by fungi on agricultural commodities in the field and during storage under a wide range of climatic conditions. About 200 different filamentous fungi species, e.g. *Aspergillus*, *Penicillium* and *Fusarium* species (sp.), have been identified. Several hundred different mycotoxins have been discovered so far, exhibiting great structural diversity, which results in different chemical and physicochemical properties. Aflatoxins and ochratoxins (produced mainly by *Aspergillus* sp.), fumonisins, trichothecenes and zearalenone (produced by *Fusarium* sp.), patulin (produced by *Penicillium* sp.), and ergot alkaloids (produced in the sclerotia of *Claviceps* sp.) receive the most attention due to their frequent occurrence and their severe effects on animal and human health (Bennett and Klich 2003; D'Mello and MacDonald 1997). Mycotoxins are potent toxins and have a wide range of actions on animals and humans, e.g. cyto-, nephro- and neurotoxic, carcinogenic, mutagenic, immunosuppressive and estrogenic effects. Although mycotoxicoses caused by direct consumption of contaminated food and feedstuffs poses the greatest risk to animals and humans, the entry of mycotoxins or their metabolites into the food chain by ''carry over'' into milk, animal tissue or eggs, for example, should not be underestimated.

National and international institutions and organisations, such as the European Commission (EC), the US Food and Drug Administration (FDA), the

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World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) of the United Nations, have recognized the potential health risks to animals and humans posed by food- and feed-borne mycotoxin intoxication and addressed this problem by adopting regulatory limits for major mycotoxin classes and selected individual mycotoxins. The FAO has compiled comprehensive worldwide regulations and directives regarding mycotoxins in food and feed as of December 2003 (FAO 2004). The Joint Expert Committee on Food Additives (JECFA), a scientific advisory body of FAO and WHO, provides mechanisms for assessing the toxicity of food additives, veterinary drug residues and contaminants, and has recently evaluated the hazards related to several mycotoxins, including fumonisins B_1, B_2 and B_3, ochratoxin A, deoxynivalenol, T-2 toxin, HT-2 toxin, and aflatoxin M_1 (WHO 2002). The report explains the nature of each toxin, including its absorption and excretion, as well as toxicological studies, and it includes general considerations of analytical methods, sampling, associated intake issues and control mechanisms.

The EC has set maximum levels for some mycotoxins, including several aflatoxins, ochratoxin A, patulin, deoxynivalenol and zearalenone, in certain foodstuffs. Maximum levels for fumonisins B_1 and B_2 came into force in October 2007. Consideration of a review of the maximum levels for deoxynivalenol, zearalenone and fumonisins B1 and B2 as well as the appropriateness of setting a maximum level for T-2 and HT-2 toxins in cereals and cereal products should be completed by July 2008 (EC 2006).

The requirement to apply these regulatory limits has prompted the development of a vast number of analytical methods for the identification and quantification of mycotoxins in various samples, such as food, feed, and other biological matrices. The chemical diversity of mycotoxins and their varying concentration ranges in a wide range of agricultural commodities, foods and biological samples poses a great challenge to analytical chemists. The different chemical and physicochemical properties of the mycotoxins require specific extraction, cleanup, separation and detection methods. Therefore, most methods target only individual mycotoxins or at best a group of closely related mycotoxins. These methods are usually based on labour-intensive sample preparation protocols followed by traditional chromatographic separation (mostly liquid chromatography, LC). Gas chromatography (GC) either with electron capture detection (ECD) or mass spectrometric (MS) detection is used in mycotoxin analysis, e.g. for trichothecene or patulin determination, but less frequently than alternative methods. In some cases, fast and accurate screening methods based on enzyme-linked immunosorbent assay (ELISA) are applied instead of the more labour-intensive LC methods. Thin-layer chromatography (TLC) provides a cheaper alternative to LC-based methods and has an important role, especially in developing countries, for surveillance purposes and control of regulatory limits (Gilbert and Anklam 2002). Modern sample clean-up techniques, such as immunoaffinity columns (IAC) or solid-phase extraction (SPE) methods, help to simplify protocols, improve selectivity and, thus, performance characteristics.

