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# Developments in mycotoxin analysis: an update for 2009-2010

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## Abstract

This review highlights developments in mycotoxin analysis and sampling over a period between mid-2009 and mid-2010. It covers the major mycotoxins aflatoxins, Alternaria toxins, ergot alkaloids, fumonisins, ochratoxin, patulin, trichothecenes, and zearalenone. New and improved methods for mycotoxins continue to be published. Immunological-based method developments continue to be of wide interest in a broad range of formats. Multimycotoxin determination by LC-MS/MS is now being targeted at the specific ranges of mycotoxins and matrices of interest or concern to the individual laboratory. Although falling outside the main emphasis of the review, some aspects of natural occurrence have been mentioned, especially if linked to novel method developments.

**Keywords**: aflatoxin, *Alternaria*, ergot, fumonisin, ochratoxin, patulin, trichothecenes, zearalenone, sampling

## 1. Introduction

This review continues from a previous paper covering mycotoxin developments over the period mid-2008 to mid-2009 (Shephard *et al*., 2010). The section on cyclopiazonic acid has been replaced by a section on ergot alkaloids, due to the emerging interest in these toxins.

Major areas of research interest remain the development of novel immune-based methods (reviewed with other technologies by Maragos and Busman, 2010) and the application of LC-MS/MS, particularly with respect to multimycotoxin analysis. Since the principle of this technology has now been established, its application to specific matrices and combinations of toxins is receiving attention. Examples of this trend are its use for analysis of distillers dried grains (Zhang *et al*., 2009), biological fluids (Ritieni *et al*., 2010), maize silage (Rasmussen *et al*., 2010), mouldy foods (Sulyok *et al*., 2010), environmental samples such as surface and waste water (Hoerger *et al*., 2009), and its extension to include pesticides and dyes together with aflatoxin in spices (Amate *et al*., 2010). More generally, a short review on mycotoxin methods has been published (Turner *et al*., 2009).

As in the previous paper, this review emphasises new methodology, although some novel natural occurrence data has been included at the discretion of the authors, especially where it demonstrates the applicability of new methods.

## 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.

The Agricultural Research Service of the US Department of Agriculture and the US Food and Drug Administration conducted a cooperative sampling study to determine the sampling and analytical variability associated with detecting aflatoxins and ochratoxin A (OTA) in powdered ginger marketed in one pound bags (Whitaker *et al.*, 2009). The sampling and analytical variability associated with the test procedure used to measure aflatoxins and OTA in two different bulk lots of powdered ginger using 5 g laboratory samples and analysis by HPLC was determined. Averaged across both lots, the sampling and analytical variances accounted for 87% and 13% of the total variance, respectively, for aflatoxins and 97% and 3%, respectively, for OTA. The sampling and analytical coefficients of variation were 9.5% and 3.6%, respectively, for aflatoxins, and 16.6% and 2.9%, respectively, for OTA when using a single 5 g laboratory sample and HPLC analysis. Even though ginger was ground, sampling was the biggest source of error and can be reduced by increasing sample size.

The fourth session of the Codex Committee on Contaminants in Foods (CCCF) met in April 2010 in Izmir, Turkey to discuss the establishment of maximum levels and sampling plans for aflatoxins in Brazil nuts and fumonisins in maize and maize products (CCCF, 2010). Concerning Brazil nuts, the committee agreed to forward levels of 10 and 15 µg/kg for shelled Brazil nuts ready to eat and for further processing, respectively, to the Codex Alimentarius Commission (CAC) for adoption at step 5/8. These maximum levels suggested for Brazil nuts are the same adopted for pistachios, almonds, and hazelnuts by the CAC in July 2008. The committee agreed that a level for in-shell Brazil nuts was not needed. CCCF further agreed that the sampling plan for Brazil nuts should be incorporated into the existing sampling plans for almonds, pistachios, and hazelnuts, which was adopted by the CAC in July 2008. Discussions concerning maximum limits and sampling plans for fumonisin was taken off the agenda pending JECFA evaluation which is scheduled in 2011.

Whitaker *et al.* (2010) developed a Monte Carlo method to calculate the probability of accepting a lot (called an operating characteristic curve) at a given mycotoxin concentration by a specific mycotoxin sampling plan design with dual limits. Methods previously developed by Whitaker to calculate operating characteristic (OC) curves for mycotoxin sampling plans could only account for a single limit. Calculation of OC curves for dual limits are important because European Union (EU) aflatoxin limits are based upon both aflatoxin $B_1$ ($AFB_1$) and total aflatoxins. The method of calculating OC curves with dual limits was developed for sampling plans used in the United States to determine the level of $AFB_1$ and total aflatoxins (AFT) in almond lots exported to the EU. An important component needed in the method to calculate OC curves for dual limits was knowledge of the distribution of the ratio of $AFB_1$ to AFT. The ratio for almonds averaged about 0.85 and was extremely variable from sample to sample.

Casado *et al.* (2010a,b) published two papers investigating the spatial distribution of mycotoxin contaminated particles in a bulk lot so that sample selection strategies could be recommended. In the first paper (Casado *et al.*, 2010a), a data set for deoxynivalenol (DON) and OTA measurements from 100 incremental samples that were taken from 100 locations in a 26-tonne lot of wheat were analysed using geostatistical methods. Results of the study implied that sampling plans need to consider the location of sampling points in addition to the number of points sampled to obtain reliable estimates of the mean mycotoxin concentration of a bulk lot. The second study (Casado *et al.*, 2010b) was more theoretical using a two-dimensional statistical modelling approach to produce detailed information on appropriate sample selection strategies for determination of mycotoxins in bulk commodities. Results indicated that, for most sample sizes, a regular grid proved to be more consistent and accurate in the estimation of the true mean concentration of DON in the lot. The accuracy of the estimation of the lot's true mean concentration increased significantly when using up to 40 to 60 incremental samples.

## 3. Aflatoxin $M_1$

As in previous years, in the period mid 2009/mid 2010 a few dozen papers were published on the occurrence of aflatoxin $M_1$ ($AFM_1$). In almost all articles the investigated matrices involved milk and dairy products. A few studies focused on special matrices such as 24-hour duplicate diets and baby food. The latter studies have in common, that also other mycotoxins were determined in the samples, and they are summarised below.

