Developments in mycotoxin analysis: an update for 2012-2013


1University of Natural Resources and Life Sciences, Vienna, Department for Agrobiotechnology (IFA-Tulln), Christian Doppler Laboratory for Mycotoxin Metabolism and Center for Analytical Chemistry, Konrad Lorenz Str. 20, 3430 Tulln, Austria; 2National Centre for Food, Spanish Food Safety and Nutrition Agency, Carretera de Majadahonda a Pozuelo km 5, 228220 Majadahonda, Spain; 3The Food and Environment Research Agency, Sand Hutton, York YO41 1LZ, United Kingdom; 4Instituto Adolfo Lutz, Laboratroy I de Ribeiro Preto, Av Dr Arnaldo 355, CEP 14085-410, Ribeiro Preto SP, Brazil; 5Institute of Sciences of Food Production, National Research Council, Via Amendola 122/o, Bari 700126, Italy; 6Trilogy Analytical Laboratory, 870 Vossbrink Drive, Washington, MO 63090, USA; 7USDA, ARS National Center for Agricultural Utilization Research, 1815 N. University St., Peoria, IL 61604, USA; 8Institute for Reference Materials and Measurements (IRMM), European Commission Joint Research Centre, Retieseweg 111, 2440 Geel, Belgium; 9Biological and Agricultural Engineering Department, N.C. State University, P.O. Box 7625, Raleigh, NC 27695-7625, USA; franz.berthiller@boku.ac.at

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Abstract

This review highlights developments in mycotoxin analysis and sampling over a period between mid-2012 and mid-2013. It covers the major mycotoxins: aflatoxins, Alternaria toxins, ergot alkaloids, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone. A wide range of analytical methods for mycotoxin determination in food and feed were developed last year, in particular immunochemical methods and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)-based methods. After a section on sampling and sample preparation, due to the rapid spread and developments in the field of LC-MS/MS multimycotoxin methods, a separate section has been devoted to this area of research. It is followed by a section on mycotoxins in botanicals and spices, before continuing with the format of previous reviews in this series with dedicated sections on method developments for the individual mycotoxins.

Keywords: aflatoxin, Alternaria toxins, ergot alkaloids, fumonisin, ochratoxin, patulin, trichothecene, zearalenone, sampling, multimycotoxin, botanicals, method development

1. Introduction

This review continues from a previous paper that covers analytical developments in mycotoxin determination over the period mid-2011 to mid-2012 (Shephard et al., 2013). As in the previous paper, this review emphasises new methodology published over the title period, although some natural occurrence data have been included, especially where it demonstrates the applicability of new methods. Also some toxicological findings are addressed at the discretion of the authors. Topics covered are sampling and sample preparation (section 2), multimycotoxin liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods (section 3), mycotoxins in botanicals and spices (section 4), aflatoxins (section 5), Alternaria toxins (section 6), ergot alkaloids (section 7), fumonisins (section 8), ochratoxins (section 9), patulin (PAT, section 10), trichothecenes (section 11) and zearalenone (ZEA, section 12).

A number of more general articles on mycotoxin developments have been published lately. Tabata (2012) summarised the development of analytical methods in particular for the determination of aflatoxins, PAT, ochratoxins and citrinin including the research for food safety from a Japanese perspective. Moreover, the current status and prospects for advanced hyphenated chromatography-mass spectrometry in mycotoxin...
determination has been reviewed extensively by Li et al. (2013a). A comprehensive overview of the current knowledge on plant metabolites of mycotoxins, also called masked mycotoxins, was recently published (Berthiller et al., 2013). The decontamination of mycotoxins, in particular by microbes or their enzymes, has been subject to a book chapter (Juodeikienė et al., 2012) and a review (McCormick, 2013). Finally, a book on the improvement of public health through mycotoxin control has been released by the International Agency for Research on Cancer (edited by Pitt et al., 2012). Dedicated chapters deal with mycotoxin producing fungi, physicochemical attributes of mycotoxins, sampling, analysis, effects of mycotoxins in animals and humans, risk assessment, economics and practical approaches to control mycotoxins.

2. Sampling and sample preparation

Improvements in sampling and sample preparation methods used to detect mycotoxins and other quality attributes in food and feed products continues to be a high priority among regulatory agencies, international organisations and commodity industries worldwide. As in recent years, more studies are investigating the effect of sample selection methods on the additional variability added to the sampling step by various selection techniques. For instance, the Codex Committee on Contaminants in Foods (CCCF) met 26-30 March 2012 to discuss harmonised aflatoxin maximum levels (ML) and aflatoxin sampling plans for dried figs traded in the international markets. CCCF delegates agreed on a ML of 10 µg/kg total aflatoxins and a sampling plan that required three 10 kg samples of dried figs to all test below the ML of 10 µg/kg total aflatoxins (3×10 kg <10 µg/kg) to accept a lot for trade. The ML and sampling plan were sent forward for adoption by the Codex Alimentarius Commission (CAC). The CAC met 2-7 July 2012 and adopted the aflatoxin ML and sampling plan for dried figs as the official Codex Standards (Codex Alimentarius Committee, 2012).

Mallmann et al. (2013) studied the efficiency of two sample selection methods to detect fumonisins in maize. The first sample selection method was a manual system using a sampling probe or spear to select whole grain samples from a lot. The second sample selection method was a continuous flow automatic system to select milled maize samples from a lot. Ten samples, one kg each (10×1 kg) were taken from each of 11 lots by each sample selection method. Using a nested design, the total variance associated with measuring fumonisins B1+B2 (FB1+FB2) among the 10×1 kg samples was partitioned into sampling, sample preparation and analytical variances. For both the manual and automatic sample selection methods, the sampling step was the largest source of error accounting for 71 and 40% of the total variation, respectively. The coefficient of variation (CV) associated with the sampling step for manual and automatic sample selection methods was 8.9 and 6.4%, respectively. The CVs associated with the sample preparation and analytical steps were about the same (approximately 3%) for both sample selection methods.

Lippolis et al. (2012) studied the variability and distribution among 100 test portions used to measure deoxynivalenol (DON) and ochratoxin A (OTA) concentrations when two 10 kg laboratory wheat samples (each naturally contaminated with DON and OTA) were prepared using dry comminution and water-slurry mixing. The DON distribution among the 100 test portions could be represented by a normal distribution for both dry comminution and water-slurry mixing. The variability, represented by the CVs, among the 100 test portions for DON measurements were 4.6 and 6.4% for water-slurry and dry comminution, respectively. The small CVs suggest that the variability associated with both sample preparation methods was negligible and was mostly due to the analytical step. The OTA distribution among the 100 test portions for dry comminution was positively skewed while the OTA distribution among the 100 test portions for water-slurry mixing could be described by the normal distribution. The variability among the 100 test portions for OTA measurements was 4 and 75% for water-slurry and dry comminution, respectively. Results clearly indicated that slurry mixing continued to reduce particle size and reduce sample preparation variability specifically for OTA, but not DON.

3. Multimycotoxin LC-MS(±MS) methods

A survey of the available literature relevant to the period 2012-2013 shows that sample preparation continues to be the most challenging task in multimycotoxin analysis. Strategies for sample preparation based on either traditional clean-up devices or innovative systems were evaluated for their contribution in satisfactory method performances, including acceptable matrix effects.

An LC-MS/MS method based on a combination of direct injection (after dilution) and extract purification using MultiSep”226 (Romer, Union, MO, USA), has been developed to monitor the co-occurrence of DON, ZEA, OTA, FB1, FB2, T-2 (T-2) and HT-2 (HT-2) toxins in animal feeds (Åberg et al., 2013). Method apparent recovery was higher than 86% for all the mycotoxins and limits of quantification (LOQs) ranged from 2.5 to 115 µg/kg. Two examples of the use of accelerated solvent extraction (ASE) coupled with solid phase extraction (SPE) clean-up for multimycotoxin extraction have been reported. An LC-MS/MS method with a pressurised liquid extraction followed by clean-up through polymeric SPE cartridges (Oasis HLB, Waters, Milford, MA, USA) has been developed for the simultaneous determination of aflatoxins and OTA in a range of animal derived foods (Chen et al., 2012). A similar
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The use of TurboFlow™ technology, a new automated on line sample clean-up system, directly coupled to an LC-high resolution mass spectrometry (HRMS) equipment, has been described by Ates et al. (2013) for the simultaneous determination of *Fusarium* toxins (DON, T-2, HT-2, ZEA, FB$_1$ and FB$_2$) in maize, wheat and animal feed. Toxins were extracted with an acidified water-acetonitrile (MeCN) mixture. Average recoveries, relative standard deviations (RSDs) and intermediate precision values were 71-120%, 1-19% and 4-19%, respectively. The main perspective of this technology, coupled with full scan analysis by HRMS, is the inclusion of masked mycotoxins and/or the identification of other metabolites by retrospective analysis.

Also this year the increasing interest in evaluating the use of HRMS for multimycotoxin analysis in comparison with triple quadrupole-based methodologies was confirmed. Rubert et al. (2013) compared two mass spectrometry strategies for multiple mycotoxin analysis in European beers. LC-HRMS (based on Orbitrap technology) was used for unambiguous identification of 18 target mycotoxins, as well as for screening non-target mycotoxins, such as enniatins, fusaproliferin and DON-3-glucoside (DON-3G), whereas mycotoxin quantification was carried out using a triple quadrupole (LC-QqQ) instrument. The authors concluded that the LC-QqQ was most satisfactory for quantification purposes, whereas LC-HRMS improved unambiguous identification and screening capabilities.

Apparent not in line with the trend of simplifying the sample preparation and of joining targeted and untargeted screening, selective clean-up based on multi-antibody immunoaffinity columns (IAC) continues to represent a valuable tool to develop robust methodologies and to achieve low detection limits. The use of multi-antibody IAC, Myco6in1™ (Vicam, Watertown, MA, USA), has been re-proposed prior to UHPLC-MS/MS for determination of regulated mycotoxins, namely aflatoxins, fumonisins, DON, ZEA, OTA, T-2 and HT-2, in grains (Tang et al., 2013). The average recovery rates in rice, maize, wheat and peanut were mostly between 70 and 120%, with RSDs less than 15%. The LOQs ranged between 0.5 and 20 µg/kg. An interesting critical comparison between the use of IAC and direct analysis of crude extracts has been published by Senyuva et al. (2012). LC-MS/MS ion ratios and peak profiles, repeatability and LOQs were used as the basis for a detailed comparison of the two approaches. The overall conclusion was that LC-MS/MS analysis of samples without clean-up is adequate for screening, but when using LC-MS/MS for definitive measurements (e.g. for food regulatory control purposes) IAC clean-up remains essential.

On the other side, the direct injection of crude extracts coupled with highly sensitive instrumentation has been used to develop relatively rapid methods. A UHPLC-MS/MS method based on direct injection of diluted extracts has been proposed for the determination of 18 mycotoxins in different food matrices (Beltrán et al., 2013). Sample preparation consisted of extraction with acidified MeCN/water. A defatting step with MeCN/hexane was introduced for oil samples. The dilution of the sample extract was applied to minimise matrix effects. As stated by the authors, this was feasible due to the high sensitivity of the modern instrumentation used. Extraction recoveries between 70 and 120% and RSDs lower than 20% were obtained for the majority of analyte-matrix combinations, whereas LOQs were ≤1 µg/kg for most of the toxins. A similar approach was developed to detect 26 mycotoxins (aflatoxins, ochratoxins, fumonisins, trichotheccenes and ergot alkaloids) in maize, rice, wheat, almond, peanut and pistachio products by LC-MS/MS (Liao et al., 2013). Homogenised grain or nut products were extracted with MeCN/water (85:15, v/v), followed by high-speed centrifugation and dilution with water. Mean recoveries were in the range 84-104%, with RSDs lower than 16%. LOQs ranged from 0.2 to 12.8 µg/kg, depending on the mycotoxin.

Based on the number of publications (more than 10) in the period 2012–2013, the use of QuEChERS (quick, easy, cheap, effective, rugged and safe) as generic sample pre-treatment is becoming a popular alternative to the dilute-and-shoot approach. The effectiveness of four extraction methods (modified QuEChERS, matrix solid-phase dispersion (MSPD), solid-liquid extraction and SPE clean-up) was evaluated for simultaneous determination of 32 mycotoxins by UHPLC-HRMS (Rubert et al., 2012). QuEChERS clean-up was fast and yielded satisfactory recoveries (from 64-93%) for the widest range of mycotoxins. A QuEChERS-based extraction procedure has also been used to develop an LC-MS/MS method for the determination of multiple mycotoxins in highly pigmented spice matrices, namely red chilli (*Capsicum annuum* ssp.), black and white pepper (*Piper nigrum* ssp.) (Yogendraiah et al., 2013). Significant matrix effects were observed but compensated using matrix-matched calibration curves. Satisfactory recoveries (from 75-117%) and intra-day reproducibility (from 4-22%) were obtained for most of the mycotoxins. The LOQs ranged from 2.3 to 146 µg/kg. A QuEChERS-like procedure prior to UHPLC-MS/MS analysis was developed for the determination of 34 mycotoxins in dietary supplements containing green coffee bean extracts (Vaclavik et al., 2013). Average recoveries of the analytes were in the range of 75-110% with RSDs below 12%. LOQs ranged...
from 2.5 to 100 μg/kg. Also in this case, significant matrix effects were observed and corrected by standard addition method. Finally, the use of QuEChERS in combination with dispersive liquid-liquid microextraction (DLLME) has been proposed for the UHPLC-MS/MS determination of 15 mycotoxins in milk thistle (Silybum marianum), including aflatoxins, fumonisins, trichothecenes, OTA, citrinin, sterigmatocystin and ZEA (Arroyo-Manzaranes et al., 2013). The method was validated in extract and seeds of milk thistle, obtaining LOQs lower than those usually required by legislation in food matrices, with precisions lower than 10%. Recoveries were between 62 and 99%, except for ZEA and citrinin.