To deal with the increasing number of sample matrices and mycotoxins of interest, fast and accurate analytical methods are needed. This demand has led to the development of rapid screening methods for single mycotoxins or whole mycotoxin classes based on immunochemical techniques (e.g. ELISA), biosensors (e.g. protein chips, antibody/protein-coated electrodes) and non-invasive optical techniques. On the other hand, highly sophisticated multi-mycotoxin methods based on LC coupled to multiple-stage MS are being developed to allow accurate and precise determination and unambiguous identification of mycotoxins without the need for tedious sample preparation and clean-up procedures.

Sample selection and representative sample collection are often underestimated as sources of error. The design of sampling procedures for various mycotoxins and sample materials has been an international concern for several years. (FAO 2004; EC 2006b; FDA 2007). To obtain comparable data, the EC has laid down certain requirements for sampling and performance criteria for analytical methods (EC 2006b). Therefore, the whole analytical method (including sampling, sample preparation, clean-up and final determination) used by enforcement laboratories for the implementation and control of legislation and regulatory limits must be subject to a validation procedure to show that the method produces reliable results and meets the set performance criteria. Several protocols and guidelines for method validation have been published (Thompson 1993; ISO 1994; Eurachem 1998). There are a multitude of analytical methods available that have been validated and accepted by official authorities, such as the European Committee for Standardization (CEN), the Association of Official Analytical Chemists (AOAC International), and the International Organisation for Standardization (ISO) (Gilbert and Anklam 2002, AOAC 2005). Each laboratory should implement quality assurance measures such as frequently checking the accuracy and precision of their methods by analysing (certified) reference materials (CRM) and by regular participation in proficiency

testing trials. Although much has been done in the past years regarding the production and certification of reference materials (RM) and calibrants for mycotoxin analysis in various matrices, there is still a need for more RMs appropriate for the different sample matrices and concentration ranges encountered in foods and feeds.

The objective of this review is to summarize recent developments in the determination of mycotoxins with a special emphasis on LC–MS/MS and emerging rapid methods.

Sampling

Sampling plays a crucial part in the precision and determination of mycotoxin levels due to the sometimes very heterogeneous distribution of the toxins in agricultural commodities and products intended for human and animal consumption. The distribution of mycotoxins in the sample material is an important factor to be considered in establishing regulatory sampling criteria. In the past, this has been recognized by many national and international authorities and organisations, such as the EC (2006a), the FDA (2007) and the FAO (2004). In Commission Regulation No. 401/2006 (EC 2006b), the EC has laid down the methods of sampling and analysis for the official control of levels of various mycotoxins in foodstuffs, repealing all former directives and amendments on this subject. It includes methods of sampling and analysis of aflatoxins, ochratoxin A, patulin and *Fusarium* toxins in cereal, dried fruit, fruit juices, must and wine, groundnuts and nuts, spices, milk, coffee, products derived from the above mentioned basic materials as well as baby foods and food for infants and young children. While it can be assumed that mycotoxins in liquid samples are homogeneously distributed, some mycotoxins, especially in fungus-contaminated grain, may be concentrated in so-called ''hot-spots''. Mycotoxins, especially those produced by *Aspergillus* sp., e.g. aflatoxins, can be distributed very heterogeneously in food products with large particle size such as dried figs or groundnuts. The number of contaminated particles may be very low, but the contamination level within a particle can be very high. To obtain the same representativeness for batches of food products with large particle sizes, the weight of the incremental sample taken has to be larger than in cases of batches with smaller particle size. Commission Regulation No. 401/2006 regulates the number of incremental samples to be taken from different places of a lot depending on the weight of the entire lot. This may result in rather large aggregate samples, up to 30 kg in the case of aflatoxin determination in dried figs, groundnuts and nuts. However, it has to be considered that handling and sample preparation of large quantities pose great difficulties in implementing such sampling plans, especially with regard to the validation process of sampling methods. The EC regulation, therefore, stipulates the division of aggregate samples intended for direct human consumption into up to three laboratory samples of $\leq$10 kg for homogenisation and analysis. This subdivision is not necessary for products intended for further sorting or processing before human consumption or for use as an ingredient (SANCO 2005).

The Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) developed general sampling guidelines for grain (GIPSA 1995), rice (GIPSA 1994) and hops (GIPSA 1998). The FDA is constantly updating their Investigative Operation Manual (IOM) that describes general procedures for field investigators and inspectors and includes information on sampling and sampling schedules for various occasions (FDA 2007). It contains a detailed sampling schedule for mycotoxin analysis that lists sample sizes dependent on the type of product and distinguishes between samples taken for surveillance (initial sample) and follow-up samples in case of positive findings. The minimum total sample size ranges from 4.5 kg up to 34 kg, depending on the heterogeneity expected of the sample type.

Kay (2001) compared a number of approaches for sampling grain, developed by different national and international authorities, and provided an interpretation of the major differences between the methods in terms of lot size, tolerances, sampling techniques, sample size, rates, etc.

Sample preparation and clean-up

Only a few analytical techniques, i.e. optical techniques based on IR spectroscopy (Kos et al. 2003), are capable of detecting mycotoxin contamination directly in ground cereal samples without the necessity of further sample preparation, such as solvent extraction or clean-up. However, the application of such techniques is still limited to screening purposes due to a high matrix dependence and lack of appropriate calibration materials.

Analytical methods based on chromatography or immunoassays usually require solvent extraction to liberate the mycotoxin from the sample matrix, and subsequent clean-up of the extract to reduce matrix effects. Various combinations of solvents, sometimes with the addition of modifiers (e.g. acids, bases, etc.), are used for extraction, depending on the

physicochemical properties of the mycotoxins, the sample matrix and the type of clean-up used afterwards (Zöllner and Mayer-Helm 2006). Accelerated solvent extraction (ASE), also known as pressurised liquid extraction (PLE) (Royer et al. 2004; Urraca et al. 2004; Juan et al. 2005; Pallaroni and van Holst 2003, 2004) or microwave-assisted extraction (MAE) (Pallaroni et al. 2002) help to speed-up and automate the extraction process, and offer a robust and time-saving alternative to classical solvent extraction techniques. So far, the high cost of an ASE apparatus has, however, limited the application of this technique in the field of mycotoxin analysis to a few laboratories. Supercritical fluid extraction (SFE), especially with supercritical CO_2 as an environmentally safe extraction medium, received a lot of attention in the 1990s. The extraction selectivity of the non-polar supercritical CO_2 is influenced by temperature and pressure and can be varied in a wide range by adding modifiers (polar solvents, complexing agents, etc.). Although in the past, SFE has received much attention regarding agricultural applications, only a few papers deal with it as an extraction method in mycotoxins analysis (Huopalahti et al. 1997; Krska 1998; Ambrosino et al. 2004; Liau et al. 2007).

Liquid–liquid partitioning of the mycotoxin containing aqueous acetonitrile/methanol sample extract with hexane is sometimes used for de-fatting or protein precipitation (Sørensen and Elbæk 2005; Kokkonen et al. 2005). For further purification and analyte enrichment, liquid samples and extracts are predominantly submitted to solid-phase extraction (SPE) protocols for which a wide variety of sorbent materials are available. A comprehensive compilation of different clean-up approaches for various mycotoxins has been published by Zöllner and Mayer-Helm (2006). Conventional SPE procedures use reversed-phase (RP) materials (e.g. C_8, C_{18}), strong cation or anion exchangers (SCX, SAX) or polymeric materials with combined properties. Modern clean-up procedures employ multifunctional MycoSep® (Krska 1998; Radová et al. 1998; Biselli and Hummert 2005; Ren et al. 2007) or immunoaffinity columns (IAC) (Krska 1998), although these methods are more expensive than conventional clean-up methodologies. MycoSep® columns contain a mixture of charcoal, ion-exchange resins and other materials and are suitable for aflatoxins, trichothecenes, ochratoxins, zearalenone, moniliformin and patulin (Romerlabs 2007). Mycotoxin specific molecularly imprinted polymers (MIPs) are also considered as a potential and cheaper alternative for clean-up, which, contrary to IACs, do not suffer from storage limitations and stability problems regarding organic solvents. MIPs have been developed with recognition properties towards several mycotoxins including deoxynivalenol (Weiss et al. 2003), zearalenone (Weiss et al. 2003; Urraca et al. 2006a, b), ochratoxin A (Baggiani et al. 2001; Jodlbauer et al. 2002; Maier et al. 2004; Turner et al. 2004) and moniliformin (Appell et al. 2007).