Bakker *et al.* (2009) described a study, in which $AFM_1$ was determined (in addition to $AFB_1$, OTA, various trichothecenes and fumonisins $B_1$ and $B_2$ in lyophilised 24-hour diets of 2-6 year old children in the Netherlands. The method of analysis to determine $AFM_1$ included the simultaneous determination of $AFB_1$ and OTA (Sizoo and van Egmond, 2005). For $AFM_1$ no levels at the limit of quantification (14 ng/g) or higher were found in the 123 samples, whereas in 10% of all samples traces (between 7 and 14 ng/kg) of $AFM_1$ were detected. The authors concluded that the $AFM_1$ levels were very low, and did not give cause for concern. Alvito *et al.* (2010) used the

same multiplex method for the aflatoxins and OTA (Sizoo and van Egmond, 2005) to determine these toxins in baby food in Portugal. Out of 27 analysed samples, $AFM_1$ could be determined in 2 samples of cereal-based food and in 2 samples of milk powder-based infant formulae, with $AFM_1$ contents ranging from 17-41 ng/kg. The authors concluded that the levels found did not constitute a public health problem. Meucci *et al.* (2010) published results of a survey on the presence of $AFM_1$ and OTA in the 14 leading brands of infant formulas marketed in Italy. $AFM_1$ was detected in only two samples (range 12-15 ng/l) from the same brand. The authors concluded that the favourable situation was probably due to the increased attention paid in European countries to the presence of aflatoxins in feeds and milk.

Whereas in all three above-described studies HPLC with fluorescence detection (HPLC-FL) was applied, this classical technique has been superseded by ELISA in surveillance studies on $AFM_1$ in milk and milk products. A review of 24 papers on occurrence of $AFM_1$ in milk and dairy products, published since mid 2009, revealed that ELISA was the most applied technique. HPLC-FL was applied to a lesser extent, whilst LC-MS/MS was applied in one survey only (Alonso *et al.,* 2010; Alvito *et al.,* 2010; Bakker *et al.,* 2009; Bilandžić *et al.,* 2010; Delialioglu *et al.,* 2010; Elzupir and Elhussein, 2010; Elzupir *et al.*, 2009; Fallah, 2010; Fallah *et al.*, 2009; Gundinc and Filazi, 2009; Hampikyan *et al.*, 2010; Heshmati and Milani, 2010; Hussain *et al.*, 2010; Kang'ethe and Lang'a, 2009; Maqbool *et al.*, 2009; Meucci *et al.*, 2010; Mohamadi *et al.*, 2010; Motawee *et al.,* 2009; Nemati *et al.,* 2010; Rahimi *et al.*, 2009, 2010; Ruangwises and Ruangwises, 2009; Velázquez *et al.*, 2009; Virdis *et al.*, 2009) (see Figure 1). This situation was different in studies on method development (Frenich *et al.*, 2009; Huang *et al.*, 2010; Neagu *et al.*, 2009; Nocentini *et al.*, 2009; Rameil *et al.*, 2010; Romero-Gonzalez *et al.*, 2009; Rubio *et al.*, 2009; Uchigashima *et al.*, 2009; Vig *et al.*, 2009) and in studies on $AFB_1$ transfer from feed into $AFM_1$ in milk and on the effect of processing (Aly and Diekmann, 2010; Pietri *et al.*, 2009a). Here HPLC-FL, LC-MS/MS and immunosensors were most often applied, followed by ELISA (see Figure 1).

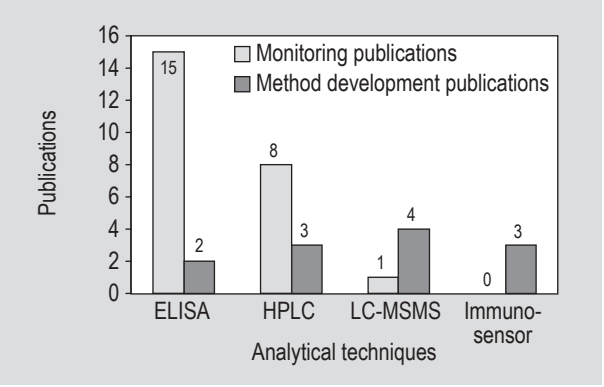


**Figure 1. Frequency of analytical techniques used in papers on monitoring and occurrence of aflatoxin $M_1$ in milk and dairy products (indicated as monitoring publications) and in papers on method development, aflatoxin $B_1$ transfer from feed into aflatoxin $M_1$ in milk and on the effect of processing (indicated as method development publications).**

The LC-MS/MS methods of Frenich *et al.* (2009), Huang *et al.* (2010) and Neagu *et al.* (2009) were reported to be able to determine $AFM_1$ in conjunction with other mycotoxins. They were not specifically developed for $AFM_1$, but for multimycotoxin determination in cereals and related foods, peanuts and beers respectively. Huang *et al.* (2010) reported the presence of small amounts of $AFM_1$ and aflatoxin $M_2$ ($AFM_2$) in unprocessed peanut butters. The authors commented that the peanuts were free of milk products and that, therefore, $AFM_1$ and $AFM_2$ might have been formed on the peanuts. This would not be surprising as some strains of *Aspergillus parasiticus* produce these toxins in minute amounts in addition to much larger amounts of B and G aflatoxins.

In the previous review on developments in mycotoxin analysis (Shephard *et al.*, 2010), mention was made of the gradual entry of sensor-based methods in the $AFM_1$ analytical field. Further applications in this area were published in the last year (Neagu *et al.*, 2009; Rameil *et al.*, 2010; Vig *et al.*, 2009). In all three papers use was made of disposable screen-printed electrodes, which were applied to the development of sensitive and specific electrochemical immunosensor systems. Moreover the systems were characterised as rapid, simple, low cost and suitable for miniaturisation. Working ranges of the methods of Neagu *et al.* (2009), Vig *et al.* (2009) and Rameil *et al.* (2010) were reported for $AFM_1$ from 5-250 pg/ml, 15-1000 pg/ml and 250-2,000 pg/ml milk respectively, whereas corresponding limits of detection for $AFM_1$ were reported as 1, 12 and 40 pg/ml milk. Whereas screening for $AFM_1$ in milk with these electrochemical immunosensors is likely to be fast and simple with all three methods, the mutual detection limits differ significantly. None of the methods have gone through the rigour of extensive (interlaboratory) validation studies yet, which makes it difficult to judge about the performance of these newcomers in practice, as compared to the popular ELISA- and HPLC-based methods, which are currently the main techniques used for monitoring (see Figure 1). One may expect however, that the immunosensor-based methods for $AFM_1$ determination will further develop and may improve in the near future, as will the LC-MS/MS based methods, and eventually they might be launched in monitoring and surveillance programmes.