Being now routinely used to compensate matrix effects, different approaches for fully isotope-labelled mycotoxins as internal standard have been studied. A novel normalisation approach of the signal response of 32 mycotoxins was investigated using the isotopically labelled versions of only three mycotoxins, i.e. DON, aflatoxin B1 (AFB1) and ZEA, as both true isotopologues and as structurally different internal standards. Mycotoxins were extracted via a single-step procedure using a mixture of MeCN/water/formic acid and the extracts were directly analysed by UHPLC-MS/MS. This approach reduced both the number of standard compounds needed for quantification and therefore cost of analysis per sample (Jackson et al., 2012a). Zhang et al. (2013b) described a procedure for quantitative determination of 12 mycotoxins (aflatoxins, OTA and Fusarium toxins) in milk-based infant formula and foods using the relative response factors of the 13C-uniformly labelled internal standards and target mycotoxins.

Still far from harmonisation, efforts are being made for method comparison and deeper understanding of performances of available LC-MS/MS methodologies for mycotoxin analysis. Within the EU network of Excellence MoniQA (www.MoniQA.eu) a proficiency test was conducted to benchmark laboratories using LC-MS/(MS) for multmycotoxin analysis and to obtain information on currently used methodologies and related method performances (De Girolamo et al., 2013; Solfrizzo et al., 2013a). The study involved 41 laboratories from 14 countries and was conducted for the simultaneous determination of up to 11 mycotoxins (aflatoxins, OTA, FB1, FB2, ZEA, DON, T-2 and HT-2) in spiked and naturally contaminated maize. Only two laboratories obtained acceptable results of z-score for all mycotoxins. In general, extraction mixtures of water with MeCN, methanol or both provided good results for quantitative extraction of mycotoxins from maize. Laboratories using extract clean-up reported acceptable results for the majority of mycotoxins. Good results were also obtained by laboratories that analysed crude extracts although a high variability of results was observed for all tested mycotoxins. Matrix-matched calibration or isotope-labelled internal standards efficiently compensated matrix effects whereas external calibration gave reliable results by injecting <10 mg of matrix equivalent amounts. It is worth mentioning that unacceptable high recovery and high variability of fumonisins results were obtained by the majority of laboratories for spiked maize. Results of an inter-laboratory study concerning relative and absolute matrix effects in multymycotoxin determination have been reported by Malachova et al. (2013). The applicability of commonly used strategies in matrix effect reduction was tested in the quantitative determination of nivalenol (NIV), DON, FB1, FB2 and ZEA in complex feed matrices. The study showed that the use of any purification technique helped to improve absolute matrix effects for some analytes, whereas other factors such as changes in LC conditions or switching of ion source polarity had a minor impact. These studies provide a great deal of information on currently used methodologies enabling a deeper understanding on the performances of different LC-MS-based approaches for multymycotoxins analysis in real food matrices.

The increasing availability of LC-MS/(MS) methods for multymycotoxin determination provides data about the co-occurrence of multiple mycotoxins in the same sample, including a wide array of less known or ‘emerging’ mycotoxins and other metabolites. LC-MS/MS has been used to investigate the co-occurrence of beauvericin and enniatins (Blesa et al., 2012; Juan et al., 2013) also in combination with other Fusarium toxins such as trichothecenes and zearalenones (Juan et al., 2013b; Serrano et al., 2012), or fusaproliferin (Serrano et al., 2013) in cereals and cereal products. Further surveys provided data on co-occurrence of major mycotoxins (aflatoxins, fumonisins, OTA, trichothecenes and ZEA) in a wide range of food matrices, such as Serbian wheat flours (Škrbić et al., 2012), peanut cakes from Nigerian markets (Ezekiel et al., 2012), compound feeds from South Africa (Njobeh et al., 2012), raw cereals and cereal foods from Tunisian markets (Oueslati et al., 2012), staple food commodities from Cameroon (Abia et al., 2013), Alternaria, Penicillium and Aspergillus toxins were surveyed in wine, cider and their cork stoppers from eight countries (Scussel et al., 2013). LC-MS/MS screening for a broader range of mycotoxins and other fungal metabolites showed the presence of up to 69 metabolites in a single feed sample (Streit et al., 2013; Warth et al., 2012a) emphasising the great variety of potential mycotoxin co-exposure.

With use of advanced LC-MS/(MS) methods, low concentrations of mycotoxins and relevant metabolites can be quantified simultaneously in biological fluids and tissues and be used as biomarkers. An overview of published LC-MS/MS-based multibiomarker approaches for the assessment of human exposure to mycotoxin has been published by Warth et al. (2013). Furthermore, a recent comparison of single and multi-analyte LC-MS/MS methodologies for mycotoxin biomarker determination in
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et al. (2013) developed an LC-MS/MS method for the determination of seven mycotoxins and their metabolites (including DON-glucuronide (DON-GlcA)) and ZEA-glucuronide (ZEA-GlcA)) in human urine. Urine samples were extracted with ethyl acetate/formic acid followed by SPE clean-up. For pig plasma, two LC-MS/MS methods have been developed for two sets of mycotoxins, namely for DON, deoxy-deoxynivalenol (DOM-1), T-2, HT-2, ZEA, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), OTA, FB₁, and AFB₁ (Devreese et al., 2012) and for beauvericin and enniatins (Devreese et al., 2013). In both cases, sample clean-up consisted of a deproteinisation step using MeCN, followed by evaporation of the supernatant and re-suspension of the dry residue in water/methanol mixture. However for toxicokinetic studies with individual mycotoxins, highly sensitive analyte-specific LC-MS/MS methods were developed within the same study. For pig urine, Song et al. (2013a) developed an LC-MS/MS method for simultaneous analysis of AFB₁, DON, FB₁, OTA, ZEA, T-2 and their metabolites. A salting-out assisted liquid-liquid extraction (LLE) procedure was used for sample preparation. The developed method was also validated for human urine as an extension of its application. Finally, Veršilovskis et al. (2012) reported on the use of LC-MS/MS for the simultaneous determination of masked forms of DON and ZEA after oral dosing in rats. A total of 21 metabolites were quantified in rat organs giving insights in metabolisms of masked mycotoxins. A multi-biomarker LC-MS/MS method was used to validate urinary biomarkers of five mycotoxins (DON, FB₁, ZEA, OTA and AFB₁) in piglets fed with boluses contaminated with increasing levels of the toxins (Gambacorta et al., 2013).

A current trend in chemical food safety control is represented by an effort to integrate analyses of various groups of food contaminants/toxicants into a single, high-throughput method. Again, the power of modern LC-MS/MS instrumentation to screen for a large number of contaminants is employed. Increasing attention has been given to the choice of optimal sample preparation procedures that represents one of the key steps to achieve satisfactory performance characteristics. As an example, Lacina et al. (2012) reported results of a study dedicated to test different sample preparation procedures: aqueous MeCN extraction followed by partition (QuEChERS-like method), aqueous MeCN extraction and pure MeCN extraction for the analysis of a list of target analytes, including 28 pesticides together with 38 mycotoxins. The QuEChERS-like method showed the best performance in terms of co-extraction of focused analytes. Indeed the QuEChERS approach is the most commonly used when developing multi-contaminant extraction, either in combination with HRMS based on TOF (time-of-flight) or Orbitrap mass analysers or with last generation QqQ enabling fast detection of multiple analytes. QuEChERS-like methods have been developed and optimised for the simultaneous determination of 22 carbamate insecticides and 17 mycotoxins in cereals (Zhang et al., 2013b) by UHPLC-MS/MS, for simultaneous analysis of veterinary drugs and mycotoxins in hen eggs by LC-MS/MS (Capriotti et al., 2012b) and for purifying bakery raw materials and finished products to detect pesticides, mycotoxins and veterinary drugs by UHPLC-HRMS (De Dominicis et al., 2012). On the other side, some authors proposed commodity-dedicated methods for the determination of a restricted number of mycotoxins together with other contaminants of interest in specific matrices. Mornar et al. (2013) developed a method for simultaneous determination of citrinin and cholesterol-lowering compounds present in red fermented rice using connected diode array and fluorescence (FLD) and MS detectors, whereas Song et al. (2013b) reported an LC-MS method for the simultaneous determination of AFB₁, OTA and PAT together with bisphenol A for regulatory purposes, in beverages and food products. A screening method for the detection and identification of undesirable organic compounds, including antibiotics, pesticides and mycotoxins in aquaculture products based on direct injection of MeCN/water extracts in the LC-HRMS system, has been reported by Nácher-Mestre et al. (2013). Retrospective analysis of accurate mass full spectra provided by QTOF-MS enabled detection and tentative identification of other undesirable organic compounds different from those included in the validated list.

4. Mycotoxins in botanicals and spices

There was an increase in published methods for mycotoxin determination in botanicals and spices this past year compared to previous years. This increase was mainly due to the multiple LC-MS/MS methods recently published. In addition, there were several new methods published along with several surveillance studies using HPLC-FLD. There were also a group of publications that described methodologies specifically for the analysis of mycotoxins in Chinese herbal medicines.

Six published methods for mycotoxins in botanicals and spices used HPLC-MS/MS. Two of the methods described the analysis of aflatoxins and OTA in liquorice roots. The first method was developed for analysing liquorice roots and fritillary bulbs for the simultaneous determination of AFB₁ and OTA using LC-MS/MS (Wang et al., 2013b). The samples were concentrated and purified using a low cost SPE clean-up prior to HPLC analysis. The precision of the method was excellent (RSD <2.8%) with analytical recoveries ranging from 93-105%. The LOD and LOQ
were 0.02 and 0.09 µg/kg, respectively. The second method (Wei et al., 2013) described the analysis of AFB₁, aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁), aflatoxin G₂ (AFG₂) and OTA in Glycyrrhiza uralnensis, one of the main sources of liquorice in China. This method used sonication extraction using methanol:water (80:20, v/v) followed by an IAC extract purification with HPLC-MS/MS detection. This purification method was used due to the complexity and high coloration of the liquorice root matrix. The LOQs of AFB₁, AFB₂, AFG₁, AFG₂ and OTA were 0.02, 0.02, 0.01, 0.05 and 0.01 µg/kg, respectively. The results of analysing a total of 15 samples of G. uralnensis showed that almost all the samples were contaminated with aflatoxins and OTA. This was the first report of the co-occurrence of aflatoxins and OTA in G. uralnensis in China. A multimycotoxin method validation was published (Škrbić et al., 2013) utilising UHPLC-MS/MS for the analysis of crude extracts of red and black pepper. This validation was performed to establish a high-throughput multi-matrix procedure for the simultaneous determination of mycotoxins included in the EU regulations for spices. The data obtained on red pepper samples fortified with AFB₁, AFB₂, AFG₁, AFG₂ and OTA were in compliance with these regulations. However, validation parameters for analysis of some mycotoxins (recoveries and LODs for AFG₁, AFG₂ and OTA) did not comply. Another publication evaluated different extraction procedures for the simultaneous determination of pesticide residues and mycotoxins in fruits, cereals, spices and oil seeds employing UHPLC-MS/MS (Lacina et al., 2012). Acceptable recoveries (70-120%) for most of the analytes were obtained by the analysis of spiked matrices. However, during the analysis of dry samples with incurred analytes, pure MeCN did not efficiently extract some common contaminants. The development and validation of an HPLC-MS/MS method for the simultaneous quantification of AFB₁, AFB₂, AFG₁ and AFG₂ in lotus seeds was published (Liu et al., 2013a). The samples were extracted with methanol:water solution (80:20, v/v) and then purified using IAC. Recoveries for samples of spiked lotus seeds were all above 66% with the RSDs all below 15%. Nineteen out of twenty samples of lotus seeds collected from different stores and markets in China were found to be contaminated with aflatoxins at different levels ranging from 0.02 to 688.4 µg/kg.