Currently, there is a strong trend towards the use of IACs in mycotoxins analysis as a clean-up and enrichment technique for sample extracts or liquid samples. IACs contain immobilised antibodies that exclusively retain a certain mycotoxin or mycotoxin class. Due to their high specificity, IACs produce cleaner extracts with a minimum level of interfering matrix components and excellent signal-to-noise ratios compared to less selective SPE sorbent materials. IACs have been developed for most major mycotoxins and mycotoxin classes such as aflatoxins, ochratoxin A, trichothecenes, zearalenone and their metabolites (Zöllner and Mayer-Helm 2006). The AOAC International and the EC have already validated a few IAC methods; however, these address only a limited number of food commodities. For some mycotoxins, such as ochratoxin A, IACs are already used in routine analysis, e.g. coupled with LC with fluorescence detection (FLD). When comparing both conventional clean-up and IAC approaches in the analysis of selected mycotoxins (aflatoxins, B-fumonisins, and ochratoxin A), discrepancies are found for certain food and feed matrices (Castegnaro et al. 2006; Sugita-Konishi et al. 2006). These problems highlight the necessity to validate methods for each complex matrix separately to provide reliable, comparable and traceable analytical data.

Careful selection of the clean-up method is, however, essential for the effectiveness of an analytical method. Immunoaffinity materials are expensive and distinctly less feasible for multitoxin analysis since they are highly specific for only one target mycotoxin (or class). Some scientists even talk about ''overkill'' when using highly specific clean-up techniques, such as IACs in combination with liquid chromatography with mass spectrometry (LC/MS), since compound-specific detection stands in contradiction to the multi-analyte detection capabilities of MS (Leitner et al. 2002; Zöllner et al. 1999). However, there are already combined immunoaffinity materials on the market that are specific to a wider range of mycotoxins (MacDonald et al. 2007). It has been shown that, in many cases, the quality of the analytical result does not suffer when conventional SPE approaches are used (Leitner et al. 2002; Reinsch et al. 2005). Of course, this also depends on the selectivity of the MS equipment itself. Single-stage MS in selected ion-monitoring mode might need selective clean-up to remove matrix interferences, while those interferences

might not be visible with multi-stage MS in selected reaction-monitoring mode (Zöllner and Mayer-Helm 2006). However, matrix-induced signal suppression or enhancement should always be taken into consideration and can normally be omitted by clean-up of the extract or by using an appropriate calibration method (e.g. matrix-matched calibration standards, standard addition, or the use of adequate internal standards, i.e. isotope-labelled standards, etc.).

Analytical techniques

Conventional analytical techniques

The term ''conventional method'' usually refers to a chromatographic separation coupled to a suitable detection system. The currently used quantitative methods for the determination of regulated mycotoxins, such as the fumonisins, zearalenone, type-A (e.g. T2-toxin) and -B trichothecenes (e.g. deoxynivalenol), ochratoxin A and the aflatoxins, in food and feed mainly use immunoaffinity clean-up with high-performance liquid chromatography (HPLC) or gas chromatography (GC) in combination with a variety of detectors, such as fluorescence detection (FLD) with either a pre- or post-column derivatisation step, UV detection, flame ionisation detection (FID), electron capture detection (ECD) or mass spectrometry (MS). Reviews of these methods have been summarized and published elsewhere (Krska et al. 2001, 2005; Krska and Josephs 2001). From the multitude of available procedures, CEN is trying to standardize methods for mycotoxin analysis. CEN establishes performance criteria for mycotoxin methods usually on the basis of collaborative studies. CEN methods are official reference methods and are used for official control and surveillance and in cases of dispute. CEN–approved methods exist for aflatoxins, ochratoxin A, fumonisins, patulin and deoxynivalenol, for example, in various foods. Further methods for various mycotoxins in feed will be issued in the near future (Gilbert and Anklam, 2002).