## 4. Aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$

Quantitative aflatoxin analysis is widely performed by sample extraction with a suitable aqueous organic solvent mixture, extract clean-up and HPLC separation and

quantification by fluorescence detection. Within this broad context, new work continues to be published. A pressurised liquid extraction system has been optimised for extraction of aflatoxins from almonds, hazelnuts and peanuts (Campone *et al.*, 2009). Optimum conditions were 60 °C, 1,500 psi, 100% acetonitrile and a single extraction cycle. No further clean-up, other than lipid removal with hexane, was required. A novel solid-phase microextraction technique for aflatoxins from cereal flours was developed and optimised with respect to fibre polarity, pH, ionic strength, and adsorption and desorption times (Quinto *et al.*, 2009). Although the method gave low limit of detections (LODs) and good repeatabilities, extraction recoveries ranged from 49% to 59%. A comparison between slurry and dry blended homogenisation for aflatoxins in dried figs demonstrated little difference between the two techniques and the latter was used for an extensive surveillance of export products (Bircan, 2009a). Aflatoxins and OTA in high-sugar-content traditional Turkish delicacies, such as baklava and halvah, were determined by grinding the sample under liquid nitrogen prior to conventional extraction and clean-up on immunoaffinity columns (IACs) (Senyuva *et al.*, 2009). The method showed no interfering co-extractives and was robust enough for routine food control. Analysis of a commercial complex dietary ingredient required modification of AOAC Official method 990.33 in which 5 g sample replaced 50 g, methanol/water replaced methanol/0.1 mole/l hydrochloric acid, and dichloromethane replaced hexane (Zahn *et al.*, 2008). Analysis of animal feeds can be problematic. In a comparison of aqueous methanol or aqueous acetone as extractants for animal feeds containing adsorbents, it was determined that methanol can result in severely reduced recoveries with a range of adsorbents, an effect also noticed with acetone in the presence of activated carbon or sodium bentonite additives (Gallo *et al.*, 2010). Other authors have validated a method for animal feeds which uses aqueous acetonitrile extractant, clean-up on a commercial multifunctional column and HPLC with fluorescence detection of preformed trifluoroacetic acid derivatives (Khayoon *et al.*, 2010). Although it has frequently been asserted that polar aflatoxins would not be found in non-polar vegetable oils, there is increasing evidence that this is incorrect for non-purified oils. Consequently, a validated method for aflatoxins in a wide range of spiked plant oils has been developed (Mahoney and Molyneux, 2010). The oil sample was dissolved in hexane and passed through a silica solid phase extraction (SPE) cartridge to capture the aflatoxins. After washing with hexane, the aflatoxins were eluted with aqueous methanol and determined by conventional HPLC.

A single laboratory validation has been reported for aflatoxins in a range of botanical roots at levels between 2 ng/g and 16 ng/g (Weaver and Trucksess, 2010). Samples were extracted with aqueous acetonitrile, purified on IACs and quantified by HPLC with postcolumn photochemical derivatisation. Recoveries ranged from 86% to 118% with repeatabilities from 1.4% to 15.9%. A similar method for foods and cereals for animal consumption was validated using aqueous methanol extraction (Muscarella *et al.*, 2009). The method satisfied requirements for official methods in the European Union.

The use of two-photon-induced fluorescence (excitation wavelength 729 nm) for the fluorescence spectroscopic determination of $AFB_1$ in alcoholic beverages was investigated and shown to produce lower LODs than one-photon-induced fluorescence (excitation wavelength 360 nm) (Rasch *et al.*, 2010). Aflatoxins in pistachio nuts have been determined by HPLC with diode-array detection after reversed-phase C18 SPE clean-up using parallel factor analysis for second order calibration methodology, which allowed quantification in the presence of various unknown HPLC interferences (Vosough *et al.*, 2010).

LC-MS/MS is a rapidly expanding technique for mycotoxin analysis in general, as well as for aflatoxins. Some of these recent applications have been mentioned in the introduction. Apart from conventional HPLC separation prior to MS detection, ultra-high performance liquid chromatography (UHPLC) has also been coupled to MS to achieve rapid chromatographic analysis times (Frenich *et al.*, 2009; Grio *et al.*, 2010; Han *et al.*, 2010a; Huang *et al.*, 2010; Romero-Gonzalez *et al.*, 2009). Whereas some authors are utilising a 'dilute-and-shoot' approach without extract clean-up, others are applying a range of clean-up techniques. For beer analysis, a conventional reversed-phase (C18) SPE cartridge was used to concentrate the aflatoxins (Romero-Gonzalez *et al.*, 2009), for peanuts a multifunctional cartridge was used (Huang *et al.*, 2010) and for Chinese medicines, a 'home-made' mixed cartridge with subsequent addition of a $[^{13}C_{17}]$-$AFB_1$ internal standard (Han *et al.*, 2010a). To overcome the complexity of animal and pet feeds, Grio *et al.* (2010) used a C18 SPE. Other authors have reported more complex systems. Monbaliu *et al.* (2010) reported a method for 23 mycotoxins in animal feed in which a multi-step clean-up was employed consisting of C18 SPE, lipid removal with hexane and the subsequent use of a multifunctional column.

The development of antibody-based methods continues in a range of assay formats. These have recently been reviewed (Li *et al.*, 2009). However, for the general analytical laboratory, these are not viable to adopt unless commercialised as a readily usable kit. A competitive assay has been described for the simultaneous determination of $AFB_1$ and OTA in which bound and free mycotoxin competes with the relevant dissolved antibodies (Huang *et al.*, 2009). Quantification is via dual-label time-resolved fluoroimmunoassay using $Eu^{3+}$ and $Sm^{3+}$ labelled anti-antibodies to separate the signals for the two mycotoxins. An organic solvent-tolerant antibody has been used in

an IAC format for purification of aflatoxins without the extensive dilution normally needed prior to application to an IAC (Uchigashima *et al*., 2009). These antibodies were reported to tolerate up to 60% methanol and 20% acetonitrile. Other developments include a surface plasmon resonance (SPR) assay for $AFB_1$ and aflatoxin $G_1$ (Cuccioloni *et al*., 2008), and a microarray for $AFB_1$ and fumonisin $B_1$ ($FB_{1)}$ (Lamberti *et al*., 2009). A novel approach to rapid methods has been the recent description of two computationally designed polymers for adsorption of $AFB_1$ and OTA (Piletska *et al*., 2010). This concept involves the adsorption of the mycotoxin on the relevant polymer and measurement of its fluorescence emission when exposed to UV light in a specially designed fluorometer, for which a prototype is in use.

