Determination of mycotoxins in human foods

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This *tutorial review* deals with the analytical methods available for the determination of mycotoxins in food commodities. As the secondary metabolites of a range of fungal species, mycotoxins possess diverse chemical structures, presenting analytical chemists with a unique set of challenges in the $\mu g\ kg^{-1}$ (ppb) range. A number of analytical methods have been applied to mycotoxin analysis. These include widely applicable HPLC methods with UV or fluorimetric detection, which are extensively used both in research and for legal enforcement of food safety legislation and for regulations in international agricultural trade. Other chromatographic methods, such as TLC and GC, are also employed for the determination of mycotoxins, whereas recent advances in analytical instrumentation have highlighted the potential of LC-MS methods, especially for multi-toxin determination and for confirmation purposes. Conventional chromatographic methods are generally time consuming and capital intensive, and hence a range of methods, mostly based on immunological principles, have been developed and commercialised for rapid analysis. These methods include, among others, enzyme-linked immunosorbent analysis (ELISA), direct fluorimetry, fluorescence polarization, and various biosensors and strip methods.

1. Introduction to mycotoxins

Mycotoxins occurring in food commodities are secondary metabolites of a range of filamentous fungi, which can contaminate food or food crops throughout the food chain. Although many hundreds of fungal toxins are known, a more limited number are generally considered to play an important part in food safety and for these a range of analytical methods have been developed. Fungal toxins of concern are generally produced by species within the genera *Fusarium*, *Aspergillus* and *Penicillium*, which frequently occur in crops in the field or during storage of major food agricultural crops, including cereals, groundnuts and various fruits. Besides the deleterious effect of the fungi themselves on agricultural productivity, the fungal toxins have a range of detrimental health effects in humans, including carcinogenesis, immune suppression, teratogenicity and growth retardation. Similarly, mycotoxin-contaminated animal feeds can lead to animal toxicoses and the possible carry-over of mycotoxins or their metabolites into the human food chain.

Fungally-contaminated feed has long been associated with animal disease and cultured fungal species have been used since the 1930's for the isolation of antibiotics. During World War II in the USSR, hundreds of thousands died of Alimentary Toxic Aleukia due to the consumption of over-wintered grain contaminated with *Fusarium sporotrichioides*. However, the defining moment for modern mycotoxicology is frequently given as the outbreak of Turkey-X disease in the United Kingdom in 1960. In this episode of mycotoxicosis, thousands of turkeys died after consumption of imported feed, which was subsequently found to be contaminated with *Aspergillus flavus*, the producing fungus of a previously unknown carcinogenic mycotoxin, aflatoxin B_1. The realization that human food could be contaminated with mycotoxins led to a great expansion in research efforts into all areas of mycotoxicology, especially into their biochemical modes of action, their implications for human health and analytical methods for the chemical determination of these newly identified compounds at the levels found in human foods. Based on their production by fungal pathogens of major crops and their significant natural occurrence and implications for human health, most research has focused on the aflatoxins, fumonisins, trichothecenes, zearalenone, ochratoxin A and patulin. These mycotoxins represent a diverse range of chemical structures. For the aflatoxins, fumonisins and tricothecenes, each group contains a number of structurally-related analogues. Table 1 lists the important toxins in each group, their main producing fungi and some typical food commodities in which they can occur.

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Table 1 The major food-borne mycotoxins, their main producing fungal species and the commodities most frequently contaminated

Mycotoxin	Fungal species	Food commodity
Aflatoxins B_1, B_2, G_1 and G_2	*Aspergillus flavus* *Aspergillus parasiticus*	Maize, wheat, rice, sorghum, ground nuts, tree nuts, figs
Aflatoxin M_1	Metabolite of aflatoxin B_1 in mammals	Milk, milk products
Fumonisins B_1, B_2 and B_3	*Fusarium verticillioides* *Fusarium proliferatum*	Maize, maize products, sorghum, asparagus
Deoxynivalenol[a]	*Fusarium graminearum* *Fusarium culmorum*	Cereals, cereal products
T-2 toxin[b]	*Fusarium sporotrichioides* *Fusarium poae*	Cereals, cereal products
Zearalenone	*Fusarium graminearum* *Fusarium culmorum*	Cereals, cereal products
Ochratoxin A	*Aspergillus ochraceus* *Penicillium verrucosum* *Aspergillus carbonarius*	Cereals, dried vine fruit, wine, coffee
Patulin	*Penicillium expansum*	Apples, apple juice

[a] Deoxynivalenol is a widely occurring group B trichothecene. It is commonly known in the USA as vomitoxin. [b] T-2 toxin is a group A trichothecene, mainly found in cereals grown in colder climates.

2. Introduction to analytical methods for mycotoxins

After the discovery of aflatoxin in 1960, the analytical methods for mycotoxins, which typically occur in the µg kg^{-1} (ppb) range, have developed and expanded along with the general advances in analytical science. The earliest analytical methods were based on solvent extraction, crude clean-up on open-ended packed-silica columns and separation of the analytes of interest by thin layer chromatography (TLC). Such methods are still valid today and in the case of aflatoxins, TLC with either visual assessment or instrumental densitometry is routinely applied in many laboratories in the developing world.

The basic requirements of extraction, clean-up and separation for mycotoxin determination in food matrices remain the same in current methods. Advances have come in the areas of sample purification techniques and in separation science with the development of high-performance liquid chromatography (HPLC) and associated detectors. For mycotoxins with suitable chromophores (patulin) or fluorophores (aflatoxins), the increased sensitivity of modern detectors has allowed analysts to achieve lower limits of detection. Other mycotoxins, which lack suitable chemical groups (trichothecenes and fumonisins), can be initially derivatised and then separated either by gas chromatography (GC) or by HPLC. Although investigated as a research topic, electrophoretic methods such as capillary electrophoresis have not found wide application in mycotoxin analysis.