Liquid chromatography/mass spectrometry (LC/MS)

Within the last 10 years, liquid chromatography/mass spectrometry has become the universal approach for mycotoxin analysis, as more or less all potential analytes are compatible with the conditions applied during separation and detection. Nevertheless, the breakthrough of this approach did not occur until the mid-1990s, when suitable interfaces, such as atmospheric pressure ionization, became accessible on a routine basis. Compared to conventional detection techniques, such as UV or fluorescence, mass spectrometry offers increased selectivity and sensitivity (although fluorescence detection might be more sensitive for certain mycotoxins, e.g. aflatoxins), unambiguous confirmation of the molecular identity of the analyte and the option to use isotopically labelled substances as internal standards. Furthermore, it is possible to investigate the molecular structure of metabolites and sugar conjugates (such as ''masked mycotoxins''; Berthiller et al. 2005b) and to omit time-consuming and error-prone derivatization and clean-up steps. However, it must be kept in mind that a reduction of the sample preparation inevitably emphasizes the Achille's heel of LC/MS, i.e. relatively poor method accuracy and precision due to the irreproducible and unpredictable influence of co-eluting matrix components on the signal intensity of the analytes.

Due to the large number of LC/MS-based methods for the quantitative determination of single mycotoxin classes, their exhaustive examination goes beyond the scope of this work and, therefore, the interested reader is referred to the reviews of Zöllner and Mayer-Helm (2006) and Sforza et al. (2006).

Multi-mycotoxin methods

In the last few years, increased efforts have been made to develop analytical methods for the simultaneous determination of different classes of mycotoxins using LC–MS/MS. This trend is a result of the discovery of co-occurrence of different toxins and related synergistic toxic effects that raise concerns about the health hazard from contaminated food and feed (Creppy et al. 2004; Speijers and Speijers 2004). In addition, it would be desirable to cover the toxins addressed by Commission Regulation 1881/2006 (aflatoxin B1, B2, G1, G2 and M1, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisin B1 and B2, HT-2 and T-2 toxin) with a single method as this increases sample throughput and decreases the costs per analysis. Although mass spectrometry offers sufficient selectivity (especially if tandem-mass spectrometry is applied) and multi-analyte capabilities, its realization in the field of multi-mycotoxin analysis has been hampered mainly by the chemical diversity of the different toxin classes, which include acidic (fumonisins), basic (ergot alkaloids) as well as polar (moniliformin, nivalenol) and apolar (zearalenone, beauvericin) compounds. Therefore, compromises have to be made in the choice of extraction solvent and mobile phase, and the conditions may be far from optimal for certain analytes.

The initial stimulus for LC/MS-based multi-mycotoxin methods came from the field of mycology, where mass spectrometry is used to identify mould species according to their metabolite profile (Smedsgaard and Frisvad 1996). Beside the development of databases dealing with qualitative LC/MS of mycotoxins (Nielsen and Smedsgaard 2003), this has led to early quantitative methods for the simultaneous determination of *Aspergillus* and *Penicillium* mycotoxins in building materials (Tuomi et al. 2001) and in an artificial food matrix (Rundberget and Wilkins 2002). While the former method suffered from low recoveries of some analytes, excellent accuracy and precision were obtained in the latter case through use of a de-fatting step applied to the raw extract, and use of matrix-matched calibration to compensate for matrix effects. Some years later, this method was applied for the simultaneous determination of aflatoxins, ochratoxin A, mycophenolic acid, penicillic acid and roquefortine C (Kokkonen et al. 2005) after a slight modification of the extraction solvent.

After this initial phase, the focus of multi-mycotoxin analysis shifted to *Fusarium* mycotoxins. Royer et al. (2004) developed a method for the quantitative analysis of deoxynivalenol, fumonisin B_1 and zearalenone in maize, including accelerated solvent extraction, a two-step SPE procedure and internal standards for each analyte. The LODs were below the maximum concentration levels permitted in the EU, but the method suffered from a low recovery for zearalenone. The next generation of methods included several A- and B- trichothecenes as well as zearalenone, and used Mycosep® columns for clean-up of the raw extracts. Zearalanone was used as internal standard for zearalenone in the method of Berthiller et al. (2005a), and Biselli and Hummert (2005) applied matrix matched calibration for this analyte. Cavaliere et al. (2005) added α-zearalenol and three fumonisins to the list of analytes and performed de-fatting and solid-phase extraction of the raw extracts of corn meal. While the efficiency of the extraction step was greater than 84% for all analytes, matrix effects were still present and required matrix-matched calibration.