Several methods were published utilising HPLC-FLD for the analysis of mycotoxins in spices and botanicals. A collaborative study was performed to validate an analytical method for the determination of OTA in liquorice and liquorice extracts (Lerda et al., 2013). The samples were extracted with a mixture of methanol and aqueous sodium bicarbonate, purified with IAC and quantified by HPLC-FLD. The mean recoveries were 87% for liquorice root and 86% for liquorice extracts. The RSDs for reproducibility ranged from 10-17% and from 11-22% in liquorice extracts and liquorice roots, respectively. An HPLC-FLD method for the determination of the AFB₁, AFB₂, AFG₁ and AFG₂ in peanuts, rice and chilli was developed (Khayoon et al., 2012). The sample was extracted with MeCN:water (90:10, v/v) and then purified using SPE. After a pre-column derivatisation, the analytes were separated within 3.7 min using a RP-18 monolithic HPLC column. The recoveries of aflatoxins that were spiked into the samples were 86-105% and the RSDs were 4-4%. Another method utilising UHPLC-FLD was developed for the determination of OTA in ginger using molecularly imprinted polymer (MIP)-SPE clean-up (Cao et al., 2013). The recoveries of OTA from spiked ginger samples ranged from 87-94%. After a regeneration procedure, this clean-up column could be reused at least forty-one times to obtain more than 80% recovery of OTA from ginger samples. Other surveillance studies from Turkey, Malaysia and Pakistan investigated the incidence of aflatoxins and OTA in spices using HPLC-FLD and IAC (Iqbal et al., 2013; Jalili and Jinap, 2012; Ozbey and Kabak, 2012).

A number of articles were published this past year specifically describing methodologies for the analysis of mycotoxins in Chinese medicinal plants. One published article reviewed the advancement of detection methods for mycotoxins in Chinese herbal medicines (Xu et al., 2012). The natural occurrence of citrinin in red yeast rice, medicinal plants and their related products was investigated for the first time (Li et al., 2012d). The samples were extracted by methanol/water, purified with IAC for citrinin and quantified by HPLC-FLD. The mean recoveries on spiked samples ranged from 73-93% with RSDs of 1.4-7.9%. Out of a total of 109 samples, citrinin was detected in 31 of the red yeast rice matrices ranging from 16.6 to 5,253 µg/kg. A gas chromatography (GC)-electron capture detection (ECD) method was developed for the simultaneous determination of T-2 and HT-2 in Chinese herbal medicines and related products (Kong et al., 2012). The method used IAC for sample extract purification and a pre-column derivatisation with N-heptfluoro-butyryl imidazole. The LODs for T-2 and HT-2 were 1.9 and 0.5 µg/kg, and the recoveries for different herbal matrices ranged from 89 to 99%. T-2 and HT-2 were not detected in all 89 Chinese herbal medicines analysed using this methodology.

5. Aflatoxins

Aflatoxins remain of interest, either as model substances to prove the concept of new immunoassays or sensors or as target analytes in a number of multimycotoxin methods. Certainly the current situation of an apparent increased aflatoxin occurrence in agricultural products will promote methods for early as well high throughput screening.

Taking note of the rapid progress of immunochemical methods, 133 publications mainly of the last 3 years have
been reviewed (Li et al., 2012a) for current development of microfluidic immunosensing approaches for mycotoxins including aflatoxins. Another review (Li et al., 2012c) summarises the developments in surface plasmon resonance (SPR) biosensors for mycotoxins, including aflatoxins. The review covers 28 publications of the years 1999-2011. A few new immunoassays and sensors have been described by various authors (Abhijith and Thakur, 2012; Dinçkaya et al., 2012; He et al., 2012; Jiang et al., 2013; Li et al., 2012b, 2013b; Linting et al., 2012; Masoomi et al., 2013; Nachi Rossi et al., 2012; Wang et al., 2013d; Xu et al., 2013; Yu et al., 2013; Zhang et al., 2012) and a number of method parameters that are specific to the test system applied are given by the authors. However, as method performance depends on the availability of the antibodies used, which are either not readily available and often raised in the laboratory of the authors, arrive as a gift, or are only temporarily available in case of polyclonal antibodies, these methods are not further reviewed for their performance and the claims made, but are mentioned for completeness.

The inhibition of acetylcholinesterase by AFB$_1$ was used (Pohanka, 2013) for its determination in a spectrophotometric assay. The author describes the system with respect to many variables for neat AFB$_1$ in solution. Ma et al. (2013a) developed a new immunoaffinity column linking the antibodies to silicagel as carrier. They report on various loading, washing and elution parameters of the developed product, which have been described in the past having the potential of being reusable. Authors considered the aspect of reusing their columns but also mention the restrictions that apply. Oasis HLB$^+$ columns (Waters) have been proposed (Wang et al., 2012b) for the purification of milk and milk powder as alternative for IAC. The method was tested on various dairy products and the authors reported recovery rates of approx. 80-90% with repeatabilities of less than 5%. The supplied chromatograms show, however, that the purification by immunoaffinity is superior, while the one by Oasis HLB appears to be sufficient for the described purpose. A similar approach was followed by Tamura et al. (2012) who used an Oasis HLB column and a MycoSep$^+$ column (Romer) for clean-up for the determination of aflatoxins next to 10 other mycotoxins in wine. After validation, the method was used to test red and white wines. Only fumonisins and OTA could be identified in a few cases. Bertuzzi et al. (2012) compared currently used methanol- and acetone-based extractants at various ratios and identified that a ratio of 6:4 (acetone:water) favours better extraction yields for total aflatoxins. Other ratios favour AFB$_1$, while one key observation was that the used IAC was found to be robust for all aflatoxins up to a concentration of 8.5% acetone after 10-fold extract dilution. The method was applied to maize and resulted in recoveries above 90% with acceptable repeatability figures.

Degola et al. (2012) described a fluorometry method for the determination of aflatoxins in culture media. The method reads the induced fluorescence of the culture media and allows the evaluation of the aflatoxins progression in the media. The method was compared with an LC method, and a good correlation between the observed fluorescence and the determined aflatoxins was achieved. The addition of cyclodextrins further facilitated the procedure. The authors showed the usefulness of the method for monitoring the production or inhibition of aflatoxins in cultures. Unfortunately, the authors did not address to what degree their procedure is of value for other fungal metabolites that also exhibit fluorescence like kojic acid (after reaction with peroxidase from the seed). Therefore, some knowledge of the metabolic products of the cell cultures tested seem to be important. Chen and Zhang (2013) reported the use of ASE for aflatoxin-fortified grains. Various extraction parameters (temperature, solvent, time) were investigated and some indicated satisfactory performance comparable to conventional extraction and clean-up procedures. The authors (like others before) did unfortunately not address the issue of the possibility of analyte migration into the matrix, rather than the assumption of matrix extraction. This is, however, an important aspect as similar procedures (parboiling) are used to promote the migration of potential analytes (niacin or calcium) into the matrix (rice), rather than extracting them. Jinap et al. (2012) described an inline IAC clean-up coupled to HPLC. The method offers the advantage that the IAC (mounted via a six port valve) can be used for a number of cycles and facilitates aflatoxin concentration and purification by direct transfer onto the chromatographic column. The method was tested for a number of performance parameters (recovery, repeatability and intermediate precision) that all met current provisions for methods in official control laboratories. Furthermore, the authors demonstrated the specificity of their IAC used and lack of cross-reactivity to other potential interfering analytes.

A clean-up procedure for aflatoxins in edible oil that is based on IAC clean-up with further manipulation by DLLME has been described (Afzali et al., 2012). The proposed method was extensively studied for various parameters and validated, however, it lacked the demonstration that it is superior to conventional IAC clean-up followed by evaporation, especially as no interferences were reported. Interesting is that the authors used an elution solvent based on diluted MeCN with an aqueous NaNO$_3$ solution. This solution allowed a direct injection of a larger aliquot of the IAC eluate onto an HPLC system. A method for a cluster of almost 40 analytes ranging from insecticides to mycotoxins in cereals has been reported by Zhang et al. (2013b). Next to some Fusarium toxins and OTA, aflatoxins were subject of investigation. The method is based on QuEChERS clean-up followed by LC-MS. Recovery values and precision data were generated over a range of 5-50 µg/kg for rice,
wheat and maize. Recovery values for all aflatoxins were above 85%, with repeatability values of less than 15% and slightly higher levels for intermediate precision. Song et al. (2013b) described an LC-MS method with SPE clean-up for the determination of AFB\textsubscript{1}, next to OTA, PAT and bisphenol A, a rather heterogeneous combination for a variety of matrices, i.e. peanut butter, cereals and fruit juices. Even though the methodological components have been described before, the authors managed to tune the method for satisfactory performance for each matrix. In particular, aflatoxin recoveries ranged from 70-93% for dry foods, while all precision experiments demonstrated figures below 15% (typically below 10%). The authors found that matrix-matched calibration is key for the performance, but only demonstrated the performance on solvent-spiked materials. This might be critical in that respect that solvent-spiked analytes (e.g. AFB\textsubscript{1}) might have different extraction behaviour depending on the matrix (e.g. peanut butter) and the way they are incurred and extracted. Wang et al. (2013b) described a method for the determination of AFB\textsubscript{1} and OTA in liquorice roots and fritillary bulbs based on an SPE clean-up with C18 material followed by LC-MS. They reported precision data of less than 3% for both analytes with a recovery rate above 90%. Moreover, the LOQs for AFB\textsubscript{1} and OTA were 0.024 and 0.095 µg/kg, respectively. The authors used the method to analyse more than 20 samples, and OTA were 0.024 and 0.095 µg/kg, respectively. The authors analysed commercial samples and confirmed the presence of mycotoxins, but not of AFB\textsubscript{1}. The method performance figures are comparable with those reported by Zhang et al. (2013b) and Afzali et al. (2012) who used similar methodologies for clean-up. However, it should be taken into account these authors covered a different range of mycotoxins and other matrices. Song et al. (2013a) developed a method for the assessment of a number of mycotoxins, including AFB\textsubscript{1} and aflatoxin M\textsubscript{1} (AFM\textsubscript{1}), as biomarkers in urine. The method made use of LLE followed by LC-MS. Recovery was assessed to range from 70-108% for all analytes with an intermediate precision of less than 25% for most analytes when tested at different concentrations. The method confirmed the presence of AFB\textsubscript{1} next to other mycotoxins in pig urine samples. Abia et al. (2013) described an LC-MS-based multitoxin method that includes aflatoxins and some of its precursors in the metabolic pathway (avеруrin and versicolorins). It was reported to be suitable for nuts, cereals and products thereof (e.g. beer). The method required no clean-up and confirmed a high abundance of aflatoxins in local products.

An clean-up for the determination of AFM\textsubscript{1} as an alternative to IAC has been proposed by Biancardi et al. (2013). The authors used liquid-liquid extraction of defatted milk with ethyl acetate and subsequent measurement by LC-MS. The method was compared with the official ISO method 14501 and proposed as an alternative one. The method did not use any proprietary products, which is an asset for analysts in infrastructural or economically challenged regions. Unfortunately, the authors only applied this new concept to LC-MS, leaving the question unanswered whether their novel clean-up procedure is also suitable for LC-FLD. The IAC purification drawbacks highlighted by the authors appear a crucial driver for the search of a clean-up alternative. This highlights how diverse the experiences of different research groups are with respect to IACs. While some scientists further implement the application of IACs in methods for improving the performance of their methods, others put effort to find alternatives for the same products for the simultaneous purification of AFB\textsubscript{1} and T-2 toxin in plasma, the authors used a self-prepared clean-up column on the basis of silica gel and florisil after testing the performance of this clean-up in comparison with other alternative clean-up materials, such as charcoal, aluminium oxide and kieselguhr. LOQs of 0.05 ng/ml, recoveries of 71-108% and RSDs below 14% were achieved for both toxins in plasma and tissue samples. A multitoxin LC-MS method has been described for a variety of mycotoxins in the botanical *Silybum marianum* (Arroyo-Manzanares et al., 2013). The method combines QuEChERS clean-up followed by DLLME prior to identification and quantification by LC-MS. The authors compared the effectiveness of both clean-up steps with respect to ion suppression. Precision figures over different levels were below 10% (typically around 5-7%) for all tested mycotoxins, including AFB\textsubscript{1}. The method was compared with the official ISO method 14501 and proposed as an alternative one. The method did not use any proprietary products, which is an asset for analysts in infrastructural or economically challenged regions.
6. Alternaria toxins

The stable isotope dilution assay (SIDA) approach was used by Liu and Rychlik (2013) to develop an LC-MS/MS method for the determination of tentoxin (TTX), dihydrotentoxin (DHT) and isotentoxin (isoTTX) in different food products. Triply deuterated internal standards were prepared via total synthesis. Samples were extracted with MeCN-water (84:16, v/v) and purified on a C18-phenyl SPE column providing a more selective binding of these phenyl-containing cyclic peptides. For the three toxins LODs ranged from 0.10 to 0.99 µg/kg, recoveries ranged between 98 and 115% and method precision was below 9%. The method was applied to 103 food samples and 85% were found contaminated with TTX at levels up to 52 µg/kg, 55% with DHT at levels up to 36 µg/kg whereas isoTTX was not quantifiable. The SIDA approach was also used by Lohrey et al. (2013) who developed an LC-MS/MS method for determination of tenuazonic acid (TeA) in tomato and pepper products. Isotopically labelled internal standard $^{13}$C$_2$-TeA was synthesised and added to samples before extraction. Samples were slurried with water, extracted with MeCN-formic acid (10:1,1, v/v) and sample extracts were purified by using a QuEChERS approach. Values of LOD and LOQ were 0.9 and 2.9 µg/kg, respectively. Acceptable values of recoveries (91-99%) and precision (1-6%) were reported. The application of this method to samples from a German market revealed the presence of TeA in all 30 analysed samples at levels ranging from 3 to 2,330 µg/kg.