As in many other branches of analytical chemistry, the introduction of bench top mass spectrometers as detectors for GC or HPLC instruments has impacted on mycotoxin analysis and has allowed detection at very low levels with simultaneous confirmation of the compounds of interest. Due to the range of chemical properties used for extract clean-up and chromatographic detection, analytical methods for mycotoxins are generally limited to a single toxin or a group of structurally-related toxins (fumonisins or aflatoxins) in a single analysis. The introduction of LC-MS instrumentation has made possible the development of multitoxin methods suitable for a range of structurally diverse toxins in a single chromatographic run. The need for such multitoxin techniques lies in the fact that a single fungal species can produce different toxins and that a single agricultural commodity can be contaminated with different fungal species resulting in the co-occurrence of a number of different toxins.

Another area of technological advance has been the introduction of solid phase extraction techniques using a range of chemistries (normal phase, reversed-phase, strong anion exchange). The development of immunoaffinity columns (IACs) containing antibodies specific to the analyte of interest has resulted in faster clean-ups and a greater degree of sample purification. This has been followed by the introduction of analytical methods which rely on direct fluorimetric measurement of the resulting purified extract (or a suitable derivatised product). When combined with HPLC separation, injected samples are cleaner and the resulting chromatograms are less complex, with attendant advantages to HPLC column life and analytical reliability.

Mycotoxin analytical methods need to have low limits of detection (generally in the µg kg^{-1} range, but in the ng kg^{-1} range for aflatoxin M_1 in milk), be specific to avoid analytical interferences, be easily applied in routine laboratories, be economical for the laboratory involved and provide a confirmatory test for the analyte of interest. For official control and implementation of mycotoxin regulations, a number of official methods, mostly based on HPLC, have been validated by interlaboratory collaborative studies conducted under the auspices of international bodies such as AOAC International. The validation process involves testing the within-laboratory repeatability, between-laboratory reproducibility, analytical recovery, and limits of detection and quantification. At the same time, the European Committee for Standardization (CEN) has published criteria for mycotoxin analytical method performance.[1] However, aside from these official methods, a need exists for rapid screening methods which can be used for control purposes and in situations where rapid decisions are required, frequently in field situations at granaries, silos and factories. For this purpose, a number of screening methods and biosensors have been developed which are mostly based on immunological principles and use antibodies raised against specific mycotoxins. These methods range from quantitative ELISAs to qualitative tests based on obtaining a simple result of contamination above or below a set control level. Such tests, which are available in various formats such as lateral flow dipsticks, are evaluated by the level of false positive or false negative results. In terms of consumer protection, a level of false positives may be acceptable in that such batches will then be subject to more comprehensive testing. Thus in selecting a method for mycotoxin analysis, it is necessary to consider the purpose for which the results are needed, the matrix to be analysed, the detection limit required and the expertise and infrastructure available.

3. Sampling of commodities for mycotoxins

The mycotoxin contamination of agricultural products and food commodities, more than most other analytical tests, represents a substantial problem with respect to representative sampling.[2] Mycotoxins are not produced homogeneously in the crop, but are the result of fungal growth in specific units, be they maize cobs, maize kernels, wheat kernels, groundnut pods, *etc*. For example, a large 360-kg crate of apples for juicing may contain only a limited number of apples that have been physically damaged and subject to growth of *P. expansum* and consequently contain high levels of patulin. The result is that there is a non-homogeneous distribution of mycotoxin and a single lot of the product will contain hot spots of contamination. This skewed distribution implies that most analytical samples drawn from the lot will contain lower mycotoxin levels than a mean formed by drawing many samples from the lot. An illustration of this is that analysis of 200 samples, each of 100 g, drawn from a batch of groundnuts found them to have a mean aflatoxin level of 1.0 $\mu g\ kg^{-1}$, whereas the range of analytical results for individual samples was 0 to 81 $\mu g\ kg^{-1}$.[2] Processing of the agricultural commodity can improve the homogeneity of the material. Nevertheless, a study on aflatoxin contamination of peanut butter produced from a single batch of groundnuts illustrates the problem in that of the 377 jars produced, 300 had levels below 5 $\mu g\ kg^{-1}$ but 7% of the jars were above 10 $\mu g\ kg^{-1}$ and one jar was above 100 $\mu g\ kg^{-1}$.[3]

The total error associated with mycotoxin testing can be split into three separate errors, namely the sampling error, sample preparation error and analytical error. The last term is the one most familiar to laboratory chemists and represents the error from the chemical analysis of the subsample extracted. The sampling error is the error introduced by withdrawing a random sample from the lot of material to be tested and the sample preparation error is the error introduced by milling or grinding the sample and removing the subsample for laboratory extraction. Each of these makes an independent contribution of variance to the total testing variability. The worst case is the determination of aflatoxin in lots of groundnuts. In a trial in which 2.27 kg samples were withdrawn from a lot of nuts and a 100 g subsample of ground material was extracted and analysed, the sampling error contributed 92.7% to the total variability, sample preparation contributed 7.2% and the actual sample analysis only contributed 0.1%.[4] The situation with respect to other mycotoxins in other agricultural commodities is not skewed to the same extent, although the problem remains. In the case of maize, 1.1 kg samples of kernels drawn from a single batch and 25 g subsamples analysed showed a sampling contribution to total variance of 61.0%[5] and for the *Fusarium* toxin deoxynivalenol in wheat, 0.454 kg samples and 25 g subsamples gave a sampling contribution of 22.0%, the same as the contribution of the analysis.[6] The reason for the difference between the mycotoxins and the commodities lies in a more general fungal contamination of the *Fusarium* species and in the fact that a unit mass wheat kernel sample represents a much greater number of kernels than the same unit mass of groundnuts.