Although aflatoxin exposure in developed countries is low, it continues to be a major issue in developing countries and a significant contributor to global disease burden (Liu and Wu, 2010; Wild and Gong, 2010). Its natural occurrence continues to be an area of intense publication. Some of the new information on contamination levels in human foods includes its occurrence in Brazil nuts (Pacheco and Scussel, 2009), in tiger nuts and their beverages in Spain (Sebastia *et al*., 2010), in common foods in Uganda (Kitya *et al*., 2010), in cereals in Croatia (Klaric *et al*., 2009), in maize in Brazil (Rocha *et al*., 2009), Turkey (Giray *et al.*, 2009) and Pakistan (Shah *et al*., 2010), in dried vegetables in west Africa (Hell *et al*., 2009), in sorghum and pistachios in Tunisia (Ghali *et al*., 2009), in rice in Austria (Reiter *et al*., 2010) and Iran (Mazaheri, 2009), in foods in Jordan (Salem and Ahmad, 2010), Cameroon (Njobeh *et al*., 2010) and Japan (Sugita-Konishi *et al*., 2010), in children's food in the Netherlands (Bakker *et al*., 2009), in vegetable oils in Sudan (Elzupir *et al*., 2010), in peppercorns and nuts in Malaysia (Jalili *et al*., 2009; Leong *et al*., 2010) and in figs in Turkey (Bircan, 2009a,b).

## 5. *Alternaria* toxins

LC-MS/MS was extensively used for both method development and screening purposes. A previously reported approach for determination of tenuazonic acid (TeA) by LC-MS/MS after derivatisation with 2,4-dinitrophenylhydrazine was used by Siegel *et al.* (2010a) for the quantification of TeA in beer. The LOD and limit of quantification (LOQ) were 2 µg/kg and 8 µg/kg, respectively. For the determination of actual TeA concentrations, a standard-addition scheme was performed for each sample above the LOQ to compensate for sample-dependant recoveries. Thirty-seven out of 43 beer samples were found contaminated by TeA up to 175 µg/kg and average concentration of 11 µg/kg. A multimycotoxin LC-MS/MS method was developed for the determination of alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ANE) and 20 other mycotoxins in cereal-based feeds. Feed samples were extracted with a mixture of acetonitrile/water/acetic acid (79:20:1). Before LC-MS/MS determination the filtered extract was submitted to extensive clean-up which included the use of C18 and Multisep226 Aflazon+ SPE and a liquid-liquid defatting step with hexane (Monbaliu *et al.,* 2010). Low levels (17-25 µg/kg) of AOH and AME were found in 4 of 82 maize samples.

A modified pH-buffered sample extraction, known as QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), was used by Rasmussen *et al.* (2010) for simultaneous LC-MS/MS determination of AOH, AME, TeA and other mycotoxins and secondary fungal metabolites in maize silage. The three *Alternaria* toxins were analysed in negative electrospray ionisation (ESI) mode and quantified by using calibration curves with matrix-matched standards. Values of LOD for AME, AOH and TeA were 6, 10 and 121 µg/kg, respectively. Good recovery and precision values were obtained for AOH and AME, 78-79% and 5-9%, respectively, whereas a recovery of only 37% was obtained for TeA with a precision of 20%. Signal suppression and enhancement (SSE) values were reported for AOH and AME of 67% and 48%, respectively, but not for TeA. AOH was found in one out of ten samples of visibly unspoiled silage at a level of 24 µg/kg, whereas one out of ten samples of hot spots with visible fungal growth contained 236 µg/kg AOH and 51 µg/kg AME.

An existing LC-MS/MS method was used for a semi-quantitative screening of 87 mouldy food samples from private households. Within the 49 mycotoxins identified in positive samples, AOH and AME were found in several samples of fruits (strawberry, cranberry, pear, blackberry) and in paprika and tomato, but their concentration exceeded 100 µg/kg only in 5 and 1 sample, respectively (Sulyok *et al.,* 2010).

*Stagonospora* (syn. *Septoria*) *nodorum* (Berk.) Castell. and Germano [teleomorph: *Phaeosphaeria* (syn. *Leptosphaeria*) *nodorum* (Müll.) Hedjar.], a major pathogen of wheat and other cereals has been reported to produce AOH for the first time. No longer can *S. nodorum* be singularly associated with inflicting yield losses and it should now be considered a health risk and treated as such (Tan *et al.,* 2009). Siegel *et al.* (2010b) studied the degradation kinetics of *l*-TeA ((5S,8S)-3-acetyl-5-sec.-butyltetramic acid) in aqueous solutions over a period of 4 months at different pH (3.5 and 7.0) and temperatures (4, 25 and 40 °C). *l*-TeA and *u*-TeA ((5R,8S)-3-acetyl-5-sec.-butyltetramic acid) were monitored by HPLC-UV whereas *iso*-DTeA (*iso*-deacetyl TeA, epimeric mixture of *l*-DTeA and *u*-DTeA) was monitored by LC-ESI multistage MS. At pH 7.0, 4 °C and 25 °C *l*-TeA was stable for 4 months whereas at 40 °C 11% degradation to *iso*-DTeA occurred. At acidic pH (3.5) 11%, 61% and 100% degradation to *iso*-DTeA occurred over 4 months at 4, 25 and 40 °C, respectively. Therefore

*iso*-DTeA might be formed in aqueous beverages such as fruit juices that have acidic pH.

The effect of different gamma radiation doses (2, 5 and 7 kGy) on the growth of *Alternaria alternata* and on the production of AOH and AME was evaluated by Braghini *et al.* (2009), who inoculated sunflower seeds with spore suspension of *A. alternata* previously submitted to gamma radiation. A 98% reduction of fungal growth was observed at 7 kGy whereas a 99% reduction of mycotoxin production was already observed at a dose of 5 kGy. New data on the toxicity of *Alternaria* mycotoxins were published by Tiemann *et al.* (2009). AOH and AME, but not TeA, specifically inhibited hormone progesterone secretion in cultured porcine granulosa cells already at 0.8 µM. *Alternaria* toxin-contaminated feed may therefore affect reproductive performance in pig and other mammalian species. Logrieco *et al.* (2009a) published an overview of *Alternaria* taxonomy, molecular phylogenetic relationships amongst *Alternaria* species, *Alternaria* mycotoxins and their toxicity, and the main plant diseases associated with *Alternaria* species and mycotoxins occurrence.