A comparison of the performances of two LC-MS/MS methods for the determination of TeA and its analogues was performed on sorghum-based infant food (Asam et al., 2013). One method involved derivatisation with 2,4-dinitrophenylhydrazine and the other no derivatisation. A good chromatographic separation of TeA and its four analogues was obtained whereas the separation of TeA and TeA analogues dinitrophenylhydrazones was only marginal, and the isobaric dinitrophenylhydrazones of TeA and its analogue derived from leucine (LeuTeA) had to be differentiated by mass spectrometric means. LODs were between 1-3 µg/kg (with derivatisation) and 50-80 µg/kg (without derivatisation). Analogues of TeA derived from valine (ValTeA) and LeuTeA were detected in sorghum-based infant food highly contaminated with TeA. Prelle et al. (2013) developed an LC-MS/MS method for the simultaneous determination of alternariol, alternariol monomethyl ether, altenuene, TTX and TeA in apple juices, beers, tomato sauces, olives and dried basil. For sample extract clean-up, four different SPE columns were selected to optimise the purification step for each food matrix. LODs and LOQs were in the range 0.2-12 and 0.5-41 µg/kg, respectively. Recoveries were generally above 70% and precision in the range of 1-38%. Thirty out of 70 samples from an Italian market were contaminated with at least one Alternaria toxin at levels up to 35 µg/kg.
Within the mandate M/520, the European Committee for Standardization (CEN, 2013) has recently launched a call for tender for the development of a standardised method for the analysis of Alternaria toxins in food. Probably due to the increasing interest for Alternaria toxins, several papers were published over the last year on the toxicity of these toxins (Fleck et al., 2012; Schwarz et al., 2012a,b; Solhaug et al., 2012, 2013; Tiessen et al., 2013).

7. Ergot alkaloids

The European Food Safety Authority (EFSA) has delivered a Scientific Opinion on ergot alkaloids in food and feed (EFSA, 2012). The opinion considered over 20,000 analytical results for ergot alkaloid levels in 1,716 food, 496 feed and 67 unprocessed grain samples, and made a risk assessment based on the major ergot alkaloids identified in sclerotia from *Claviceps purpurea*. The EFSA Panel on Contaminants in the Food Chain (CONTAM Panel) considered the main *C. purpurea* alkaloids (ergometrine, ergotamine, ergosine, ergocristine, ergocryptinine, ergocornine and their corresponding -inine epimers) as the most significant. Considering estimates of chronic and acute exposure for various age groups in European countries, a group acute reference dose of 1 µg/kg body weight (b.w.) and a group tolerable daily intake of 0.6 µg/kg b.w. were established. The opinion gives a comprehensive overview of analytical methods. The CONTAM Panel recommended that efforts should continue to collect analytical data on occurrence of ergot alkaloids in relevant food and feed commodities. The alkaloids monitored should include at least the *C. purpurea* compounds listed above, along with monitoring of dihydroergosine and agroclavine from *Claviceps africana* and *Claviceps fusiformis*. The need for commercially available reference standards was reiterated (the most important are now available), and in particular the need for isothe labelled internal standards and certified reference materials, which are not so freely accessible.

The most important analytical contribution over the current period was a comprehensive study of the variability of matrix effects encountered in LC-MS/MS analysis of ergot alkaloids in cereals (Malyshева et al., 2013a). In a systematic investigation of matrix effects, several approaches to the elimination or minimisation of matrix effects were investigated for rye, wheat, triticale, oat and barley. These included variation of ionisation techniques, chromatography and sample preparation for a range of different grain types and varieties. Signal suppression or enhancement could not be eliminated but could be reduced by the use of UHPLC methods and choice of sample preparation procedure. Sample preparation conditions (pH and extraction buffer concentration, shaking time, drying temperature and extraction volumes) did not affect the recoveries of ergot alkaloids significantly, but subsequent clean-up and detection methods did. Matrix effects differed significantly between the grain types with up to 80 to 90% signal suppression of ergometrine in barley and oats, and 50% suppression of ergocryptinine and ergocristinine in oats and barley. There was almost no suppression or enhancement of the later-eluting compounds ergocryptinine and ergocristinine in rye, wheat and triticale. There were also differences in matrix effects between three varieties of rye. These results emphasise the need for a careful and correct choice of blank matrix for the preparation of matrix-matched standards. Sample preparation procedures strongly affected the matrix effects, the highest signal suppression being observed for ergometrine and ergometrinine. Samples cleaned up using MycoSep® multifunctional columns eliminated matrix interferences better than other SPE techniques for the majority of ergot alkaloids, although recovery of ergometrine was poor. Atmospheric pressure chemical ionization gave a tremendous signal enhancement for most of the ergot alkaloids compared to electrospray ionisation (ESI), leading to an overestimation of the levels of ergot alkaloids in some cereal samples. Overall, a method based on liquid-liquid extraction combined with UHPLC-MS/MS in ESI+ mode was recommended.

Malyshева et al. (2013b) also developed a rapid LC-MS/MS method to determine ergot alkaloids in buffered solutions from *in vitro* studies. Analytes were obtained using liquid-liquid extraction under alkaline conditions and determined by LC-MS/MS. Recoveries of the six major alkaloids and their epimers were good (91-123%). The method was applied to a study of the efficacy of a clay-based mycotoxin binder towards ergometrine, ergosine and ergocornine. The samples were adjusted to pH 10 and the ergot alkaloids extracted with ethyl acetate. The extract was evaporated to dryness, reconstituted in a methanol:water:MeCN mixture and analysed by LC-MS/MS in positive ESI mode. Two selected reaction monitoring transitions were selected for each of the analytes. LOQ values between 0.19 to 1.45 µg/l and recoveries above 70% were achieved. The method showed the clay to have a binding efficacy above 93% for the peptide ergot alkaloids, and 24% was observed for ergometrine. Liao et al. (2013) measured six ergot alkaloids as part of a simple and rapid LC-MS/MS method to detect 26 mycotoxins (aflatoxins, ochratoxins, fumonisins, trichothecenes, and ergot alkaloids) in a maize, rice, wheat and three nut products. The analytes were extracted with MeCN:water (85:15, v/v), centrifuged and diluted with water. Separation and identification of epimers was not included in the method. The extraction procedure was optimised for the maximum number of analytes but the acid conditions could have led to degradation of the ergot alkaloids. Recoveries of the ergot alkaloids were typically above 75%, lower in pistachio spiked at 10 µg/kg and higher (>100%) in peanuts. LOQs were above 1 µg/kg.

In a typical clinical application, Nakamichi et al. (2012) measured the postnatal uterotonic drug methylergometrine...
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in human breast milk using solid-phase extraction and HPLC-FLD. The analysis was complete in 8.5 min, performed well and had an LOD of about 50 ng/l.

Two significant alternative approaches have been published. Lenain et al. (2012) gave the first report of the use of a MIP that recognised six ergot alkaloids and their epimers. Metergoline was used as a template for the production of suspended polymerised beads that acted as a selective sorbent in a solid-phase extraction column.

Rouah-Martin et al. (2012) produced the first report of the production and characterisation of aptamers for ergot alkaloids for use in a biosensor. Aptamers for the ergot alkaloids metergoline and lysergamine were produced using the iterative selection procedure SELEX (Systematic Evolution of Ligands by EXponential enrichment). The aptamers were isolated, amplified and sequenced before being characterised by SPR. The selected aptamers had a high sensitivity for lysergamine. A colorimetric reaction could be achieved with an aptamer linked to gold nanoparticles producing a specific colour change in the presence of lysergamine and small ergot alkaloids. The system was considered suitable for development with regard to producing a lateral flow-based dipstick test.

A complete procedure for detecting ergot bodies in cereals based on near-infrared (NIR) hyperspectral imaging was developed by Vermeulen et al. (2013). NIR spectral characteristics were used to build relevant decision rules based on chemometric tools and on the morphological information obtained from the NIR images. The procedure was transferred from a pilot online system to the industrial level. The discrimination between cereals and ergot was dependent on the different fat and starch contents of cereal kernels and ergot bodies. Validation experiments carried out using ergot and wheat, rye, rapeseed, straw, barley straw and cardboard showed the method to be quite stable and repeatable, with good correlation with the reference method based on stereomicroscopy. No false positives were produced. It could detect an ergot body concentration of 0.02%, less than the EC limit for intervention cereals of 0.05%.

Merkel et al. (2012) studied the fate of ergot alkaloids on baking cookies made from contaminated rye flour and also monitored the action of in vitro digestion. Baking of the cookies caused a 2-30% degradation of ergot alkaloids and formation of an increased proportion of (S)-epimers. The digestion model showed a selective increase in the proportion of toxic (R)-epimers in the case of ergotamine-type alkaloids (ergotamine and ergosine). Ergotoxine type alkaloids (ergocornine, alpha- and beta-ergocryptine and ergocristine) were predominantly transformed into their biologically inactive (S)-epimers.

Wakimoto et al. (2013) offered the first report of the isolation of an ergopeptine from a marine creature, the sea slug Pleurobranchus forskalli. An ethanolic extract of the frozen slug was evaporated and partitioned between water and chloroform, and the chloroform-soluble material subjected to flash chromatography on a silica gel using a gradient of methanol (0-20%) in chloroform. The collected fractions were analysed by HPLC with diode array ultra violet (UV) detection at 210 and 311 nm. The major peak was identified as ergosinine by means of both time of flight mass spectrometry in ESI mode and 1H-NMR.

Ergot alkaloid formation pathways via the intermediate chanoclavine-I were studied in the non-toxin-producing Aspergillus nidulans inoculated with fragments of genomic DNA from the ergot alkaloid producer Aspergillus fumigatus (Ryan et al., 2013). New pathway intermediates and an ergot alkaloid-like compound were detected but not identified. Precursors of ergot alkaloids were extracted from A. nidulans colonies by washing with methanol, centrifugation and evaporation, prior to HPLC with fluorescence detection. Ergot alkaloids were determined by LC-MS using positive ESI and detected by scanning for ions with a m/z between 200-400.

8. Fumonisins

Various aspects of fumonisins, including occurrence, toxicity, analysis and chemoprotection of plant extracts were recently reviewed (Waskiewicz et al., 2012; Yang et al., 2012), and sampling plans for maize (Mallmann et al., 2013) and workplace air (Jargot and Melin, 2013) were recently described. The absence of a good chromophore in the fumonisin structure influences the methods used for their detection. Widely used methods for these toxins generally fit into one of three groups: those based upon physical properties, such as mass/charge ratio (i.e. various forms of MS), those based upon derivatisation with more easily detectable labels (such as fluorescence) and those based upon indirect detection. The later usually involve the interaction with selective binding materials, such as antibodies, or more recently synthetic materials, such as aptamers. Research effort in these areas has changed over time and the literature is not evenly distributed among these groups. Over the past several years, there has been a pronounced shift towards the use of LC-MS/MS for fumonisins detection, particularly in the context of multitoxin methods, while the number of literature reports developing new LC-FLD methods has declined. The indirect assays, such as immunoassays and biosensors, continue to be well represented. As with the other technologies, there has been a trend towards multitoxin quantification.

By far the greatest research effort, as reflected in the published literature, has been towards the development of MS-based assays. The large number of papers dictates that,
with few exceptions, they can be only briefly summarised here. Because most of the MS methods have LODs well below current regulatory limits, in general the LOD for these methods will not be provided here. The MS methods fall into two categories: those for which the fumonisins are the sole analytes and those for which fumonisins are one class of analytes among many tested (multi-analyte assays). The distinction is important, because the extraction conditions that are optimal for the fumonisins may not be the same as the extraction conditions that are optimal for multi-analyte assays. Multiple mycotoxin detection using MS was recently reviewed (Capriotti et al., 2012a). In work focused upon fumonisins, Marschik et al. (2013) tested extraction solutions previously described for multimycotoxin LC-MS/MS methods in their own LC-MS/MS method and compared the results to a reference method (EN 14352:2004; CEN, 2004) that combined methanol:MeCN:water (25:25:50, v/v/v) extraction and LC-FLD. Extraction solutions examined included: (a) methanol:MeCN:water (1:1:2); (b) MeCN:water:formic acid (80:19.9:0.1, v/v/v) followed by MeCN:water:formic acid (20:79.9:0.1, v/v/v); (c) MeCN:water (75:25, v/v) containing 50 mM formic acid; (d) MeCN:water:acetic acid (79:20:1, v/v/v); and (e) MeCN:water (80:20, v/v). Overall, the most appropriate extractant for spiked or naturally contaminated maize was methanol:MeCN:water (1:1:2). Other LC-MS/MS methods focused on fumonisins in matrices such as red cargo rice (Tansakul et al., 2012), wheat having black point disease (Busman et al., 2012), culture media and mouldy maize (Falavigna et al., 2012b), and maize and wheat-based products (Bryla et al., 2013). The method described by Falavigna et al. (2012b) also included fumonisins beyond the commonly tested for FB₁, FB₂ and fumonisin B₃ (FB₃), specifically the fumonisin A and C series. The method described by Bryla et al. (2013) is notable because of the use of a MIP-SPE to isolate FB₁, FB₂ and FB₃ from cereal products. The MIP-SPE was a commercial product that showed almost equal selectivity for FB₁, FB₂ and FB₃. For these three fumonisins, the recoveries from spiked samples averaged from 95-106%, with repeatabilities less than 17%.