The problem of sampling has been addressed by the development of sampling plans, which are based on statistical evaluations to balance consumer protection (by not accepting contaminated lots) and producer protection (by not rejecting clean lots).[2] Such plans are a compromise between the statistical need for large samples and the practicalities and costs of such samples. For official food safety testing, sampling plans, which specify commodity type, number of increments sampled in a lot, size of the increments, place in the lot taken and total aggregate sample collected, are frequently specified by government regulation and can involve aggregate samples of up to 30 kg.

An important aspect of a sampling plan is the operating characteristics curve, which is generated from statistical evaluation of the mycotoxin distribution and the analytical variability. Such a curve plots the probability of a lot's acceptance against the mycotoxin level. It demonstrates that as the actual level of contamination increases and approaches the regulated level, so the probability of its rejection increases. This is known as the producer risk (of acceptable lots being rejected). Similarly, lots contaminated at increasing levels above the regulated level have a diminishing probability of being accepted. This is known as the consumer risk (of contaminated lots being accepted). The ultimate aim of a good sampling plan is to reduce both risks, although they cannot ultimately be eliminated.[2]

4. Extraction of food matrices

Parameters of importance in mycotoxin extraction are solvent type and composition of a mixture, solvent to sample ratio, type of matrix (processed or unprocessed), extraction method and physical aggregation of the sample. For optimum extraction efficiency, the analytical sample should be ground to a fine powder. Mycotoxins are polar compounds which occur naturally in the $\mu g\ kg^{-1}$ up to $mg\ kg^{-1}$ range in diverse and complex food matrices, from which the analytes of interest must be extracted. Hence, mycotoxins are potentially extracted by a range of polar solvents or mixtures of solvents. For example, in the case of aflatoxins, a widely used early method relied on chloroform extraction of peanut products, which after shaking and filtration could be purified on packed silica columns.[7] Similarly, current methods for patulin determination rely on the use of an ethyl acetate–*n*-hexane mixture to extract the mycotoxin from apple juice.[8] Due to the cost and environmental implications of chlorinated solvents, the use of aqueous mixtures of methanol, acetonitrile or acetone have come to the fore. In the case of deoxynivalenol in cereals and cereal products, pure water has been used as an extraction solvent.[9] For the other mycotoxins, the ratio of organic to aqueous solvent must be tailored to the toxin–matrix combination to achieve optimum efficiency. In the case of fumonisins, both pure methanol and pure water will extract fumonisins from maize, but the optimum is generally found at a methanol–water ratio of 3 : 1 (v/v).[10] Alternatively, mixtures of water, methanol and acetonitrile have also been successfully employed.[11]

Investigations of optimal extraction solvents for aflatoxins from a range of matrices highlighted a series of potential problems which need to be considered.[12] The presence of salts or sugars in certain matrices has the potential to cause phase separation of an extractant mixture with a non-uniform distribution of the toxin between the layers. Similarly, the

extraction of very dry materials can lead to a variability associated with water uptake by the dry matrix, an effect that depends on factors such as the matrix, the organic solvent and its ratio in the aqueous extractant and the solvent-to-sample ratio used for the extraction experiment. A further consideration in selection of extractants is the extent to which the chosen mixture will also extract interfering matrix impurities. For this reason, acetone has been preferred over methanol for aflatoxin extraction from matrices containing citrus pulp. Further consideration must also be given to the next step of the analytical process, namely sample clean-up. It is desirable that the extractant mixture should be compatible with the extract purification process. Pure organic extractants are suitable for clean-up on silica columns (ethyl acetate–*n*-hexane for patulin from apple juice[8]), whereas aqueous mixtures are suitable for reversed-phase or ion exchange clean-up.[10] In the case of toxins for which immunoaffinity columns exist, aqueous methanol extractants allow, after suitable dilution, larger volumes to be used on the antibody column than would be advisable for acetonitrile or acetone.[12]

The type of matrix that is analysed can strongly influence the analytical recoveries from the extraction process. This is particularly a problem with fumonisins. Extraction of fumonisins from maize is readily achieved as described above.[10,11] However, once maize has been processed into a variety of commercial products such as breakfast cereal, muffins, extruded products and maize chips, analytical recovery can be a serious problem. Despite the application of a range of solvent mixtures and the use of acidic and alkaline extraction solvents, no single extraction solvent mixture appears to be of universal applicability to all these processed products.[13] More recently, researchers have drawn attention to the fact that certain matrices contain both free mycotoxin and mycotoxin bound to protein or sugars such as glucose, which is not analysed or included in the conventional methods.[14] Determination of such bound mycotoxins may require additional processing to release the toxin.

The physical process of extraction is generally achieved by shaking of the matrix and extractant or by blending with a homogenizer for a shorter time period. The use of accelerated solvent extraction under increased pressure[15] and supercritical fluid extraction (SFE)[16] has been investigated, but the results have not justified the cost of adopting this instrumentation in place of simple shaking. In the case of SFE, the polar nature of mycotoxins and their poor solubility in carbon dioxide is a major problem which requires the addition of organic solvent modifiers, such as methanol or acetonitrile. Problems also arise with respect to extraction times, analytical recoveries and co-extracted impurities.