## 6. Ergot alkaloids

The major source of ergot alkaloids is the fungus *Claviceps purpurea,* which infects cereals, notably rye, replacing the grain with alkaloid-containing bodies called sclerotia. The ergot alkaloids most commonly encountered in food and animal feed are ergometrine, ergotamine, ergosine, ergocristine, ergocryptine and ergocornine. The related ergovaline is produced by the fungal endophyte *Neotyphodium,* which infects important forage grasses such as tall fescue (*Festuca arundinacea*), causing toxicosis in grazing animals. The sclerotia of *Claviceps* contaminate cereal grains and consequently cereal-based feed and food, causing adverse health effects in humans and animals. Following a risk assessment of feed, the European Food Safety Authority asked that data should be collected on ergot alkaloids in feed and that validated analytical methods should be developed (EFSA, 2010). Ergot alkaloids from *Claviceps* can undergo rapid epimerisation at the C8 ergoline-peptide bond to form a mixture of -ine and -inine epimers. The latter are thought to be physiologically inactive but since they can interconvert it is important to determine both isomers.

An overview of ergot alkaloids and their analysis provided by Krska and Crews (2008) provides an introduction to the current situation. The major modern analytical methods for ergot alkaloids are based on simple extraction procedures allied to LC-MS/MS with demonstrable separation of the C8-isomers or epimers, with a trend towards reduced analysis time and/or inclusion within multi-mycotoxin methods.

An existing LC-MS/MS method for multi-mycotoxin determination was extended and used in a semi-quantitative analysis of 87 food samples visibly infected with fungus (Sulyok *et al.,* 2010). Mycotoxins were extracted into acetonitrile/water/acetic acid, centrifuged and analysed by positive and negative ionisation electrospray LC-MS/MS with a 5 µm C18 column and selected reaction monitoring (SRM). Ergot alkaloids were present in all samples of dark breads at low µg/kg levels and both epimeric forms were separated. Strong matrix effects were seen on the ionisation process. Similar levels were reported in dark rye breads analysed by LC-MS/MS after extraction into acetonitrile and ammonium carbonate buffer followed by a rapid clean-up using dispersive solid-phase extraction with a primary-secondary amine phase (Crews *et al.,* 2009). Kokkonen and Jestoi (2009) determined mycotoxins including epimers of ergot alkaloids and their epimers in wheat, barley and oats. Automated accelerated solvent extraction was used, with the extract filtered and concentrated before LC-MS/MS in both positive and negative electrospray ionisation. The method was semi-quantitative, with LOQs mostly between 20 and 60 µg/kg, and recoveries between 48% and 122%. Matrix suppression was observed but compensated for by using matrix-matched standards. Uhlig *et al.* (2009) developed an HPLC method for the determination of 24 ergot alkaloids and also *Claviceps paspali* derived indole-diterpenes in simple acetone extracts of Bermuda grass (*Cynodon dactylon*) and *C. paspali* sclerotia. The extracts were filtered and injected on to a 2.5 µm C18 column and alkaloids detected by MS/MS in positive ion electrospray mode. Molecularly imprinted polymers have been proposed as a clean-up method for ergot alkaloids prior to solid phase extraction LC/MS-MS (Lenain *et al.,* 2010).

Müller *et al.* (2009) used an HPLC-FL to determine 12 ergot alkaloids in rye and rye products. Samples were extracted with ethyl acetate/methanol/aqueous ammonia, followed by centrifugation and purification through basic alumina before HPLC analysis on a phenyl-hexyl column. Recoveries were 89% to 100% with LOQs of 3.3 µg/kg or better. Repeatability precision (RSD) ranged from 2.8% to 12.4%, within-laboratory reproducibility from 6.5% to 14.9%. HPLC-FL and UV absorption was used by Blaney *et al.* (2009) to screen ergot sclerotia from Australian rye grasses, grain screenings and feed. Samples were extracted by homogenising in methanol. The fluorescence was measured with an excitation wavelength of 235 nm and emission wavelength of 370 nm and the UV at absorption maxima of 206, 238 and 312 nm. Epimers of all of the major ergot alkaloids could be measured. Sclerotia alkaloid profiles and concentrations were similar regardless of region or type of grass or grain. Ergocristine levels were very low in contrast to US and European ergots. A novel screening method for detecting ergot was developed by Franzmann *et al.* (2010) who determined ricinoleic acid (12-hydroxy-9-cis-octadecenoic acid) in sclerotia lipid. The acid was measured

by saponification, methylation, and gas chromatography with flame ionisation detection. Ergot alkaloid contents were measured by HPLC-FL according to Müller *et al.* (2009) but using a 5 µm C18 column. The ricinoleic acid content correlated well with the ergot alkaloid content in the sclerotia, but less so for processed rye foods, where it could nevertheless be used as a marker of contamination.

Lezica *et al.* (2009) used thin-layer chromatography and spectrophotometry to test for ergot alkaloids in extracts of endophyte-infected ryegrass. The grass was extracted with ethanol and concentrated before solvent exchange into diethyl ether, or extracted with chloroform/methanol/ammonia and partitioned into dilute aqueous acid. The silica TLC solvents were sequential applications of chloroform/methanol/water and ethyl acetate/dimethylformamide/ethanol. Colorimetric reactions (p-dimethylaminobenzaldehyde) were used for detection and spectrophotometric quantification. Although no ergot alkaloids were detected in the grass (LOD 1 µg/kg) their presence was inferred by bioassays using the contractile response of rat uterus.