Most of the published methods for fumonisins involve their inclusion among other analytes in multi-analyte assays. As noted previously, methods for detecting multiple groups of analytes employ either compromise solvent mixtures to extract analytes of widely different polarities, or involve multi-step extractions with different polarity mixtures of solvents. Extraction techniques continue to evolve and recent trends in the use of MSPD for clean-up have been reviewed (Capriotti et al., 2013). Several of the multi-analyte papers use forms of QuEChERS for analysis of various matrices including milk thistle (Arroyo-Manzanares et al., 2013), dietary supplements (Vaclavik et al., 2013), spices (Yogendrarajah et al., 2013) and cereals (Zhang et al., 2013b). The multitoxin assays have generally been applied to multiple commodities or foods. Extraction conditions vary according to the clean-up desired and the detection technique used. Most of the LC-MS/MS methods that include fumonisins extract using mixtures of MeCN:water, which is often acidified (Åberg et al., 2013; Abia et al., 2013; Ates et al., 2013; Liao et al., 2013; Nácher-Mestre et al., 2013; Soleimany et al., 2012; Varga et al., 2013a; Warth et al., 2012a). While most of the multitoxin methods that include fumonisins were developed for cereals or cereal-based feeds, several of them dealt with other commodities such as nuts (Abia et al., 2013; Liao et al., 2013; Varga et al., 2013a), fish feeds (Nácher-Mestre et al., 2013), and wines and beers (Ali-Taher et al., 2013; Pérez-Ortega et al., 2012; Rubert et al., 2013; Tamura et al., 2012). Solfrizzo et al. (2013a) reported results of a proficiency test for a multimycotoxin method that included fumonisins. From the 41 laboratories involved in the performance test, 30 results from spiked maize were reported for FB₁ and 29 results were reported for FB₂. For contaminated maize, 32 results were reported for FB₁ and 31 for FB₂. The percentage with satisfactory z scores (those with an absolute value less than or equal to 2) in spiked maize were: 30% (for FB₁) and 38% (for FB₂). For contaminated maize the results were: 50% (FB₁) and 52% (FB₂). Poor performance (z scores with an absolute value above 3) were seen for 31% (FB₁) and 32% (FB₂) of the results when testing the contaminated maize, and 63% (FB₁) and 52% (FB₂) for spiked maize. Mean recovery results were acceptable for all the toxins tested, except for the fumonisins, which were unacceptably high (159% for FB₁, 163% for FB₂). This suggests that many of the multi-analyte assays are likely compromised when it comes to fumonisin quantification.

As described earlier, fumonisins can also be detected by derivatising them to yield fluorescent products. Methods based upon LC with either pre- or post-column derivatisation are still widely used. Several derivatisation reagents have been previously described and investigations on improving fluorescence detection continue. Recently four reagents were compared for pre-column derivatisation of FB₁, FB₂ and FB₃ and separation by UHPLC (Wang et al., 2013a). The reagents were: o-phthalaldehyde (OPA), naphthalene-2,3-dicarboxaldehyde, dansyl chloride and 6-aminquinolyl-N-hydroxysuccinimidyl carbamate (AQC). From among the four, OPA was selected for development of a method. The LOQs in maize were 25, 14 and 17 µg/kg for FB₁, FB₂ and FB₃, respectively (Wang et al., 2013a). The reagent AQC was also used in a pre-column derivatisation method for measuring FB₁ and FB₂ in Chinese rice wine (Ma et al., 2013b). The AQC derivatives were stable for 5 days and the LOD for FB₁ and FB₂ standards was 6 µg/L. Observed recoveries were in the range of 88-95%. A reference LC-FLD method (EN 16006; CEN, 2011), based upon pre- or post-column derivatisation, was used to determine the extent to which analytical performance was affected by the addition of mycotoxin binders in the formulation of animal feed (Kolosova and Stroka, 2012). Twenty commercial
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The third group of fumonisin assays involve indirect detection mediated by a binding event, such as with antibodies in immunoassays. While antibodies against the fumonisins have existed for many years, improving immunoassays using a variety of formats (ELISAs, LFDs, biosensors) continues to be an active area of research. While sensitive monoclonal antibodies and ELISAs for fumonisins continue to be developed (Sheng et al., 2012), much of the literature now concerns either novel fumonisin binding elements, or multiplexed assays. A novel ELISA based upon a mimotope peptide was reported and applied to cereals (Liu et al., 2013b). The mimotope peptide, which bound to a fumonisin antibody (analogous to FB₁), was attached to bovine serum albumin (BSA) and used as a solid phase antigen for the ELISA (e.g. instead of a FB₁-protein conjugate). The LOD was 1.2 µg/l, with an IC₅₀ of 6.1 µg/l. The assay compared well to a commercial ELISA and an LC method for 32 cereal samples (R² of 0.95 and 0.90 respectively). Interestingly, in competitive assays 1,289 µM of the free peptide (CT-452) was equivalent to 1 µM of FB₁ (Liu et al., 2013b). Fumonisins were among the analytes measured in multymycotoxin assays in formats that included an immunochromatographic strip (Wang et al., 2013), a flow-through sepharose gel (Njumbe Ediage et al., 2012b), a chip-based microarray (Oswald et al., 2013), a fluorescence energy transfer (FRET) aptasensor (Wu et al., 2012a), and several microsphere-based assays. The chip-based microarray was essentially an antigen-immobilised ELISA, conducted on glass slides functionalised with polyethylene glycol diamine (Oswald et al., 2013). A substrate yielding a chemiluminescent product was used for detection. Assays took 19 min. The response to FB₁ was linear from 168 to 2,215 µg/kg, with an IC₅₀ of 645±59 µg/kg. Chips could be regenerated 50 times. Recoveries of 55-80% were observed from spiked oat, wheat, rye and maize-pollen. Another assay format involved FRET between aptamers tagged with fluorescent (donor) unconversion nanoparticles (UCNPs) and a graphene oxide (GO) acceptor (Wu et al., 2012b). This very novel format used aptamers that, in the absence of toxin, bound to GO that quenched the fluorescence of the UCNPs. In the presence of toxin, the aptamer-UCNPs did not bind to the GO and were not quenched. In this fashion, the signal from the aptasensor was proportional to toxin level. The sensor had a linear range of 0.1 to 500 µg/l for FB₁ and an LOD of 0.1 µg/l. Recoveries of FB₁ were between 84-124%. Results from the multiplexed FRET were correlated to those of a commercial ELISA for 15 naturally contaminated samples. Several other formats for multiplexing immunoassays used microspheres of various forms (Czeh et al., 2012; Deng et al., 2013; Peters et al., 2013). The first of these used three different colours of silica photonic crystal microspheres (SPCMs), each coated with a different toxin-BSA conjugate. The immobilised toxin competed with toxin from samples for fluorescein isothiocyanate-labelled antibody. After washing, the SPCMs were applied to a slide surface. The SPCMs were categorised by reflectance spectrometry and the fluorescence (inversely proportional to toxin content) was determined. The LOD for FB₁ was 1 ng/l, with a dynamic range of 0.001 to 10 µg/l. Spiked over the range from 1 to 10,000 pg/g, the recoveries ranged from 87-117% (peanuts), 88-124% (maize) and 79-119% (wheat). Another way that microspheres have been used is in formats based upon flow cytometers, e.g. Luminex (Austin, TX, USA) or similar instruments (Czeh et al., 2012; Peters et al., 2013). One such assay, termed a competitive fluorescent microsphere immunoassay (CFIA), used colour-coded polystyrene microspheres coated with anti-toxin antibodies (Czeh et al., 2012). Competition was between phycocerythrin-labeled (fluorescent) toxin and unlabelled toxin for binding to the microspheres. The multiplex assay was used to detect six groups of mycotoxins, including FB₁, using a single extraction (with MeCN:water, 84:16, v/v). Interested readers are referred to the original manuscript for the recoveries from spiked wheat or peas, as the data were presented in the form of bar graphs rather than tabular form. Another type of microsphere assay used paramagnetic colour-coded beads coated with anti-toxin antibody (Peters et al., 2013). Competition for binding to the microspheres was between phycocerythrin-labelled toxin and unlabelled toxin (i.e. from samples). The beads were isolated and the fluorescence (indirectly proportional to toxin content) was detected. Two Luminex flow cytometers were evaluated and the assays were compared to an LC-MS/MS method. When applied to naturally contaminated wheat, maize or feed, the assay worked well for certain of the mycotoxins, but severely...
overestimated fumonisin content. Despite this fact, it is clear from the aforementioned papers that the detection of fumonisins in the context of multiplexed immunoassays is making significant progress, with several of the assays able to quantify fumonisins in relevant matrices.

Increased interest in fumonisin metabolism and potential hidden or ‘masked’ mycotoxins has lead to several reports of fumonisin biomarkers and masked fumonisins in foods. Application of chromatographic and immunochemical methods for detecting masked mycotoxins were recently reviewed (Cirlini et al., 2012; Goryacheva and DeSaeger, 2012). The masked fumonisins were further subdivided into two categories: ‘hidden’ or ‘bound’ (Falavigna et al., 2012a). The authors of that work suggested the distinction be made based upon whether the attachment was covalent (e.g. bound) or non-covalent (e.g. hidden). The terminology gets entangled because the ‘hidden’ group is described as ‘bound non-covalently’, which is contradictory to the definition. Despite problems with terminology, the importance of detecting such materials is well recognised. Both hidden and bound fumonisins were found to co-occur in thermally processed maize-based products (Falavigna et al., 2012a), suggesting further study is needed to determine the extent to which these are found in commodities and foods. In related topics, the fate of fumonisins following extrusion of maize and biomarkers of fungal contamination were also reviewed (Jackson et al., 2012b; Turner et al., 2012). Several methods were developed to detect fumonisins in biological fluids, such as plasma (Devreese et al., 2012) and urine (Song et al., 2013a; Njumbe Ediage et al., 2012a).

There are also several recent reports of finding fumonisins in commodities in which they were not commonly expected, including pineapples (Stepień et al., 2013), onion bulbs (Varga et al., 2012) and banana beer (Shale et al., 2012).

The methods described in this section have all involved methods requiring extraction of fumonisins for detection. Methods have also been explored that avoid extraction by measuring spectroscopic properties of solids. One such method used data from Fourier transform NIR to develop a regression model with 15 principal components. The model was used to predict $\text{FB}_1 + \text{FB}_2$ content of maize meal (Gasparo et al., 2012). A set of 143 maize meal samples was used to define the principle components. The developed model was then used to predict fumonisin contamination of an additional 25 samples that were also tested by a reference LC-FLD method. Content of the samples as measured by the reference method were correlated with values predicted by the model, suggesting the technology may have value for screening maize meal.

### 9. Ochratoxins

Apparently, LC-FLD with IAC clean-up continued being the most widely used technique in routine analytical activities for the determination of OTA in food and feed along the last year. As an example, in a proficiency test (PT) conducted on OTA in cocoa, 29 out of 36 participants used IAC clean-up and only 2 participants used SPE. 27 out of 31 participants declared the use of LC-FLD with only 2 participants using MS/MS detectors and 2 participants using UV detection (FAPAS, 2012). Nevertheless, some PT organisers (FAPAS) have launched also specific rounds for users of ELISA, what may be an indication about the increasing number of laboratories using this type of screening technique in the routine analysis of OTA.

Regarding procedures based on LC, Llerda et al. (2012) published the results of a collaborative study for the determination of OTA in liquorice and liquorice extracts by LC-FLD with IAC clean-up. The test portion was extracted with a mixture of methanol and aqueous sodium bicarbonate solution; the extract was filtered and diluted with phosphate-buffered saline and purified by IAC. Twenty laboratories, all from different countries, took part in this study. The tested contents of OTA ranged from 7.7 to 141 µg/kg OTA in both types of matrices. Mean recoveries were 87% for liquorice root and 84-88% for liquorice extracts; reproducibility values ranged from 10-17% and from 11-22% in liquorice extracts and root, respectively. A novel approach for microextraction of OTA from cereal matrices was reported by Campone et al. (2012). The procedure used the so-called pH-controlled DLLME, which was based on two successive steps conducted at opposite pH values. One ml of the raw methanolic extract was re-extracted with 400 µl $\text{CCl}_4$ at pH 8 in order to eliminate the hydrophobic matrix interferences. Further, the pH of the aqueous phase was adjusted to pH 2 and the toxin was extracted with 150 µl $\text{C}_4\text{H}_8\text{Br}_2$. The final determination of OTA was accomplished by conventional LC-FLD. The LOQ estimated was 0.06 µg/kg and recoveries were in the range of 81-90%. Among procedures based on LC-MS/MS and dealing specifically with OTA, Duarte et al. (2013) presented a new analytical methodology for determination of OTA in pork. The method used acidified methanol, ultrasound treatment and centrifugation before the IAC clean-up, followed by LC-ESI-MS/MS in positive mode using SRM scanning. The LOQ was estimated 0.2 µg/kg with recoveries varying between 99 and 101%. The validation study was carried out according the guidelines from the European Commission Decision 2002/657/EC (EC, 2002) for this type of products.