5. Extract clean-up

Original methods for mycotoxin analyses frequently relied on extract clean-up on open columns packed with materials such as silica or diatomaceous earth, which were eluted with various organic solvent mixtures.[7] In some cases, liquid–liquid partition was used for sample clean-up and is still applied in a widely used method for the determination of patulin in apple juice, in which the original ethyl acetate extract is washed with a sodium carbonate solution to remove acidic impurities.[17] These techniques are non-specific and require large volumes of organic solvents for column packing, clean-up and mycotoxin elution. In some instances, these methods can also involve multiple steps of defatting with hexane or petroleum ether and impurity precipitation with solutions of lead acetate. A number of alternative and simpler clean-up techniques have been developed and are available to the analyst. Solid phase extraction (SPE) using small prepacked cartridges containing up to 500 mg sorbent has been applied for a number of years to mycotoxin analysis. The general mechanism of this purification is the physical adsorption of the toxin on the packing surface, appropriate washing of the cartridge for removal of as many impurities as possible without loss of the analyte and then finally the complete elution of the analyte from the SPE cartridge. Packing materials are mostly based on silica particles, although a number of polymer resin packings have been developed commercially and applied to purification of mycotoxins. The SPE packings cover a range of separation chemistries, such as normal phase (usually unmodified silica), reversed-phase (usually octadecylsilica, C18) or ion exchange. This last category can comprise strong or weak anion or cation exchangers bound to a silica support material and can provide a more specific purification than silica or C18.

The sample extract solutions need to be compatible with the SPE chemistry to be used for clean-up. The example of patulin clean-up on silica has already been mentioned.[12] A further example is the conversion of an aflatoxin method from large open column to a SPE column containing 500 mg silica sorbent.[18] Extracts from maize and peanut products were cleaned-up by application in dichloromethane solution onto the silica SPE columns, which resulted in a twenty-fold reduction in organic solvent use over the original open column method. The use of aqueous mixtures of methanol and acetonitrile, which have largely replaced polar organic solvents as extractants, results in extracts which are easily tailored for reversed-phase or ion exchange SPE clean-up. The fumonisin mycotoxins provide an example of this type of purification. These toxins are diesters of propane-1,2,3-tricarboxylic acid (tricarballylic acid) and various 2-amino-12,16-dimethylpolyhydroxy-eicosanes in which the hydroxyl groups on C14 and C15 are esterified with the terminal carboxyl group of the tricarballylic acid. They are extracted from maize with methanol–water (3 : 1, v/v) and can be retained on a strong anion exchange (SAX) SPE cartridge, provided the pH of the extract is such as to allow ionization of the carboxylic acids.[10] This is generally achieved at around pH 6 and may require adjustment of the extract prior to application to the SPE cartridge. Such a system allows impurities to be washed off the cartridge with methanol and the fumonisins are only released on elution with dilute acetic acid, which suppresses the ionization of the carboxylic acid groups and allows elution of the mycotoxin. However, the fumonisins can also be purified on reversed-phase C18 SPE cartridges.[19] This clean-up is not as efficient as the SAX SPE method and requires the sample extract to be diluted with water so as to retain the fumonisin on the C18 cartridge.[19] Elution of the mycotoxin occurs with methanol, so care must be exercised in the washing of impurites from the cartridge with aqueous methanol mixtures. The toxin is a diester, so under various

conditions (nixtamalization with alkali in tortilla production[20]), hydrolysis of one or both of the tricarballylic acid groups can occur. For samples of this nature, SAX clean-up alone is no longer possible and C18 cartridges are used to adsorb all the chemical forms.[20] Alternatively, the two types of SPE cartridge have been consecutively employed to achieve a chemical separation, firstly the SAX for fumonisin itself and then the eluate is passed onto a C18 cartridge for isolation of the hydrolysed forms.[21]

The development of antibodies raised against individual mycotoxins led to the introduction of immunoaffinity columns (IACs) in which a specific antibody is immobilized on a gel contained in a small column. The analyte is generally extracted with aqueous methanol and the resulting extract diluted with water or phosphate-buffered saline (PBS) prior to application to the IAC. The antibodies on the column will recognise and bind the specific mycotoxin and allow impurities to pass through the column, which is subsequently washed with PBS. The mycotoxin is eluted with a small amount (usually a few mL) of methanol, which denatures the antibody and releases the bound analyte. IACs have been commercially developed for most of the major mycotoxins of agricultural, trade and health interest. Recent developments have been the combination of different antibodies into one column which allows the determination of more than one mycotoxin per single sample extract and clean-up. The resulting solution can be analysed separately for each toxin[22] or a suitable gradient HPLC separation can be developed to achieve a multitoxin determination in a single chromatographic analysis.[23] IACs achieve a superior purification to that obtained with SPE cartridges, although it should be noted that impurities can still be retained on the column despite the specific nature of the antibody–toxin interaction.[24]

In all the above examples of mycotoxin extract clean-up, the mechanism involved has been the linking of the toxin to the column or cartridge packing and the washing away of impurities prior to elution of the analyte with a stronger solvent. However, an alternative scenario is the passing of the extract through the cartridge without adsorption of toxin, but with adsorption of the interfering impurities. This system has been the basis for a method developed for moniliformin, a highly polar acidic compound, which due to a low pK_a occurs naturally as an alkali salt rather than a free acid.[25] In this method, the defatted sample extract in methanol is passed through a C18 reversed-phase SPE cartridge, immediately eluted with water, evaporated to dryness and the residue, after redissolving in HPLC mobile phase, is passed through a small alumina column. The multifunctional column is a similar concept, in which the extract is passed through a column containing a mixture of adsorbents such as charcoal, alumina, silica and Celite. This model has been commercialized into an easy to use test tube system for rapid purification. Specific commercial products are available for a range of mycotoxins in different matrices.[31]

Molecularly imprinted polymers (MIPs) represent an area of research interest and have been investigated as a potential novel clean-up system for food analysis. The technique involves the creation from suitable monomers of a three-dimensional network (polymer) that retains a memory of the shape and functional groups of the template or analyte molecule around which the polymerization occurs. Once the template is removed, the resulting MIP is able to recognise the template (analyte) within a mixture, effectively functioning as an artificial antibody (biomimetic receptor). Although there has been some interest in developing MIPs for mycotoxin analysis, such methods are still confined to a limited number of research laboratories.[26]

6.   Thin layer chromatography (TLC)

Separation of the mycotoxin analyte from various impurities and interferences that may still be present after extract clean-up is mostly performed by chromatography. As TLC was a well known technique at the time of the first development of many analytical methods for mycotoxins, it was initially used for this purpose. In suitable cases, gas chromatography (GC) also found application in mycotoxin analysis, whereas HPLC methods were gradually developed as this separation technique itself matured. Despite the development of these instrumental techniques, TLC still has a place in some analytical laboratories, especially in developing countries. TLC offers the advantage of testing a number of samples simultaneously and can also be used as a screening test prior to more sophisticated instrumental methods.