Progress has been made in the development and application of analytical methods to animal tissue samples. LC-MS was used to determine ergot alkaloids added to venous tissue from cattle (Klotz *et al.,* 2009; Smith *et al.,* 2009). Alkaloids were extracted from the freeze-dried tissue with methanol and redissolved in mobile phase. The LC system used a 2.5 µm C18 stationary phase with water/acetonitrile/formic acid. The MS was operated in positive ion electrospray mode with single ion monitoring. Correlation of the data with biomonitored contractile responses indicated that ergovaline may have a greater potential than lysergic acid for inducing toxicosis from contaminated fescue grass. The metabolic fate and gastric transport of ergot alkaloids in ruminants, were studied in vivo by analysis of ruminal fluid and urine (Ayers *et al.,* 2009). Total ergot alkaloids were determined by ELISA, and ergovaline by HPLC-FL. Ergovaline was not detected but lysergic acid was found in urine. Schumann *et al.* (2009) analysed sclerotia, feed, serum, milk, urine and faeces by a SPE method (Wolff *et al.,* 1988) in which extracts obtained with a mixture of dichloromethane/ethyl acetate/methanol and aqueous ammonia were transferred into mixtures of toluene, methanol, and hexane, and placed on an acidified diatomaceous earth column. The column was washed with solvents, made basic with gaseous ammonia, and the alkaloids eluted with dichloromethane. The detection limit was 5 µg/kg (ergometrine 10 µg/kg). About 24% of alkaloids were excreted in faeces but none were found in milk. Despite its relative complexity the method is applicable to a range of sample types, especially aqueous media.

Roberts *et al.* (2009) used near infrared (NIR) spectroscopy to measure ergovaline and total alkaloids in hay prepared from fescue grass. Ergovaline levels decreased sharply on storage but remained above a level (250 µg/kg) toxic to livestock. Vermeulen *et al.* (2009) used NIR spectrometry to screen cereal grains for ergot bodies. A database of ergot body and wheat kernel spectra was built using an NIR spectrometer (1,100-2,400 nm range) and a hyperspectral NIR imaging system (900-1,700 nm range). Calibration and validation sets built from the database provided a method to discriminate between ergot bodies and wheat kernels.

Overall, there have been significant analytical developments over the past 2 years covered by this review. The availability and stability of standards continues to be a problem. Whilst many ergot alkaloids are readily available, sources of pure epimers are difficult to find. This situation should improve with increased interest in the topic prompted by regulation. Isotopically labelled internal standards useful in LC-MS quantification will probably remain unavailable for some time. Synthetic or semi-synthetic alternatives such as methylsergide intended for pharmaceutical use have been employed (Smith *et al.,* 2009; Uhlig *et al.,* 2009).

## 7. Fumonisins

HPLC with either fluorescence, mass spectrometry or tandem mass spectrometry continues to predominate in laboratory-based methods for detecting fumonisins. The use of fluorescent labels in the detection of fumonisins is well established, and several recent articles reported adaptations of the concept. One such adaptation has been to use a fluorescent label (*o*-phthaldialdehyde; OPA), but monitor the ultraviolet (UV) absorbance of the product rather than the fluorescence (Ndube *et al*., 2009). This was done so that laboratories that lack a fluorescence detector might still be able to monitor for fumonisins. In the comparison using fluorescence for detection was about 20-fold more sensitive than using UV for detection (Ndube *et al*., 2009). The fluorescence detection of OPA-derivatised fumonisins was also included in a multi-toxin assay combining UV and fluorescence detection in series. Absorbance in the UV was used to detect DON, while fluorescence was used to detect aflatoxins, zearalenone (ZEA), OTA and the (derivatised) fumonisins in maize (Ofitserova *et al*., 2009). Method performance was reported to be within specifications set by the Feed Additives and Contaminants group of the AOAC International. Fluorescence detection was also the basis for a report describing problems with widely used IACs for isolation of the fumonisins. In particular the authors suggest that there may be an underestimation of fumonisins in samples cleaned-up with the IAC, if the volume and organic solvent content used in the elution are not sufficient (Oh *et al*., 2009). An OPA-based method for measurement of fumonisins in maize products for infants and young children also underwent a validation study in three laboratories (De Girolamo *et al*., 2010).

The use of LC-MS and LC-MS/MS for fumonisin analysis is becoming increasingly widespread. During the past year the development of HPLC with MS detection was the most widely reported method development. Many of the reported methods involved electrospray ionisation (ESI) with MS/MS. Some of these methods were novel because they allowed the detection of multiple mycotoxins within a single chromatographic run, others because they used innovations in the chromatography, and still others because they examined unusual matrices or fumonisin analogues. With regard to detection of multiple mycotoxins or metabolites in a single assay, several groups reported successful development of methods. Among these were included a LC-ESI-time of flight (TOF) and an LC-ESI-ion trap method for detecting $FB_1$ and 28 isomers (Bartók *et al.*, 2010). An LC-MS/MS assay using the QuEChERS approach to isolate 27 secondary metabolites from maize silage was reported (Rasmussen *et al.*, 2010), although the validation results for the fumonisins were relatively poor. Other multi-toxin LC-MS/MS methods that were reported included an assay for detection of 23 toxins in feed samples (Monbaliu *et al.*, 2010), a multi-toxin assay for mouldy foods (Sulyok *et al.*, 2010), and an assay for 186 bacterial and fungal metabolites in indoor matrices such as house dust (Vishwanath *et al.*, 2009). A comparison of LC-MS, LC-MS/MS, and LC-fluorescence was reported (Silva *et al.*, 2009). Maize products were extracted with aqueous methanol, the fumonisins were isolated by IAC clean-up and detected using each of the three methods. The LC fluorescence method used naphthalene 2,3 dicarboxaldehyde (NDA) as the labelling reagent. LC-MS/MS had the highest sensitivity, followed by LC-fluorescence, and LC-MS. The same group also reported an LC-MS/MS method for $FB_1$ and fumonisin $B_2$ ($FB_2$) in human urine (Silva *et al.*, 2010).

UHPLC, using chromatography columns with smaller particle sizes to increase the efficiency of the chromatographic separation, has steadily advanced and applications to fumonisin detection continue to be developed. This has been particularly true for the development of multi-toxin assays. In the past year examples of the latter that involve UHPLC include an assay for multiple mycotoxins in maize, pasta and baby food (Beltrán *et al.*, 2009), a multi-toxin assay for fumonisins in beer (Romero-González *et al.*, 2009), and the previously mentioned method employing QuEChERS clean-up (Zachariasova *et al.*, 2010). The changes in chromatographic conditions can significantly reduce the separation time. As an example 11 mycotoxins were separated within 4 min (Beltrán *et al.*, 2009). UHPLC was also applied to the detection of 23 mycotoxins, including $FB_1$, $FB_2$, and fumonisin $B_3$ ($FB_3$) in food supplements such as Maca (Di Mavungu *et al.*, 2009). The technology has also been applied to the detection of 12 mycotoxins, including $FB_1$ and $FB_2$, in maize, walnuts, biscuits, and breakfast cereals (Frenich *et al.*, 2009). As with many analytical technologies components of the sample matrix have the potential to interfere with MS detectors, for example by suppression or enhancement of the ionisation process. To minimise these effects many MS-based methods incorporate clean-up and/or chromatographic steps to reduce the amount of matrix present during elution of the analyte. The impact of matrix effects can also be controlled through the use of internal standards, or matrix-matched external standards. An example is the incorporation of stable isotope labelled internal standards (Rychlik and Asam, 2009; Trebstein *et al.*, 2009; Zachariasova *et al.*, 2010). Methods using matrix-matched standards or standard addition approaches have been widely reported (Beltrán *et al.*, 2009; Di Mavungu *et al.*, 2009; Frenich *et al.*, 2009; Rasmussen *et al.*, 2010; Trebstein *et al.*, 2009; Zachariasova *et al.*, 2010).