A method for the simultaneous determination of *Fusarium*, *Aspergillus* and *Penicillium* toxins (ochratoxin A, zearalenone, α- and β-zearalenol, α- and β-zearalanol, fumonisins B1 and B2, T2- and HT2-toxin, T2-triol, mono- and diacetoxyscirpenol, deoxynivalenol, 3- and 15-acetyldeoxynivalenol, deepoxy-deoxynivalenol and aflatoxin M1) was reported by Sorensen and Elbæk (2005). Bovine milk samples were defatted and after adjustment of the pH, an SPE procedure was applied. Signal suppression/enhancement was minimized and recoveries >76% were obtained. However, the major drawback of this method was the necessity of using two chromatographic runs with different columns and eluents. The two most recent reports, which include a clean-up of the raw extract using MultiSep #226 cartridges, introduced instrumental improvements to multi-mycotoxin analysis in foodstuffs. A time-of-flight mass spectrometer was used by Tanaka et al. (2006), while, in the method of Ren et al. (2007), analysis time was significantly decreased through the application of ultra-performance liquid chromatography. In both methods, recoveries >70% were obtained for all analytes and no significant matrix effects were reported.

As all these methods rely on some sort of clean-up, certain toxin classes are excluded as they are not compatible with the clean-up and/or extraction conditions (for example, the fumonisins are not determined by the methods of Tanaka et al. (2006) and Ren et al. (2007). In particular, neither ergot alkaloids, moniliformin, enniatins nor masked mycotoxins are included in any of these reports. To overcome these problems, some existing methods omit a clean-up of the sample and inject raw extracts into the LC/MS. This clearly increases the demands on the selectivity of the detector as well as on the investigation of matrix effects, especially if complicated food matrices are analysed. Spanjer et al. (2005) determined 22 mycotoxins (including the ergot alkaloid ergotamine) in different food matrices. Samples were extracted with an acetonitrile/water mixture and were diluted with water prior to injection. Matrix effects were investigated for every analyte/matrix combination and validation data obtained that suggested that the analysis of diluted raw extracts is indeed feasible and at the same time sensitive enough for determining most mycotoxin levels set in the legislation. Our own contribution in this field was the quantitative determination of a set of 39 analytes (including moniliformin, beauvericin, enniatins and masked mycotoxins) in wheat and maize (Sulyok et al. 2006). In both matrices, linear calibration curves were obtained (with the exception of moniliformin) after spiking blank matrices at multiple concentration levels, with coefficients of variance of the overall process of <5.1 and <3.0%, respectively. Significant matrix effects were observed for maize, but these could be overcome by matrix-matched calibration. LODs ranged from 0.03 to 220 $\mu g\,kg^{-1}$ and the trueness of the method was confirmed for deoxynivalenol and zearalenone though the analysis of certified reference materials. Very recently, this method has been extended to the simultaneous determination of 87 mycotoxins and has successfully been applied to mouldy food samples (Sulyok et al. 2007).

In the near future, a strong trend towards multi-mycotoxin methods, which do not involve a clean-up of the sample, can be expected, as these methods can be relatively easily adapted to new analytes and matrices, and the obvious time- and cost-savings compensate for the expense of initial validation. Advances in the technology and in the instrumental design in mass spectrometry will further decrease the influence of matrix effects, which certainly constitute the main drawback of this approach at the moment.

Fast screening methods

Immunochemical techniques

Rapid methods based on immunochemical techniques often have the advantage of not requiring any clean-up or analyte enrichment steps. ELISAs have become routinely used tools for rapid monitoring of most mycotoxins, especially for the screening of raw materials (Gilbert and Anklam 2002; Fremy and Usleber 2003). Although ELISA tests may show a high matrix dependence and possible overestimation of levels, the advantages of the microtitre-plate format are speed, ease of operation, sensitivity and high sample throughput. ELISA test kits are commercially available for most of the major mycotoxins (EMAN 2007).