Mycotoxins are polar compounds and have mostly been separated on normal phase silica TLC plates using a range of organic solvent mixtures as mobile phase.[27] The four major aflatoxin analogues (B_1, B_2, G_1 and G_2) are readily separated by TLC and are easily observed under long wavelength UV light at levels that are useful for quantification of naturally contaminated food samples.[7] Quantification can be achieved by visual comparison of the intensities of sample spots with those of standards. A number of improvements over the conventional TLC analysis have been introduced and applied to aflatoxin analysis. These include the use of densitometry to improve quantification, the introduction of high-performance TLC plates (HPTLC) and the use of bi-directional TLC to improve the separation of the aflatoxin analogues from interfering impurities. In the case of mycotoxins that do not fluoresce, the plate must be sprayed after TLC separation to yield visible spots. In this manner a toxin such as deoxynivalenol is rendered visible after the drying of the developing solvent, spraying with $AlCl_3$ and heating at 120 °C.[27] Similarly, the fumonisins can be visualised on normal phase silica TLC plates by spraying with p-anisaldehyde solution.[28] This type of reagent was suitable for the detection of fumonisins in fungal cultures, but did not allow the determination of fumonisins at levels suitable for investigations of natural contamination. To achieve this, reversed-phase (C18) TLC plates were used and the mycotoxin was either separated and then derivatised to a fluorescent product by the spraying of borate buffer and fluorescamine onto the plate or a pre-derivatisation with fluorescamine was performed and then the fluorescent derivatised products were separated by reversed-phase TLC.[29] This latter method achieved detection levels as low as 20 ng fumonisin B_1 spotted on the plate, allowed estimation of contamination levels down to

500 μg kg^{-1} and avoided the analytical interference from an unknown compound of similar R_f value to fumonisin B$_1$.

Given the low levels at which mycotoxins occur, confirmatory analyses are frequently conducted to provide confidence in the analytical result. In the case of TLC analyses, confirmatory methods have mostly involved alternative or additional spray reagents or the development of the TLC plate in an alternative solvent system.[27] In the case of aflatoxins determined in cottonseed, blue fluorescent TLC spots can frequently be mistaken for aflatoxins G$_1$ and G$_2$. However, a sulfuric acid spray which turns the aflatoxins yellow or yellow–blue can be used to distinguish the aflatoxins from the interferences. Similarly, for the mycotoxin zearalenone, which shows greenish-blue fluorescence under shortwave UV light (256 nm), a confirmatory spray of AlCl$_3$ can be used. After heating for 5 min at 130 °C, the putative spot for zearalenone should become visible under long wavelength UV light (365 nm) as blue fluorescence.[27] For ochratoxin A, sodium bicarbonate, AlCl$_3$ and NH$_3$ vapour can all be used for this purpose. Ochratoxin A itself fluoresces greenish blue and under alkaline conditions of the confirmatory spray, changes to blue with an increase in intensity. Ochratoxin A has another confirmatory method in which the mycotoxin, which contains a carboxylic acid, can be derivatised by esterification with ethanol to the resultant ethyl ester. Confirmation is achieved by TLC separation of the derivatised solution and observing the disappearance of the OTA spot and the appearance of a spot corresponding to the ester.

7. Gas chromatography

GC has been applied to the analysis of a range of mycotoxins, although for many of these compounds, which possess strong fluorescence or UV properties, HPLC methods have been more successful. Nevertheless, for the trichothecenes, of which the B group possesses weak UV absorption and the A group does not have a suitable absorption band, capillary GC has been extensively used. The interplay between GC and HPLC methods is exemplified by the discovery of fumonisins and the need for a reliable and sensitive analytical method. The initial attempts to use GC analysis required the hydrolysis of the diester and the subsequent analysis, after suitable sample preparation, of either the resultant tricarballylic acid or the aminopolyol backbone.[28] The development of HPLC techniques with fluorescence detection for derivatised fumonisins caused the emphasis to shift away from GC and all natural occurrence data on fumonisins has been generated by HPLC methods.

The analysis of trichothecenes by GC has been extensively studied. These are oxygenated polar compounds which need to be derivatised prior to injection into a GC column. Most common GC detectors such as flame ionization (FID), electron capture (ECD) and mass spectrometry (MS and MS/MS) have been used. The conjugated carbonyl group in group B trichothecenes and the use of fluorine-containing derivatising agents for group A compounds make them sensitive to ECD detection at low levels. Typically, the hydroxyl groups are converted to their corresponding trimethylsilyl (TMS) ethers or trifluoroacetyl (TFA), pentafluoropropionyl (PFP) or heptafluorobutyryl (HFB) esters. These derivatization

reactions needed to be optimized so as to avoid multiple reaction products such as mono-, di- and tri-TMS derivatives of deoxynivalenol. Other aspects to consider include removal of excess reagents, removal of excess water and the stability of the derivative formed.[30]

The fungal producers of trichothecenes frequently generate a number of these compounds simultaneously. Hence, a GC method capable of separation and analysis of a range of toxins in a single chromatographic run is desirable. Thus methods have been developed to simultaneously determine deoxynivalenol and nivalenol or members of the group A trichothecenes, T-2 toxin, HT-2 toxin and diacetoxyscirpenol in cereals or in some cases members of both groups, as well as the 3- and 15-acetyldeoxynivalenol toxins.[30,31] Mass spectrometry or tandem mass spectrometry provides the advantage of selective, multitoxin, quantitative data from a single analytical run. The ability of the MS detector not only to quantitate at low levels (down to approximately 5 μg kg^{-1} for deoxynivalenol), but to confirm the identity of the chromatographic peak by the production of characteristic fragment ions, has significant advantages for analytical chemists.