Various innovative approaches were developed for the analysis of OTA in wine, specifically. Arroyo-Manzanares et al. (2012) proposed the use of DLLME and capillary LC coupled to laser-induced fluorescence detection allowing...
the quantification of OTA at the level of 0.2 µg/l with an intermediate precision of 4.9% and recoveries >90% when applied to white, rose and red wines. The method was based on the treatment of an aliquot of 5 ml wine by a mixture of chloroform as extraction solvent and MeCN as the disperser solvent. Other feature was the addition of an anionic surfactant (0.2 M SDS) to the isotropic mobile phase for increasing the fluorescence intensity. Mao et al. (2013) reported the quantification of OTA in red wines by conventional LC-FLD using a commercial column packed with 2.6 µm core-shell particles. Core-shell particles are different from the totally porous particles in that they have a solid core surrounded by a thin porous shell. This type of columns can provide similar speed and efficiency while maintaining low back pressure, thus allowing their use on conventional LC instruments. Under optimised conditions, the authors were able to analyse extracts from red wines after IAC clean-up, with recovery rates between 81 and 83% and a retention time for OTA of less than 5 min. Longobardi et al. (2013) described a new method in red wine that used a double extract-clean-up and fluorometric detection. The prepared sample was first cleaned-up by IAC and the eluted extract was further purified through a commercial aminopropyl silica SPE cartridge. The OTA in the final extract was measured fluorometrically and quantified after deconvolution of fluorescence spectra. This mathematical treatment allowed significant recovery improvement by removing the contributions arising from interfering substances eluting with OTA. As an example, the recovery went down from 118 to 95% in samples spiked at the level of 2.0 µg/l. The repeatability values ranged from 4-15% at spiking levels between 0.5 and 3.0 µg/l.

In spite of the availability of commercial MIPs for selective SPE of OTA in foodstuffs, very few articles on its use were published along the last year. Cao et al. (2013) proposed a method based on the use of MIP as an alternative to IAC for the determination of OTA in ginger by UHPLC-FLD. The method was optimised after checking the influence of several parameters, such as the pH value of loading solution, the volume and composition of washing solution and volume of eluting solution: pH 1.0, 5 ml 0.1 M HCl:MeCN (60:40, v/v) and 2 ml of methanol:acetic acid (98:2, v/v) respectively, were found the best conditions. The recoveries ranged from 88-95% in ginger spiked at 5, 15 and 25 µg/kg with an inter-day precision ranging from 2.2-3.7%. More interferences were observed in chromatograms after clean-up with MIP compared to IAC, although they had no adverse effect on the separation of OTA. It is worth mentioning, that MIP columns were found to be reusable up to forty-one consecutive times after a single wash step with 10 ml methanol.

The use of QuEChERS methods for the extraction of mycotoxins is starting to appear in an increasing number of articles in the scientific literature. Paiga et al. (2012) investigated the possibility to apply QuEChERS extraction for OTA determination in bread samples by checking the performance of a number of different types of QuEChERS. After establishing the optimal parameters in terms of extraction time, volume of extraction solvent and sample mass, the authors concluded that salt mixtures from three different suppliers were adequate for OTA extraction from bread samples. The use of that type of QuEChERS for the analysis of OTA by LC-FLD in bread samples obtained recoveries ranging from 95-97%, repeatabilities ranging between 1.0 and 10% with an LOQ at the level of 0.05 µg/kg. Sirhan et al. (2012) also described the use of the QuEChERS method combined with LC-FLD in the analysis for OTA in cereal and cereal products with recoveries >85% and a chromatographic separation of the toxin performed within 4 min by using a reversed-phase C18 column at 30 ºC under isocratic conditions. Lorent-Martinez et al. (2013) reported the use of this extraction approach in a sequential injection analysis coupled to two different flow-through opsensors for comparison between them. One of the sensors was based on fluorescence detection whereas the other one used terbium-sensitised luminescence (TSL) detection. The instrumentation was formed by an automatic analyser unit in line with a luminescence spectrometer. In each case, the flow-cell was filled with the appropriate solid support: Sephadex QAE A-25 (GE Healthcare, Little Chalfont, UK) in the fluorescence detection and C18 silica gel in the TSL sensor. Taking into account the better sensitivity and sample throughput, the TSL system was chosen to perform the analysis of some cereals and pet feed with recoveries in the 86-112% range at spiking levels around 250 µg/kg.

Some attention also deserves the use of immunochemical methods for OTA detection. Anfossi et al. (2013) adapted a lateral flow immunoassay previously developed for OTA detection in cereals for measuring OTA in wine and grape must (Anfossi et al., 2011). Red wines and grape juice needed special treatment in order to avoid the matrix interference with red-purple lines arisen from gold nanoparticles used in most common LFDs. Wines were diluted 1:2:2 with NaHCO₃ (0.15 M, pH 9.0) and a water solution of PEG (4%, w/v) and pipetted into the sample well. Grapes musts were treated in the same way, except for the fact that 12% (v/v) of absolute ethanol was added before dilution. Optimal concentration of reagents for the test and control lines and gold nanoparticles-labelled antibodies for detection was redefined to achieve the best sensitivity. The LOD of the assay was 1.0 µg/l. Despite that intra- and inter-day precision were found to be high comprising between 20 and 40%, the authors proposed this method for the reliable first level monitoring of OTA. Lippolis et al. (2013) gave an account of the development of a fluorescence polarisation immunoassay applicable to the determination of OTA in wheat samples. The authors described the synthesis of a fluorescent-labelled OTA tracer and studied its binding response with three monoclonal antibodies tested, showing
an IC50 value of 0.5 µg/l in the most sensitive case with negligible cross-reactivity for ochratoxin B (1.7%) and no cross-reactivity for other mycotoxins that may be frequent in wheat. Samples were extracted and purified by SPE using an aminopropyl column prior to the assay. The authors claimed an overall time of analysis of 20 min, the LOQ was estimated at 2.0 µg/kg with average recoveries of 87% in spiked samples (3 to 10 µg/kg) and RSDs lower than 6%.

Also aptamers have been used by some research groups. Thus, Wu et al. (2012a) reported the development of a single-strand aptamer functionalised electrochemical aptasensor able to detect the presence of OTA at levels of less than 0.01 ng/l. The preparation of the electrochemical aptasensor was described after optimisation of some key parameters, such as the density of the aptamer on the surface of the electrode, the reaction time between the aptamer and OTA, and the effect of metal ions including Mg2+ and Ca2+.

The aptasensor was also applied for detection of OTA in wine samples spiked at different levels (1 to 950 ng/l) and dilution with phosphate buffered saline as the only sample pre-treatment. Recoveries were always >94% and results compared well with those obtained by LC analysis conducted on the same samples. Ma et al. (2013c) also described the development of another ultrasensitive aptasensor specific to OTA allowing its detection at femtogram measurable levels. The whole procedure was accomplished in a single PCR tube. A single-strand OTA-specific biotin-labelled aptamer and its complementary DNA fragment were incubated forming a double-strand DNA immobilised on the surface of a previously streptavidin-coated PCR tube. In the presence of OTA, the aptamer had a conformational change induced by the target binding and the single-stranded DNA complementary to the aptamer was released. The OTA concentration was measured by the change in the amplification signal generated in real-time quantitative PCR. The assay proved to be highly specific when replacing OTA by four mycotoxins (ZEA, DON, AFB1, and FB1) as target molecules, which were not recognised by the biotin-labelled aptamer. The application of this assay to the analysis of red wines required just a simple pre-treatment to remove the wine sediments; recoveries in the range of 99-112% were obtained at seven spiking levels ranging from $5 \times 10^{-6}$ to 5 µg/l, demonstrating the potential to the analysis of trace amounts of the toxin. Hun et al. (2013) reported a method based on the combination of cleavage of nicking endonuclease with target-induced strand release. The OTA aptamer and OTA aptamer complementary oligonucleotide were loaded onto a magnetic bead. When OTA was present, the aptamer preferentially bound it and caused the release of some strands from complementary oligonucleotide that acted as the primer for a subsequent polymerisation and nicking endonuclease scission process, producing a large amount of short single-stranded linker oligonucleotides. A chemoluminescence nanoparticle probe previously prepared was captured by hybridisation with the linking oligonucleotide and then measured after being separated with an external magnetic field. The method was applied to the determination of OTA in wheat samples spiked at 1.0, 3.7 and 9.2 µg/kg with recoveries >97% and RSDs <4.8%. Another innovative approach was followed by Zhang et al. (2013a) who incorporated the use of Tb3+ and magnetic beads in the aptasensor. Some biotinylated anti-OTA aptamer was captured onto streptavidin-coated magnetic beads and two single-stranded complementary oligonucleotides were added in order to allow their hybridisation to the aptamer. The resulting magnetic beads were magnetically separated and the resulting supernatant liquid contained no single-stranded oligonucleotides and could not increase the emission of Tb3+ in the absence of OTA. In the presence of OTA, the OTA-aptamer binding was produced while releasing the single-stranded oligonucleotides, which finally were present in the supernatant and could enhance the fluorescence intensity of Tb3+. This aptasensor was used for determination of OTA in spiked wheat samples in order to evaluate its practical applicability and results were found to be consistent with those obtained with ELISA.

As a final remark, a new call for tenders for project leaders for the development of standardised methods for the analysis of mycotoxins in food (Mandate M/520) was issued very recently by the European Committee for Standardization (CEN, 2013) in the framework of the Regulation (EC) 882/2004 (EC, 2004). The execution of the mandate was divided into eleven projects. Two of them dealt with OTA, namely: determination of OTA in liquorice and spices (for which an EU ML has been established) and in cocoa and cocoa products and determination of OTA in meat, meat products and edible offal. In both cases, the methods need to be interlaboratory validated, in accordance with ISO 5725 (ISO, 1994) or with AOAC International guidelines. The techniques to be used could be either fluorimetric or MS detection. In principle, it was recognised that there were methods based on LC-FLD, which could serve as good basic methods, suitable for interlaboratory validation of the missing matrices, i.e. OTA in chilli and paprika, and OTA in pig and poultry serum and blood derivatives. Those two projects may be considered as an indication about what is nowadays demanded to analytically support the food legislation.

10. Patulin

New and improved methods for PAT determination using a range of analytical techniques continue to be published. Xiao and Fu (2012) developed and validated a GC-MS method for the detection of PAT in apple juice. The method used NaHCO3 during LLE and acetic acid into the organic extract with 3-nitrobenzyl alcohol as internal standard. The LOD and LOQ were 2 and 5 µg/l, respectively. The extraction efficiency was 86% with an RSD of 14%; for
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Güray et al. (2013a) developed and validated a method for determining PAT in apple juice using capillary zone electrophoresis. The run buffer was 25 mM of sodium tetraborate and 10% MeCN at pH 10. The optimal conditions included an applied voltage of 25 kV (normal polarity), a temperature of 25°C and an injection time of 10 s at 50 mbar. The signal of PAT and phenobarbital was used to derive PAT prior to GC-MS in order to increase the sensitivity of the method. The recovery ranged from 80-88% with an LOD of 0.4 µg/l and an LOQ of 1.3 µg/l, with an RSD lower than 9.5%.

Song et al. (2013b) reported a method for the simultaneous determination of bisphenol A, AFB1, OTA and PAT in food matrices by LC-MS/MS. Analytes were extracted from food matrices, such as cereal, peanut butter, cereal-based baby formula and fruit juices, and were enriched by SPE before LC-MS analysis using negative ESI with selected reaction monitoring and matrix-matched external calibration for quantitation. The LOQs for PAT were 1 µg/l in juices and 0.8-1.5 µg/kg in solid matrices, while the recoveries were 95-127%. Malyshova et al. (2012) demonstrated the formation and use of a methanol adduct of PAT in ESI+. A study of the fragmentation pathway confirmed the authenticity of the PAT adduct, while the use of an ion trap and high resolution Orbitrap mass spectrometry allowed reliable assignment of the PAT fragment ions. Exploiting the formation of the methanol adduct, PAT has been successfully included in a single run multymycotoxin LC-MS/MS method in support of ex vivo-in vitro biomedical studies.

Chanique et al. (2013) developed a method based on square wave voltammetry for the quantitative determination of PAT in both fresh and commercial apple juices in the presence of 5-hydroxymethylfurfural. The electro-reduction of PAT and 5-hydroxymethylfurfural at glassy carbon electrodes in MeCN, tetrabutylammonium perchlorate, with and without different aliquots of trifluoroacetic acid, was also reported. The electrochemical techniques were cyclic and square wave voltammetry and controlled potential bulk electrolysis. Ultraviolet, visible and infrared spectroscopy was used to identify PAT electro-reduction products. A binary solvents-based DLLME method was developed by Maham et al. (2013) for the determination of PAT in apple juice followed by LC quantification. The method involved the use of an appropriate solvent to form fine droplets of extractant in a sample solution. The authors optimised the parameters that affect the extraction efficiency: (1) the type and volume of high-density extraction solvent; (2) the volume of ethyl acetate; (3) the kind and volume of disperser solvent; and (4) salt addition. The LOD and LOQ were 2.0 and 10.0 µg/l, respectively. The RSD for five measurements of 25 µg/l of PAT was 3.8% and the relative recoveries at spiking levels of 25, 50 and 75 µg/l ranged from 91-95%. Shirasawa et al. (2013) used a cyclodextrin-based polymer for clean-up of PAT from apple juice and subsequent HPLC-UV analysis. After optimising the procedure, recoveries of the method were 67-78% with RSDs of 3-20%, while the LOQ was 10 µg/l.