Alternatives to ELISAs include a number of immunosensors as well as upcoming methods using immunochemical platforms, such as fluorescence polarization immunoassays (FPI) (Ngundi et al. 2005) or surface plasmon resonance (SPR) with mycotoxin–protein conjugates immobilized onto a sensor chip surface (Tüdös et al. 2003). Immunosensors are emerging as a cost-effective alternative for screening and quantitative determination of mycotoxins (Maragos 2004). Array biosensors have been developed using competitive-based immunoassays for the simultaneous detection of multiple mycotoxins, including ochratoxin A, fumonisin B, aflatoxin B1, and deoxynivalenol, on a single waveguide surface by imaging the fluorescent pattern onto a CCD (charge-coupled device) camera (Sapsford et al. 2006). Other formats with fluorescence detection include automated flow-through immunosensors with enzyme-labelled mycotoxin derivatives (Urraca et al. 2005). Electrochemical immunosensors with surface-adsorbed antibodies on screen-printed carbon electrodes have been fabricated for the detection of aflatoxin M1 in milk (Micheli et al. 2005) and, in an array configuration, for the detection of aflatoxin B1 (Pemberton et al. 2006). Affinity-based surface plasmon resonance sensors (SPR) have the advantage of not requiring any labelling of the target mycotoxin (Tüdös et al. 2003) and may become an alternative method for rapid screening, which also enables the simultaneous detection of multiple mycotoxins using serial connected flow cells (van der Gaag et al. 2003). In a further label-free immunochemical approach for the detection of aflatoxin B1 and ochratoxin A, optical waveguide lightmode spectroscopy (OWLS) was used with integrated optical waveguide sensor chips measuring the resonance incoupling angle of polarized light, thus determining the surface coverage (Adányi et al. 2007).

A complementary tool for the screening of cereal samples may be DNA microarray-based chips using PCR followed by microarray colorimetric detection, which has been developed for the fast detection and identification of 14 trichothecene- and moniliformin-producing *Fusarium* species occurring on cereals (Kristensen et al. 2007).

In recent years, interest in rapid membrane-based immunoassay methods, such as flow-through immunoassays and lateral flow devices (LFDs), has strongly increased due to the need for rapid on-site (pre)-screening. A flow-through enzyme immunoassay was developed for the detection of ochratoxin A in roasted coffee (Sibanda et al. 2002). Requiring no sample preparation other than an extraction step, LFDs allow qualitative or semi-quantitative determination of mycotoxins on one-step strip tests within a few minutes. Such LFDs have been developed for selected mycotoxins, such as aflatoxin B1 (Delmulle et al. 2005) and fumonisin B1 (Wang et al. 2006). The strong interest is furthermore reflected in the increasing number of commercially available test kits for field use, based mostly on direct competitive assays.

Non-invasive techniques

Optical methods, such as Fourier Transform mid-infrared spectroscopy with attenuated total reflection (Kos et al. 2003) or near-infrared transmittance spectroscopy (Pettersson and Aberg 2003), are promising techniques for the fast and non-destructive detection of mycotoxins in grains. The approaches allow sample preparation to be reduced to an absolute minimum and to be integrated into on-line monitoring systems. Nevertheless, since rapid data interpretation is based on the output of chemometric analysis, the high matrix dependence and the lack of appropriate calibration materials are still major restrictions.

Similarly, electronic noses, featuring an array of electronic chemical sensors with pattern recognition systems, have also been developed (Logrieco et al. 2005). In this approach, volatile organic compounds of low molecular weight, which are released by many fungi as products of secondary metabolism, are

adsorbed onto the sensor surface and measured with a variety of transduction systems based on electrical-, optical-, or mass-transduction, such as metal oxide sensors (MOS) and surface acoustic wave sensors (SAW), for example (Olsson et al. 2002).

The high demand for rapid screening methods for mycotoxin analysis reflects the need for fast and cost-effective on-site determination of the level of mycotoxin contamination in food and feed. Immunochemical-based screening methods have shown great potential and are increasingly applied in routine analysis and monitoring of mycotoxins. Nevertheless, although rapid and selective, a loss of sensitivity may have to be taken into account in easy-to-use-assays due to the necessary simplification of the system which usually employ no washing step. Future trends in screening methods include the further development of fast and simple tests requiring no instrumentation and improved detection capability for the simultaneous measurement of multiple mycotoxins.

Quality assurance

Method validation

As mentioned above, a multitude of methods have been published for the determination of mycotoxins in food and feed over the years. However, only a limited number of these publications include performance characteristics data obtained by method validation, which is a prerequisite for the production of reliable results in terms of comparability and traceability. Typical performance characteristics to be evaluated for the validation of a quantitative method are the limit of detection and quantification (LOD/LOQ), linearity, precision (repeatability and reproducibility), selectivity (interference of other compounds and/or matrix components), robustness/ruggedness, working range and trueness/bias (Josephs et al. 2004). Several protocols and guidelines for method validation have been published, e.g. the ISO standard 5725 (ISO 1994) or the guide ''The fitness for purpose of analytical methods'' (Eurachem 1998).