While immunoassays continue to be widely used for screening commodities and foods for fumonisins, there were few reports of new developments in antibodies and immunoassays during the past year. Perhaps this is due to the widespread availability of commercial immunoassays and lateral flow test strips (LFD). A LFD for fumonisins was reported that gave a 4 min assay time, with a limit of detection of 199 µg fumonisin/g (Molinelli *et al.*, 2009). Colour at specific lines on the test strip, resulting from binding of an antibody-colloidal gold conjugate, was related to toxin content. The commercial use of such kits, and potential harmonisation issues, have been described (Alldrick *et al.*, 2009). Detection of standard solutions of $FB_1$ using an antibody-based microarray was reported (Lamberti *et al.*, 2009). The LOD, for standard solutions, was 43 ng $FB_1$/ml. A new monoclonal antibody and a recombinant single chain variable fragment (scFv) for binding $FB_1$ were reported (Min *et al.*, 2010). The solubility and the binding affinity of the scFv were much lower than those of the intact antibody when tested by ELISA.

Contamination of maize and maize-based products with fumonisins occurs worldwide. The prevalence of fumonisins in maize has been thoroughly established, leading to considerable efforts to detect these toxins in maize and maize-products. Summarising the occurrence of fumonisins in maize is beyond the scope of this report. As an indication of the widespread nature of the problem, during the past year reports on fumonisins in maize products were received from Brazil, Bulgaria, Cameroon, Croatia, Czech Republic, India, Iran, Italy, Japan, Kenya, Spain, Switzerland, Tanzania, Tunisia, Uganda, United States, and United Kingdom. Strategies to reduce fumonisins in stored maize were recently reviewed (Chulze, 2010). While contamination of maize continues to be a problem, fumonisins have also been found recently in other commodities and foods as well. These include wheat (Kushiro *et al.*, 2009; Manova and Mlandenova, 2009; Monbaliu *et al.*, 2010), cassava (Manjula *et al.*, 2009), horse feed (Liesener *et al.*, 2010), grapes and grape products such as wine (Logrieco *et al.*,

2009b; Mogensen *et al.*, 2010), beer (Aoyama *et al.*, 2010; Romero-González *et al.*, 2009), figs (Moretti *et al.*, 2010), soybeans and millet (Aoyama *et al.*, 2010), asparagus (Aoyama *et al.*, 2010; Waśkiewicz *et al.*, 2010) and milk (Gazzoti *et al.*, 2009). In the case of figs, strains of *Fusarium ramigenum* isolated from rotted figs were able to produce $FB_1$ and $FB_2$ (Moretti *et al.*, 2010). In the case of dairy milk a method with a LOQ of 0.1 µg/kg was developed. Eight of 10 samples analysed contained $FB_1$ above the LOQ, with the highest sample containing 0.26 µg/kg (Gazzotti *et al.*, 2009). An exposure assessment for $FB_1$ in milk estimated mean, simulated, exposures of 327 pg/kg body weight/day for males and 351 pg/kg/day for females (Coffey *et al.*, 2009). This is very low compared to the tolerable daily intake established by the European Commission of 2 µg/kg body weight/day. $FB_2$ has been found to be produced by strains of *Aspergillus niger* isolated from grapes and has been found in grape must (Logrieco *et al.*, 2009b). A study of 77 wine samples from 13 countries using LC-MS/MS found that 23% contained detectable $FB_2$, with the highest level being 25 µg/l (Mogensen *et al.*, 2010). $FB_1$ was also found to persist in the liver of ducks fed a mixture of $FB_1$ and $FB_2$ at levels of 10 or 20 mg/kg feed (Tardieu *et al.*, 2009).

In addition to exposures to the fumonisins themselves, there has been interest in the extent to which the fumonisins exist in foods in the form of conjugates with other food components such as sugars or proteins. These 'hidden', 'bound', or 'masked' fumonisins may not be detected by methods that screen only for the fumonisins that are readily extractable in free form. The formation and determination of conjugated mycotoxins was recently reviewed (Berthiller *et al.*, 2009a). Significant amounts of bound fumonisins were detected when a digestion protocol was incorporated into a method for maize (Dall'Asta *et al.*, 2009). The difference was suggested to be due to the association of fumonisin with matrix, rather than due to covalent attachment. The bioavailability of bound $FB_1$ in cornflakes was estimated to be between 37% and 64% (Motta and Scott, 2009). The formation of bound fumonisins may occur while the fungus infests the plant, during heating, or during processing steps. Free fumonisins in milling fractions (Pietri *et al.*, 2009b; Scudamore and Patel, 2009), and distillers dried grains (Zhang *et al.*, 2009) were also reported. A new fumonisin, $B_6$ ($FB_6$), a positional isomer of $FB_1$ and *iso*-$FB_1$ having hydroxyl groups at C3, C4, and C5 was also reported (Månsson *et al.*, 2010).

## 8. Ochratoxins

The European Commission has established maximum levels for OTA in some spices and liquorice (EC, 2010). As regards liquorice, Raters *et al.* (2010) reported the development and validation of a new harmonised HPLC-based method for the analysis of OTA in liquorice products. This procedure used an aqueous extraction solution and was collaboratively studied by 21 laboratories in ten countries throughout Europe and one laboratory in the USA. The performance of the method was found to be acceptable to EU regulation for the analysis of mycotoxins, the between-laboratory RSD ranged between 17.39 and 29.08%. In relation to the presence of OTA in liquorice, Pietri *et al.* (2010) detected high OTA concentrations in all of 28 samples of dried liquorice extract with a mean value of 89.6 µg/kg and a maximum value of 990 µg/kg; they also found 61% positive out of 54 samples of liquorice confectionery with a mean value of 0.96 µg/kg and a maximum of 8.3 µg/kg. Herrera *et al.* (2009) analysed 44 confectionery samples (16 hard candies and 28 soft candies) in which the incidence of OTA varied between 75% and 39% and the mean values ranged from 2.96 to 0.34 µg/kg for hard and soft candies, respectively.