The analysis of trichothecenes by GC has produced a large number of publications in which researchers have varied the matrix (mostly cereals), the extraction solvents (mostly aqueous mixtures of methanol or acetonitrile), the clean-up methods (various adsorbents such as florisil, charcoal, alumina or commercial mixtures in multifunctional cartridges), derivatization methods, GC columns and detectors (mostly ECD or MS).[31]

8. High-performance liquid chromatography

HPLC has found extensive application in the field of mycotoxin analysis. The polar nature of mycotoxins and their solubility in water and organic solvents such as methanol and acetonitrile implies that they are readily amenable to separation on reversed-phase HPLC columns and this has resulted in a diverse array of methods. The extent to which HPLC is suited to mycotoxin separation can be gauged from the compilation of a database of retention times, retention indices, UV absorption maxima and predominant mono-isotopic ions for 474 fungal metabolites.[32] Chromatographic detection has mostly been achieved with UV and fluorescence detectors, although the relatively recent successful application of atmospheric pressure ionization techniques has resulted in the development of a range of LC-MS or LC-MS/MS methods capable of very low detection limits. In addition, the evaporative light scattering detector has been applied to solutions of relatively high concentration, for example in determining the purity of standards or the levels of mycotoxins in fungal cultures.[33]

Although aflatoxin mixtures can be separated on normal phase silica columns using solvent mixtures consisting of chloroform, acetonitrile, cyclohexane and ethanol, reversed-phase columns have found a much wider application for these compounds.[30] In an intercomparison study of methods conducted among European laboratories, only one out of the 24 laboratories involved reported a normal phase separation of the aflatoxins, whereas the majority were using

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columns with C18 packing material.[34] In the case of the aflatoxins, which are most frequently detected by fluorescence, the quenching that can occur with chlorinated solvents is an additional factor in the selection of reversed-phase chromatography.[35] Other column chemistries, such as phenyl and C8 modifications of silica packing material, have found limited application.[30] In the case of the mycotoxin moniliformin, which has a pK_a value of about 1.7 and is thus ionized at the pH levels normally used for HPLC, two other chromatographic approaches have been used. An older method employed a strong anion exchange HPLC packing with a sodium dihydrogen phosphate eluent at pH 5.0, whereas more recent methods have used ion pair chromatography on C18 columns with aqueous methanol or acetonitrile mobile phases.[36,37] In general, the mobile phase composition for mycotoxin determination by reversed-phase C18 chromatography is chosen so as to match the column chemistry and the carbon loading of the column. In the case of the little studied mycotoxin tenuazonic acid, which is a metal chelating β-diketone, a high carbon loaded C18 packing material was required to achieve adequate peak shapes and prevent peak tailing.[38] In the case of the separation of carboxylic acids such as the relatively nonpolar fumonisin analogues, which require an eluent of about 80% methanol and a buffer salt, the pH of the mobile phase is also adjusted so as to suppress ionization of the carboxylate moieties.[10] For highly polar, uncharged mycotoxins such as patulin, eluents contain high percentages of water and typically have aqueous compositions with less than 10% acetonitrile in order to achieve adequate retention on reversed-phase packing materials.[17]

For mycotoxins with useful UV absorption bands, UV detection has been universally applied. Thus mycotoxins such as patulin (wavelength maximum 276 nm),[17] deoxynivalenol (wavelength maximum 219 nm)[9] and moniliformin (wavelength maximum 229 nm)[37] are routinely quantified by UV detection. However, a number of other mycotoxins, such as the aflatoxins, ochratoxin A and zearalenone, possess fluorescence bands. Fluorescence detection has a number of advantages. In measuring light emitted rather than absorbed, it can frequently achieve lower detection limits than UV detection and as analytical interferences may not absorb and fluoresce at the same wavelengths as the analyte of interest, the fluorescence chromatograms are frequently less prone to interference from co-eluting compounds.

A number of mycotoxins do not absorb in the UV range and for these, suitable derivatization methods have been developed to allow UV or fluorescence detection. Examples of these are T-2 toxin and the fumonisins. In the case of fumonisins, a number of fluorescent derivatives such as fluorescamine, o-phthaldialdehyde (OPA) and naphthalene-2,3-dicarboxaldehyde have been used.[39] In the case of fluorescamine, two distinct chromatographically separable derivatives are formed. The majority of workers have settled on OPA as the most suitable derivatising agent, despite its inherent instability.[39] The stability of the resulting compound can be improved by replacing the 2-mercapto-ethanol reagent with N-acetylcystein.[40] Although the aflatoxins are inherently fluorescent, quenching can occur in certain eluents.[35] For optimum detection limits of the aflatoxin B_1 and G_1 analogues in

reversed-phase chromatography, they are frequently derivatised either in a pre-column method with trifluoroacetic acid or post-column by a number of techniques. These include reaction with an elemental solution of iodine in a post-column reaction coil at 60 °C, reaction with bromine generated from potassium bromide in a post-column electrochemical cell (Kobra cell), reaction with pyridinium hydrobromide perbromide added post-column or hydrolysis using a post-column UV lamp and reactor coil.[41–43]

As was mentioned in the section on TLC (see above), analytical confirmation is an important aspect of mycotoxin determination. The use of diode array UV detectors, which can collect spectral information of the chromatographic peak, enables a comparison to be made with the UV spectrum of the toxin standard for confidence in the analytical result. In the case of aflatoxins B_1 and G_1, if the method involves post-column derivatisation, then confirmation can be achieved by a switching off of the post-column pump, electrochemical cell or UV light. As was previously described for ochratoxin A by TLC, the derivatisation of ochratoxin A to its ethyl ester and re-injection allows confirmation by observation of the disappearance of the chromatographic peak due to the toxin and the appearance of one corresponding to the retention time of the authentic ethyl ester standard.