There are various methods for mycotoxin analysis available that are validated and have been accepted by official authorities such as CEN, the AOAC, and ISO etc. (AOAC 2005; Gilbert and Anklam 2002). CEN usually evaluates the performance criteria of a method on the basis of collaborative studies. Most CEN methods are also AOAC- and ISO-approved. The latest edition of the Official Methods of Analysis (OMA) from the AOAC is available online and contains about 60 validated methods for mycotoxin analysis (AOAC 2005). Gilbert and Anklam (2002) have compiled validated and official analytical methods for the determination of several mycotoxins, including aflatoxins, ochratoxin A, patulin, fumonisins, deoxynivalenol and zearalenone, in various matrices, such as cereals, nuts, milk, fruits and juices, as well as their products, intended for human consumption and animal feed.

Reference materials and intercomparison studies

Reference materials (RM) or certified reference materials (CRM) are materials with a defined sample constitution and known or certified content of analyte(s) along with its uncertainty. (C)RMs play an important role not only during the validation process of a method but also as a measure to assure the quality of analytical data during routine analysis (in terms of trueness, comparability and traceability). (C)RMs can be classified as pure substances (standards), standard solutions (calibrators) or matrix materials (spiked or naturally contaminated). Until recently, the Institute of Reference Materials and Measurements (IRMM) of the EC has been the only provider of mycotoxin CRMs (IRMM 2007). These include RMs for aflatoxins in peanut (BCR 263, BCR 264, BCR 401), compound feed (BCR 375), milk powder (ERM-BC 282, ERM-BC 283, ERM-BC 284), ochratoxin A in wheat (BCR 471), deoxynivalenol in maize (BCR 377, BCR 378) and wheat flour (BCR 396), and zearalenone in maize (ERM-BC 716, ERM-BC 717). The IRMM also provides standard solutions for calibration purposes (calibrators) of deoxynivalenol (IRMM 315) and nivalenol (IRMM 316) in acetonitrile. The US National Institute of Standards and Technology (NIST) has recently issued a standard RM (SRM 2387) for aflatoxin determination in peanut butter (NIST 2007a). A full compilation of (C)RMs currently available and in production can be found on the homepages of various institutions, e.g. COMAR (2007), CORDIS (2007), IAEA (2007), IRMM (2007) and NIST (2007b). Despite past and current efforts to produce (C)RMs and calibrators for mycotoxin analysis, there is still an eminent need for more RMs that are appropriate for the different sample matrices and concentration ranges, especially with regard to the implementation and monitoring of regulatory limits (maximum levels) of mycotoxins in food and feed set by national and international authorities.

Intercomparison studies (collaborative studies or proficiency testing schemes) play an important role in the validation of analytical methods and the production of RM (Gilbert and Anklam 2002; Josephs et al. 2004) as well as acting as quality assurance tools for laboratories. To improve the

comparability and traceability of analytical data in Europe, several intercomparison studies in the field of mycotoxin analysis, especially for *Fusarium* mycotoxins, have been performed in the past 10 years within projects funded by the EC (Josephs et al. 2004; Krska et al. 2005). Additionally, the Food Analysis Performance Assessment Scheme (FAPAS) continually organises proficiency testing schemes in mycotoxin analysis (FAPAS 2005).

Conclusion and outlook

In the laboratory, sample extracts are preferably purified and enriched in a clean-up step procedure using mainly SPE and IAC, with multifunctional IACs currently being put to the test. TLC and LC are still the most frequently employed analytical methods for the (official) determination of mycotoxins; however, LC–MS/MS is increasingly used for the simultaneous determination and identification of large numbers of mycotoxins, currently up to 87. The use of CRMs and certified calibrants is one of the key issues of quality assurance in the analytical laboratory.

The established state-of-the-art chromatography-based methods for mycotoxin analysis are increasingly being complemented by a number of new screening methods, including LFDs, biosensors and IR-screening techniques, that are fast and cost-effective. Nevertheless, these techniques will have to compete with both classical confirmatory methods and MTP-ELISAs, which are now widely used for mycotoxin screening.

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