There were several surveys of PAT in food published this past year from different countries. In Italy, Pattonoa et al. (2013) reported a survey of the presence of PAT and OTA in traditional hand-made, semi-hard cheeses. OTA was present in both the rind and the inner part of the cheeses, while PAT was mainly detected in the rind and the inner part of only one cheese sample. The observed PAT concentration ranged from 15 to 460 µg/kg, while the observed OTA concentration ranged from 1 to 262 µg/kg in the rind and from 18 to 146 µg/kg in the cheese interior. In Tunisia, a total of 85 apple juice, baby food and mixed juice samples collected during 2011 from major supermarkets and stores located in Tunisia were analysed (Zaied et al., 2013). Results showed that the incidence of PAT contamination was 35%. The levels of contamination determined in the total samples ranged to 167 µg/l with a mean value of 20 µg/l and a median of 13 µg/l. 18% of the total juice samples (apple juices and mixed juices) and 28% of the baby food samples exceeded the EU maximum levels, which are 50 and 10 µg/l, respectively. A study of the occurrence of PAT in organic and conventional apple-based food marketed in Catalonia and an exposure assessment was reported (Piqué et al., 2013). A total of 93 apple-based products marketed in Catalonia were analysed, 49 of which were derived from conventional farming and 44 from organic farming. The results showed a higher incidence of positive samples and a higher concentration of PAT in organic apple purees as compared with conventional ones. In the case of juices, significant differences were found between conventional and organic samples, but applying a multivariate analysis the type of agriculture did not seem to have a relevant contribution to PAT occurrence, cloudiness being the main factor involved.
11. Trichotheecenes

In the past year, there have been a significant number of publications about trichotheecenes, with the focus increasingly on LC-MS/(MS) methods. In March 2013, the European Commission published Recommendation 2013/165/EU about T-2 and HT-2. This requested member states to collect data on T-2 and HT-2 occurrence, as well as co-occurrence with other Fusarium toxins where possible, including the masked forms of these toxins, in particular the mono and di-glycosides of T-2 and HT-2 (EC, 2013).

There was little novel in the way of HPLC methods for DON. However, two papers were of note, as both could detect DON with other trichotheecenes without using MS. An HPLC method with UV detection was used to separate and quantify DON, 15-acetylDON (15-ADON) and 3-acetylDON (3-ADON) in cereals. The analytes were separated on a reversed-phase C18 column using gradient elution and detection at 224 nm. 15- and 3-ADON showed effective baseline separation. The method gave good linearity and excellent precision for inter- and intra-day determinations. Average recovery rates for the tested matrices ranged from 71-92%. The LODs and LOQs ranged from 16 to 25 μg/kg and 48 to 60 μg/kg, respectively (Yang et al., 2013). In addition, Muscarella et al. (2012) reported an analytical method for the quantitative determination of DON and NIV in cereals. The method, based on HPLC-FLD, used automated 2 channel post-column derivatisation. Separation was achieved using a C18 column with isocratic elution, with FLD using excitation and emission wavelengths of 360 nm and 470 nm, respectively. Complete separation of DON and NIV was obtained in less than 20 min. LODs were as low as 14 μg/kg. The method was validated and the analytical performances of linearity, selectivity, precision and recovery were determined. An interlaboratory study to evaluate LC methods with UV or MS detection for simultaneous analysis of DON and NIV in wheat was conducted (Aoyama et al., 2012). DON and NIV were purified using a multifunctional column and the concentrations determined using LC-UV or LC-MS/(MS). No internal standards were used. Three fortified wheat samples (0.1, 0.5 and 1 mg/kg), one naturally contaminated wheat sample and one blank wheat sample were used. Eleven laboratories produced recovery data in the range from 90-110% for DON and from 76-83% for NIV. Regardless of sample and detector, the HorRat values for DON and NIV ranged from 0.4 to 1.4. The study demonstrated that both LC-UV and LC-MS/(MS) methods can be considered suitable for use as official methods.

Very few papers using GC were published, with little in terms of novel developments. A method of note for the simultaneous determination of trichotheccene mycotoxins T-2, HT-2, DON and NIV in grain and mixed feed by gas-liquid chromatography with ECD was reported (Amelin et al., 2013a). The mycotoxins were extracted from the samples with MeCN and the extracts were cleaned up using the QuEChERS method. A novel approach was to produce derivatives of the target components with trifluoracetic anhydride (TFAA) simultaneously with additional purification and pre-concentration of the extract by DLLME. This led to a much reduced analysis time of less than 1 h, giving a major time efficiency saving for this analysis.

Rapid test kits for T-2 and HT-2 and DON have been evaluated (Aamot et al., 2012, 2013). The studies were carried out to evaluate rapid test kits performance and applicability. For HT-2 and T-2, the combined toxins were analysed in naturally contaminated oat samples using three test kits: two ELISA kits and a LFD. Mycotoxin analysis by LC-MS/MS was used as a reference method. The LFD offered the best reliability, with the greatest agreement with the reference method. In addition, it also showed cross-reaction of 100% with both T-2 and HT-2, whereas for the ELISA kits, cross-reactions were below 100% with HT-2. Similarly in the DON study, oats and wheat were tested by three rapid kits. Again, the LFD showed the best reliability, achieving detection that was stable across toxin levels in both matrices, while the ELISA method achieved the highest accuracy at a DON level of 1,750 μg/kg in oats. For both toxins, it was noted that ELISA is more applicable for laboratory use as it allows multiple samples to be tested, while the LFD formats are well-suited for on-site detection. Goryacheva and De Saeger (2012) reviewed recent literature on possible applications of antibody-based techniques for masked mycotoxins. They concluded that for many immunochemical methods cross-reactivity of the antibodies to masked mycotoxins is unknown or poorly characterised. This can lead to overestimation of target mycotoxins, however, the methods may be able to measure the sum of the target compounds with co-existing analogues, which can be useful for risk assessment studies. However, they did also note that progress in immunochemical methods for masked mycotoxins is hindered due to a lack of analytical standards.

Two papers that could help improve analysis focussed on different aspects of method performance. Köppen et al. (2013) described the preparation and certification of a reference material for Fusarium mycotoxins in wheat flour. The material is the first to be certified for the determination of DON and NIV as well as ZEA. The certified values and corresponding expanded uncertainties were assigned in compliance with the requirements of ISO Guide 35 (ISO, 2006). The material should be of great benefit for internal quality control in addition to facilitating the validation of new analytical methods. Asam and Rychlik (2012) reviewed the synthetic routes to stable isotope-labelled 3-ADON and 15-ADON that can be used as internal standards in SIDAs. They concluded that the benefits of SIDAs in terms...
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of precision and reliability are great making the effort to produce labelled standards worthwhile.

The bulk of analytical method papers have focussed on the use of LC-MS/(MS), with increasing numbers of authors reporting the use of HRMS or Orbitrap technologies. The use of LC-MS/(MS) lends itself to the detection of many different types of mycotoxins in one analysis, which has resulted in many papers being published as multitoxin methods. These are reviewed in their own section. However, many authors report methods for Fusarium toxins as a group or include ZEA with trichothecenes due to their common co-occurrence. Therefore, some of the papers here also include ZEA due to their relevance to this review. The use of HRMS methods has also led to another increasing trend, which is the rise in the number of papers reporting methods for masked mycotoxins, including some novel masked forms that were previously unreported. A comparison was made between IAC clean-up and no clean-up for animal feed analysis by LC-MS/MS. It was reported that, while IAC improved repeatability and reduced interference, there was little difference in quantification. Nonetheless, the authors concluded that analysis without clean-up is suitable for screening, but for regulatory purposes the use of IAC is recommended (Senyuva et al., 2012). An LC-MS/MS method was developed and validated for the simultaneous determination of DON, ZEA, T-2, HT-2 and metabolites, including 3-ADON, 15-ADON, DON-3G, α-ZOL, β-ZOL, ZEA-14-glucoside (ZEA-14G), α-ZOL-14-glucoside, β-ZOL-14-glucoside and ZEA-14-sulphate in maize, wheat, oats, cornflakes and bread (De Boevre et al., 2012). A sample clean-up procedure was not included because of the low recoveries of free and masked mycotoxins and their differences in polarity. The method was validated for several parameters such as linearity, apparent recovery, LOD, LOQ, precision, expanded measurement uncertainty and specificity. The LODs varied from 5 to 13 μg/kg, and the LOQs from 10 to 26 μg/kg. The method was subsequently applied to collect information on human exposure to these mycotoxins in Belgium (De Boevre et al., 2013).

There were many publications reporting the use of LC-MS/MS for the analysis of various trichothecene mycotoxins in a variety of cereal matrices. Many authors applied the methods to obtain large amounts of occurrence data. Malachova et al. (2012) reported the first triple quadrupole LC-MS/MS method for the determination of DON, DON-3G and 3-ADON in beer. The method was applied to conduct a survey of 374 samples of beer. While DON and DON-3G were detected at a high frequency, concentrations were generally low and it was concluded that beer is not a major risk factor to moderate beer drinkers (Varga et al., 2013b). Dall’Asta et al. (2013) reported a method for DON and DON-3G in durum wheat that was used to analyse 150 samples from Italy. A high incidence of DON-3G was recorded, with 85% of the analysed samples containing this mycotoxin at levels up to 842 μg/kg. Yoshinari et al. (2012) reported a method based on LC-MS/MS for the identification of DON, DON-3G, 3-ADON and 15-ADON. Comparison of five multifunction columns revealed that DON and its acetylated derivatives were recovered well (96-120%) by all columns, but DON-3G was recovered adequately (94%) by only one column, InertSep VRA-3 (GL Sciences, Torrance, CA, USA). Samples of maize grits and maize flour were analysed. DON, DON-3G and 15-ADON were the major contaminants in the samples harvested in 2009, but only DON was detected in the samples harvested in 2010. A sensitive UHPLC-MS/MS method for type A and type B trichothecenes in rice with LOQs from 0.5 to 10 μg/kg was described (Wang et al., 2012a). The method used Oasis HLB cartridges for clean-up and gave recoveries of 71-103%. A new LC-MS/MS method for the simultaneous analysis of T-2 and HT-2 in cereals was reported by Tölgyesi and Kunsági (2013). Extracted samples were cleaned-up on Strata-XL solid-phase extraction cartridges and analysed with LC-MS/MS equipped with multimode ion source. The key point of note was that the HPLC separation was tested with both fully porous (Gemini C18; Phenomenex, Torrance, CA, USA) and core-shell type (KineteX XB C18; Phenomenex) columns, the core-shell column gave enhanced peak shapes for both toxins.

A novel water-based slow injection ultrasound-assisted emulsification microextraction (SI-USAEME) method followed by UHPLC-MS/MS analysis was developed for the rapid pre-treatment and determination of DON and its metabolite DOM-1, in maize and pork samples. A study comparing IAC clean-up, SPE clean-up and the developed SI-USAEME method was presented. In addition to providing comparable concentration and purification efficiency to the IAC and SPE methods, the SI-USAEME method was simple and considerably lower in cost than the IAC method, demonstrating that it could become a simple, low-cost alternative method. The method was successfully applied to the analysis of commercial maize and pork products (Cui et al., 2013).

In addition to foodstuffs, LC-MS/MS methods have been developed to analyse trichothecenes and their metabolites in biological systems. An assessment of DON exposure using an LC-MS/MS biomarker method was reported by Warth et al. (2012b). The method was used for a pilot survey to investigate the level of DON exposure in Austrian adults by measurement of DON and its glucuronide conjugates, as biomarkers of exposure, in first morning urine. DON-GlcAs were directly quantified by LC-MS/MS and the results were compared with indirect quantification after enzymatic hydrolysis that confirmed the suitability of the direct method. The in vivo metabolism of DON in humans was studied and two closely eluting DON-GlcAs in naturally contaminated urine samples were determined for the first time. In contrast to previous findings, DON-15-glucuronide
was tentatively identified as a major DON metabolite in human urine based on the analysis of these samples.