The coupling of HPLC and MS *via* atmospheric pressure ionization (API) techniques, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), and the development of commercial bench-top instrumentation, has opened new methodologies for routine analysis of mycotoxins. Whereas TLC and HPLC techniques frequently require derivatization for sensitive detection, LC-MS provides a detection method independent of the formation of chemical derivatives or of the UV absorption or fluorescence properties of the molecule. The API ion sources generate quasi-molecular ions in positive or negative modes and can accommodate HPLC mobile phase flow rates. This method also combines sensitive quantification of mycotoxins with a confirmatory technique. The observation of specific fragment ions produced by collision induced dissociation (CID) of the molecular ion furnishes unequivocal confirmation. The selectivity and sensitivity of MS and MS/MS methods are enhanced by selected ion monitoring or selected reaction monitoring modes, which eliminate much of the chemical background interference found in other detectors. Methods have been developed for all the important mycotoxins using ion-trap and triple quadrupole instruments. As the detection method relies only on the ability to ionize the molecule in the API source, renewed interest has been shown in multitoxin methods.[44] As a universal extract purification method is not available, use has been made of the sensitivity of the LC-MS instrument to avoid such clean-up and inject a diluted extract directly into the instrument. In avoiding the clean-up step, care must be exercised that impurities in the injected sample co-eluting with the analytes do not suppress the ionization of the compounds of interest. This technique has been applied to the determination of 39 mycotoxins in wheat and maize, with a single extraction step of acetonitrile–water–acetic acid $(79 + 20 + 1)$ and subsequent analysis by LC-MS/MS.[44] Because of the diversity of chemical structures, samples were run in both positive and

negative ESI modes. Although ion suppression was negligible for wheat, 12 mycotoxins gave significant signal suppression in maize. This could be overcome by running matrix-matched standards. Further development of the method increased the number of metabolites detected to 87, including most myco-toxins for which commercial standards are available and other metabolites produced by fungi involved in food storage.[44]

9. Immunological methods

A range of analytical methods have been developed for mycotoxins that rely on immunological principles, *i.e.* the interaction between an antigen (the analyte of interest) and an isolated antibody raised against the antigen. Antibodies are IgG or IgY immunoglobulins with specific binding sites for the antigen. Recognition of the molecule is based on spatial complement of specific chemical groups (the epitope) on the antigen, not on the whole antigen. Cross-reactivity of anti-bodies results from different chemical compounds containing similar chemical groups interacting with the antibody to varying degrees. For the production of antibodies, mycotoxins are haptens (too small to elicit an immunological response), so they are conjugated to polypeptides or proteins to form an immunogen. Polyclonal antibodies are raised in selected animal species such as rodents and rabbits, whereas mono-clonal antibodies are recovered from cloned cell lines. Because of these differences, polyclonal antibodies are easier to pro-duce, but contain limited amounts in each batch and show significant batch to batch variations, whereas for monoclonal antibodies, it may be difficult to initially obtain the correct specificity, but they are preferred for commercial use as they have uniform affinity and specificity and can be produced repeatedly in sufficient quantity.[45]

Enzyme-linked immunosorbent assays (ELISAs) have found a wide application for mycotoxin analysis and test kits have been commercially developed for the economically important mycotoxins. ELISAs can be developed in a number of formats, including direct assay, competitive direct assay and competitive indirect assay.[45] In the competitive direct format, the antibodies are coated (immobilized) on the surface of wells in a microtitre plate or in strips. Crude extracts (unpurified, but usually diluted) or standards are then mixed with analyte that has been chemically conjugated with an enzyme and the mixture is allowed to interact with the bound antibody in the microtitre wells so that competition occurs for binding sites between the mycotoxin of unknown level in the extract and the known (fixed) amount of conjugated mycotoxin standard. After reaction, the excess mixture is discarded, the wells are washed and substrate is added for reaction with the enzyme conjugate to produce a colour, whose intensity is dependent on the level of bound conjugate. In this format, the greater the sample toxin level, the less conjugate binds to available sites and the lower the colour intensity. In the competitive indirect format, the microtitre well contains bound toxin and a mixture of sample extract and specific antibody is incubated in the well. The analyte in the sample (or standard) reduces the amount of antibody available for binding to the immobilized toxin in the well. The bound antibody is detected by adding a second anti-antibody labelled

with enzyme, together with substrate. The colour signal obtained is again inversely related to the mycotoxin level in the sample. ELISAs can provide rapid quantitative and semi-quantitative analytical results, although the presence of matrix effects can cause limitations. Matrix effects can arise from a number of factors including other co-extracted compounds (such as lipids, carbohydrates,tannins, poly-phenols and pigments), extract pH, extraction solvent compo-sition and sample processing (ELISAs for grains do not necessarily work for processed food commodities). These effects can elevate or decrease the analytical result and can be overcome by measures such as extract clean-up, extract dilution or addition of detergents. For this reason, detection limits of ELISA kits cannot be improved by concentration of sample extract.[45]

As was described in previous sections, immunoaffinity columns have been developed in which specific antibodies are immobilized on a gel in a short column. Besides being used for purification of extracts prior to chromatographic separation, the eluate from the column may be used directly, or after suitable derivatization, for quantification in a fluori-meter. In such a system, mycotoxins which occur as a group of compounds (aflatoxins and fumonisins) are quantified as a total toxin level. This is the same situation as prevails for ELISA kits and the result depends on the cross-reactivity of the antibody for the different toxin analogues, *i.e.* if the antibody is specific for the major aflatoxin or fumonisin analogue (aflatoxin B_1 or fumonisin B_1), then the analytical result will not reflect the total but rather the level of that individual compound. For certain matrix–mycotoxin combi-nations, results compare well with HPLC determinations, although care needs to be exercised when investigating matrices for which the test has not been validated.[24,46]

Mycotoxin antibodies have been used to develop methods for some mycotoxins based on fluorescence polarization.[47] In this technique, the orientation of the fluorescence emission, which is related to the rate of molecular rotation, rather than total fluorescence is measured. The change in fluorescence polarization signal is obtained by competition for available binding sites on the antibody between the mycotoxin in the extract and an added fluorescently-labelled toxin standard.