The use of HRMS has increased, as the technique offers many advantages, not least the ability to analyse for both known compounds for which standards are not available, but also to identify unknown compounds. The method was used to good effect to identify masked mycotoxins derived from type A trichothecenes. These novel glucosides were identified as T-2-glucoside and HT-2-glucoside on the basis of accurate mass measurements of characteristic ions and fragmentation patterns using LC-HRMS (Orbitrap) analysis. Although their absolute structures were not clarified, 3-OH-glucosylation appeared to be the most probable route for T-2 (Nakagawa et al., 2012). In addition, the existence of a di-glucosylated derivative of T-2 in plant (maize powder) was confirmed for the first time in addition to that of HT-2. These masked mycotoxins were identified as T-2-di-glucoside and HT-2-di-glucoside based on accurate mass measurements of characteristic ions and fragmentation patterns using LC-Orbitrap MS analysis. In addition, 15-monoacetoxy-scirpenol-glucoside was also detected in the same sample (Nakagawa et al., 2013). Veprikova et al. (2012) also reported a new analytical procedure for monitoring T-2/HT-2 conjugates in cereal samples. They used dedicated immunofinity cartridges for the selective isolation and pre-concentration of the target conjugated analytes. The samples were analysed by UHPLC-HRMS that allowed the confirmation of the presence of conjugated T-2 and HT-2. In addition to monoglycosylated forms of T-2 and HT-2 detected in naturally contaminated barley, wheat and oats, diglucosides of HT-2 were found in barley. Suman et al. (2013) reported the development of an LC-linear ion trap-MS method capable of determining DON-3G in different processed cereal-derived products. Chromatographic separation was performed using a core-shell C18 column. An LC-HRMS method was used to carry out untargeted screening for the detection of biotransformation products of DON in plants using stable isotopic labelling (Kluger et al., 2013). Flowering ears of wheat were treated with a mixture of labelled and non-labelled DON. In addition to DON and DON-3G, eight further DON bio-transformation products were found by the untargeted screening approach, including DON-glutathione conjugate, which was reported in wheat for the first time. This demonstrates what a powerful tool the LC-HRMS approach can be, particularly in metabolomics studies, and no doubt the area of untargeted analysis will continue to develop in the mycotoxin field.

The final example of the usefulness of LC-HRMS was reported by De Dominicis et al. (2012). A fast method for extracting and purifying bakery matrices for analytes including pesticides, aflatoxins, trichothecene toxins and veterinary drugs was developed using UHPLC coupled to a Orbitrap Exactive HRMS. Method performance showed that accurate mass targeted screening using current single-stage HRMS analytical instrumentation can be used for screening for multiple classes of contaminants in both bakery raw materials and finished products.

12. Zearalenone

Already the majority of last year’s publications describe the concurrent detection and quantification of ZEA in food and feed among other mycotoxins by LC-MS(/MS)-based multitoxin methods, which are covered in another section of this review. Still, dedicated chromatographic methods for ZEA and its metabolites are continuously developed. For instance, Rodriguez-Carrasco et al. (2012) developed a method for the determination of ten mycotoxins including ZEA in wheat semolina using a QuEChERS-based purification and GC-MS measurement. Derivatisation was performed with a mixture of N,O-bis(trimethylsilyl)acetamide, trimethylchlorosilane and N-trimethylsilylimidazole to silylate all hydroxyl groups. For ZEA, recoveries of 60-67% have been gained. The intra- and inter-day precision was at around 10 and 20% RSD, which is still very much acceptable despite a rather poor linearity of the calibration ($r^2=0.93$). An LOQ of around 10 μg/kg was calculated in wheat semolina. A similar method was developed for the determination of OTA, T-2, DON and ZEA using GC-ECD (Amelin et al., 2013b). This method is applicable to various grains, feed mixes and also meat. Again, mycotoxins were extracted with MeCN and purified by QuEChERS. Trifluoroacetic anhydride was used as derivatising agent, introducing highly electronegative fluorine atoms into the analytes. RSDs below 8% were gained and the LOQ for ZEA was around 50 μg/kg. Capillary zone electrophoresis coupled with UV detection was developed and used to determine ZEA in cereals and feed (Güray et al., 2013b). A sodium tetraborate buffer with MeCN was used and 15 kV of potential difference was applied to the capillary. Phenobarbital was found to be a suitable internal standard and increased the repeatability of the overall method. LOQs of 25 μg/l have been achieved in solution. HPLC-FLD was used to determine ZEA in beer after DLLME using chloroform as extraction solvent and MeCN as dispersive solvent (Antep and Merdivan, 2012). This way, ZEA could be enriched by a factor of 40 while the extraction recovery was 83%. Sub-μg/kg LOQs were achieved for the quantification of ZEA in beer, which was detected in 11 out of 13 tested samples. Another HPLC-UV-FLD method for the determination of DON and ZEA has been developed (Sebæi et al., 2012). Cereal samples were extracted with water and MeCN and the extracts were purified by QuEChERS and in the case of DON additionally by SPE. At a spiking level of 100 μg/kg, 85, 93 and 91% of ZEA was recovered in maize, rice and wheat, respectively. RSDs of 7% were calculated, while LOQs of 20 μg/kg were determined for all tested matrices.
An alternative to the determination of ZEA in food and feed is the use of LC-MS-based biomarker methods to quantify ZEA or its metabolites in biological fluids like urine or plasma. In the last years, a variety of such methods has been developed and published. For instance, an LC-MS/MS method for the quantification of seven mycotoxins (and their metabolites), including ZEA, α-ZOL, β-ZOL and ZEA-GlcA, in human urine was developed (Njumbe Ediage et al., 2012a). Samples were extracted with acidified ethyl acetate. This extract was combined with the acidified aqueous fraction after SAX-SPE clean-up before LC-MS/MS measurement in positive electro spray mode. LOQs of around 2 µg/l (or 7 µg/l for ZEA-GlcA) could be reached, with intra-day precision of 8-13%. ZEA was detected in 4 out of 40 analysed samples. Three samples were contaminated with β-ZOL, while no α-ZOL was detected. In a follow-up paper by the same group, a method for the detection of 6 mycotoxins and their metabolites (ignoring potential glucuronidation) in pig urine was developed (Song et al., 2013a). A salting-out assisted LLE procedure was used for sample preparation before LC-MS/MS analysis. Therefore, urine was treated with MgSO4 and consecutively extracted with ethyl acetate and MeCN. Compared to dilute-and-shoot techniques, the used extraction technique resulted in less matrix effects and better method sensitivity. LOQs of around 1 µg/l were achieved for both pig and human urine for ZEA, α-ZOL and β-ZOL. Recoveries reached from 75-121% depending on the analyte, matrix and spiking level, while the intra-day RSDs were below 18%. Matrix-matched calibration was used for quantification. Another LC-MS/MS method was developed for the determination of ZEA, α-ZOL, β-ZOL, zearalanone (ZAN), α-ZAL and β-ZAL in bovine and porcine urine (Matraszek-Zuchowska et al., 2013). After gluconidase treatment, LLE and two SPE stages, samples were injected into an LC-MS/MS system, operating in negative electrospray mode. Recoveries ranged from 76-116% with repeatabilities below 20%. As LODs were unrealistically low, detection capabilities were calculated being 0.3 µg/l for ZEA and even lower for all other analytes. Nine hormones, including ZEA, α-ZAL and β-ZAL can be detected with a newly developed method using a UHPLC-quadrupole-orbitrap hybrid system (Kumar et al., 2013). Urine was treated with β-glucuronidase and subjected to SPE and LLE. Extraction recoveries for the three ZEA metabolites ranged from 79-83% and detection capabilities from 0.5-0.8 µg/l. At or above 2 µg/l, repeatabilities below 9% were obtained. Animal blood plasma is also a suitable matrix for ZEA biomarker methods. De Baere et al. (2012) developed two LC-MS/MS-based methods to quantify ZEA, α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL in chicken and pig plasma using an LC-ESI-QqQ and a UHPLC-ESI-Orbitrap MS. After protein precipitation with MeCN, urine samples were injected and separated within 10 min. For quantification, matrix-matched calibration was applied. Recoveries ranged from 82-96% on the QqQ and from 70-117% on the Orbitrap, and excellent LODs below 0.1 µg/l for all analytes were achieved on both instruments. Repeatabilities were in the range of 3-22% on the QqQ and from 1-15% on the Orbitrap. Besides ZEA, α-ZOL and β-ZOL, ZEA-GlcA was also detected in pig urine using the HRMS. The same group also published a multibiomarker method that was very similar in setup (and performance regarding ZEA and its metabolites) to the previous method, but also contained DON, T-2, HT-2, OTA, FB1 and AFB1 (Devreese et al., 2012).

Besides chromatographic methods, also a wealth of immunoanalytical methods were developed and employed for the determination of ZEA in cereals and other matrices. For instance, a group-specific monoclonal antibody against ZEA was produced and applied in two direct competitive ELISA systems (Cha et al., 2012). Cross-reactivities ranged from 101-152% for α-ZOL, β-ZOL, α-ZAL and β-ZAL, rendering the system suitable for the concurrent quantification of ZEA and its metabolites. LODs of 10 µg/kg could be achieved with recoveries of 106-123% and RSDs below 10%. Another ZEA ELISA was developed by Pei et al. (2013). Their raised antibody showed cross-reactivities of 24% for β-ZOL and 189% for α-ZOL in the assay, recovery rates of 80-128% from spiked maize samples, and an LOQ of 0.5 µg/kg. Wang et al. (2013g) developed a chemiluminescence immunoassay to detect ZEA in food using biotinylated ZEA conjugates and gold nanoparticles labelled with streptavidin-horseradish peroxidase for signal amplification. Low cross-reactivities below 32% were shown for ZEA metabolites. Including extraction and dilution, the linear working range was 0.4-10 µg/kg. Very good recoveries (97-117%) and repeatabilities (<10%) were achieved. The same group also developed an electrochemical ELISA for ZEA determination in grains (Wang et al., 2013h). Here, the enzymatic conversion of the substrate is detected by a microplate reader and the signal subsequently converted into an electric signal. The detection range of this immunoassay is wide (0.1-240 µg/kg) and the test showed excellent recoveries (92-113%) and RSDs (<8%). Quantum dot-based rapid tests for ZEA determination have been developed (Beloglazova et al., 2012). CdSe/ZnS core/shell quantum dots were used as the label in a fluorescent-labelled immunosorbent assay. The application of this label resulted in better IC50 values and LOQs (about 1 µg/kg) compared with classical ELISA, but in a small linear range (factor of 5). A non-enzymatic electrochemical biosensor for ZEA determination was developed (Feng et al., 2013). The biosensor was prepared by immobilising nitrogen-doped graphene sheets capturing the primary antibodies on a glassy carbon electrode. Extracted ZEA binds to the primary antibodies and is further captured by secondary antibodies, labelled with nanoporous bimetallic PtCo alloy, which can reduce H2O2. A linear range of 0.005-25 µg/l was achieved for standard solutions of ZEA. A suspension array for quantifying ZEA, AFB1, DON and T-2 in maize and...
peanuts was introduced (Wang et al., 2013c). Using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, mycotoxin antigens were attached to microspheres. Mycotoxins were then indirectly competitively quantified. Linear working ranges of about three orders of magnitude and sub-µg/kg LOQs were achieved for both matrices. 80-116% of ZEA was recovered in the two matrices, with intra-assay RSDs <15%.

Easy-to-use LFDs become more and more popular for mycotoxin detection and several ones have been developed for the determination of ZEA along with other mycotoxins. Within the last 12 months alone, test strips for the quantification of ZEA and DON (Huang et al., 2012), ZEA and FB₁ (Wang et al., 2013f), ZEA, OTA and AFB₁ (Li et al., 2013c), ZEA, DON, OTA and AFB₁ (Njumbe Eđiage et al., 2012b) as well as ZEA, T-2, HT-2, DON and fumonisins (Lattanzio et al., 2013) were developed. All these tests are suited for the analysis of various cereals and the cut-off levels for ZEA were in the range of 10-175 µg/kg.

The application of aptamers instead of antibodies also gains attention in mycotoxin determination. Chen et al. (2013) isolated and identified single-stranded DNA aptamers recognising ZEA. After repeated selection, 12 representative sequences were assayed for their affinity and specificity towards ZEA. The best aptamer was then applied in the specific detection of ZEA in real samples. The method recovered 85-105% of spiked ZEA in beer and showed a linear range of 4 orders of magnitude from 1 to 10 mg/l.

Finally, the determination of conjugated or masked forms of ZEA also improved significantly. So far, a main disadvantage was the non-availability of analytical standards. This has been largely overcome as (E)-6-(1-alkenyl)-substituted β-resorcylic acid esters have been synthesised as ZEA mimics (Mikula et al., 2013a) and used to optimise the synthesis of several ZEA conjugates. In this context, ZEA-14-β,D-glucuronic acid, which is a major metabolite of ZEA and occurring in urine of various animals, was synthesised (Mikula et al., 2012). The same group was successful to produce the Fusarium metabolite ZEA-14-sulphate as ammonium salt (Mikula et al., 2013b) and glucosides and glucuronides of α-ZOL and β-ZOL (Mikula et al., 2013c). ZEA-14G detection techniques based on acidic or enzymatic hydrolysis to ZEA and immunochemical determination of total ZEA were developed (Beloglazova et al., 2013). While trifluoromethanesulphonic acid yielded about 70% of cleavage to ZEA, several enzymes – including β-glucosidase from Aspergillus niger – performed even better, cleaving around 85% of the masked mycotoxin in cereal samples. Direct LC-MS/MS methods for the determination of ZEA and DON conjugates have also been developed and used to verify their stability after oral dosing of rats (Versilovskis et al., 2012). Already after 55 min, ZEA was found in the digestive tract, proving its release from ZEA-14G. This is in line with a study showing that masked mycotoxins can be hydrolysed by human colonic microbiota (Dall’Erta et al., 2013). Both ZEA-14-sulphate and -glucoside released ZEA already after 30 min of intestinal fermentation.

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