Mycotoxin biosensors use antibodies attached to the surface of the sensor. These interact with the mycotoxin and produce a change in surface properties. This change can be monitored by evanescent wave techniques such as surface plasmon resonance or by fluorescence coupling with the evanescent wave using optical fibres. Both techniques have been used for the determination of fumonisins.[48,49]

Immunoassay-based lateral flow devices or dipsticks allow rapid screening for detection of contamination levels above or below a pre-set limit.[50] The mycotoxin sample extract is applied at the base of the dipstick to a conjugate pad contain-ing colloidal gold conjugated anti-mycotoxin antibodies. Mycotoxin in the extract is bound by the antibodies and then both bound and unbound antibodies move up the dipstick membrane. On passing the test line of immobilized mycotoxin, any unbound colloidal gold conjugated antibody becomes immobilized and hence visible as a pink line, *i.e.* contamina-tion levels below the set limit will be identified by the test line

becoming visible. In order to check on dipstick performance, a control line, containing anti-antibodies, is included after the test line to ensure that the colloidal gold conjugated antibodies migrated along the strip.[50]

10. Other analytical methods

Apart from the range of analytical methods that have been described above, a number of other techniques have been investigated as potential methods for mycotoxin determination. However, these have found little application outside the research environment.

Capillary electrophoresis (CE) is an instrumental technique which achieves separation of components based on charge in solution rather than chromatographic interactions between solute and stationary phase. Separation of non-charged species can be achieved by the introduction of micelles in the technique known as micellar electrokinetic capillary chromatography (MECC). CE is performed in small volumes of aqueous buffers and thus avoids the large volumes of organic solvents frequently required for chromatographic separations. A wide range of mycotoxin standards, including aflatoxins, deoxynivalenol, fumonisins, moniliformin, ochratoxin A and zearalenone, have been separated by CE.[51] As the separation mechanism differs from chromatography, CE separations can have their own problems with analytical impurities, as well as with achieving low limits of detection due to the limited sample amount that can be introduced into the CE capillary. The introduction of suitable lasers for detection, combined where necessary with appropriate derivatisation of the mycotoxin analyte, has lowered the detection limits to levels suitable for analysis of contaminated food samples. For example, a CE laser-induced fluorescence (CE-LIF) method has been developed for the determination of zearalenone in maize in which the addition of heptakis-(2,6-di-O-methyl)-β-cyclodextrin enhanced the natural fluorescence of zearalenone and achieved a detection limit of 5 μg kg^{-1}.[52]

All the methods discussed above have required the extraction of the mycotoxin from its matrix. This constitutes a time consuming and expensive exercise. In situations where rapid decisions are required, a method not requiring extraction but analysing the mycotoxin in situ would be advantageous. Because of this, researchers have investigated the possibility of using infrared (IR) analysers and principal component analysis for screening of mycotoxins directly from a grain sample. The advantages of these methods are the ease of operation, rapid result and nondestruction of the sample. Kernel rots and fumonisin contamination in maize were detected using near-IR reflectance spectroscopy which enabled a distinction to be made between contaminated and clean lots.[53] Deoxynivalenol contamination in wheat and maize has been investigated using IR spectroscopic techniques and the potential of the technique demonstrated.[54] In the case of deoxynivalenol in maize, samples with levels as low as 310 μg kg^{-1} could be separated from uncontaminated samples.[54] However, the application of near- or mid-IR presents a number of challenges, including the nonhomogeneous distribution of mycotoxin, the detection limits of the method, particle size distribution of the ground grain and the large calibration sets required. A further

advance has been the use of mid-IR spectroscopy to sort dried vine fruit into different batches depending on the levels of ochratoxin A contamination.[55] In this application, samples with high contamination (>20 μg kg^{-1}) were separated from those with lower contamination (10 μg kg^{-1}) and from uncontaminated samples.

11. Conclusion

Mycotoxin analysis in food commodities continues to represent a challenge to analytical chemists. In recent years, the implementation of lower regulatory levels in the European Union has necessitated the development of methods with ever lower limits of detection and their validation by international interlaboratory collaborative study with the purpose of setting official methods. The necessity of wider import controls has also encouraged the move to multi-toxin methods using LC-MS for quantification and analyte confirmation. The greater awareness of the mycotoxin problem has also led to an increased array of rapid screening methods that can be used for testing and control on a frequent monitoring basis and which can be incorporated into HACCP (hazard analysis and critical control point) plans.

Despite the plethora of analytical methods, a key component of laboratory performance, irrespective of the degree of sophisticated instrumentation, lies in the quality assurance programmes to ensure accurate and reliable analytical results, which are crucial to food safety programmes. For this purpose, a number of certified reference materials for selected mycotoxins are available, as are commercial proficiency testing services.

The filamentous fungi and the mycotoxins they produce have existed for all recorded history. It may thus be supposed that the determination of mycotoxins in human food will remain a necessity for a long time in the future and that general advances in analytical science will be reflected in similar advances in mycotoxin analysis.

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