Two European standards have been published by the European Committee for Standardization (CEN) for the determination of OTA in currants, raisins, sultanas, mixed dried fruit and dried figs (CEN, 2010a), and in cereal based foods for infants and young children (CEN, 2010b), respectively. Both procedures were based on HPLC-FL with IAC clean-up and were validated in collaborative studies via the analysis of both naturally contaminated and spiked samples ranging from 1.0 µg/kg to 11 µg/kg in the case of currants, raisins, sultanas, mixed dried fruit and dried figs, and ranging from 0.050 µg/kg to 0.217 µg/kg for cereal based foods for infants and young children.

HPLC-FL using IAC clean-up continued being the most widely used technique in routine analytical activities for the determination of OTA in food and feed. Nevertheless, the investigation of various innovative approaches has been once again the subject of a number of papers published during the last year. Yuan *et al.* (2009) reported the development of a sensitive SPR assay using gold nanoparticles for signal enhancement on a mixed self-assembled monolayer surface. This competitive OTA immunoassay was based on the immobilisation of target OTA through its ovalbumin conjugate with a polyethylene glycol linker. Grain samples were simply extracted with 50% methanol and liquid samples treated with poly(vinylpyrrolidone) without any sample clean-up or pre-concentration step prior to analysis. The LODs in oats and corn were 0.3 and 0.5 µg/kg, respectively, while in wine and other beverages ranged from 0.058 to 0.4 µg/l. Alonso-Lomillo *et al.* (2010) reported the immobilisation of horseradish peroxidase in a polypyrrole matrix onto disposable screen-printed carbon electrodes for the selective detection of OTA. The optimisation of the chronoamperometric determination of the toxin allowed the application of this biosensor in spiked beer and roasted coffee, yielding recoveries of 103% and 99%, respectively. The LOD was established at 0.1 µg/l. Lai *et al.* (2009) reported the development of a lateral flow colloidal gold strip assay for rapid detection

of OTA; this assay used a mimotope peptide instead of OTA-protein conjugate and was capable to detect 10 µg/kg of OTA in 10 min. Supramolecular solvent made up of reverse micelles of decanoic acid, dispersed in a continous phase of tetrahydrofuran/water, was proposed for the microextraction of OTA in wheat prior to HPLC-FL, with no clean-up of the extracts or solvent evaporation. OTA recoveries ranged from 84% to 95%, precision (RSD) was 2% and the LOQ was 1.5 µg/kg (García-Fonseca *et al.*, 2010).

Molecularly imprinted polymers (MIPs) are considered as polymeric materials that mimic the functionality of antibodies. They pose great potential as artificial antibodies to be used for the selective detection of different analytes. In a study by Ali *et al.* (2010), SPE using MIP obtained recoveries close to 100% for OTA in wheat samples and had a significantly higher capacity when compared with IACs. The use of MIPs for the extraction and analysis of OTA was reviewed by Yu and Lai (2010). This review, with 22 other papers, was published as a special issue of Toxins entitled 'Ochratoxins'.

The development and characterisation of a monoclonal antibody against ochratoxin B (OTB) and its application in an indirect competitive ELISA was described by Heussner *et al.* (2010). A LOQ of 3.7 µg/l, a very low cross reactivity to OTA (2.7%) and its applicability to the analysis of beer, coffee and wine were reported in this study. Regarding OTB, Han *et al.* (2010b) reported a method for the simultaneous determination of both OTA and OTB in traditional Chinese medicines by UHPLC-MS/MS; the recoveries ranged from 86.3 to 114.2 % and the LOQs ranged from 0.03 to 0.19 µg/l, precision (RSD) was ≤13.1%. The study found only 4 out of 51 samples to be contaminated with ochratoxins at low levels.

Determination of OTA in wine continued deserving the attention of some research groups. Thus, Zezza *et al.* (2009) reported the development of a fluorescence polarisation (FP) immunoassay, based on a monoclonal antibody and an OTA-fluorescein tracer, for rapid screening of the toxin in red wine. The wine samples were diluted with methanol and passed through aminopropyl SPE columns prior to the FP assay. Average recoveries were 79% with a RSD of 11%. The LOD was 0.7 µg/l and the whole analysis was performed in less than 10 min. The method showed a good correlation (r = 0.9222) when compared with a HPLC-IAC clean-up method. No false-negative or positive results were observed using this FP immunoassay.

Tessini *et al.* (2010) studied the analytical parameters for sample pre-treatment in red wine using different IACs as well as C18 cartridges with three solvent combinations. Although repeatability and accuracy were statistically comparable, higher recoveries were obtained with the C18 cartridges when using acetonitrile as extraction solvent; lower limits of detection were obtained using IACs (0.01 µg/l) in comparison with C18 pre-treatment (0.09 µg/l). The results obtained by applying both methods in the analysis of 154 wine samples were also statistically comparable. Finally, the authors stated that considering cost, sensitivity and selectivity, the combination of C18 cartridges and HPLC-FL was the most appropriate method for being used in broad prospective programmes. The authors also evaluated the direct HPLC injection of wine without previous clean-up but concluded that this procedure was not useful at the concentration levels usually found in wine. Other approaches tested for the analysis of OTA in wine were the application of a direct competitive ELISA based on an OTA-horseradish peroxidase (OTA-HRP) synthesised conjugate (Radoi *et al.*, 2009) and the use of both gel-based and membrane-based flow-through immunoassay formats, combined with a clean-up column for the rapid non-instrumental detection of the toxin, with a cut-off level of 2 µg/l in wine (Rusanova *et al.*, 2009).

Apart from the wide application of HPLC-FL in the analysis of OTA in wine, Welke *et al.* (2010) reported the suitability of HPTLC using a charge-coupled device (CCD) for acquisition of fluorescence images of OTA, as a tool for its determination in red wine; the mean recovery and the LOQ were 92% and 0.1 µg/l, respectively. Romero-González *et al.* (2010) described the use of an optimised method for the determination of OTA and T-2 toxin (T-2) in wine and beer by hollow fibre liquid phase microextraction and UHPLC-MS/MS; recoveries were reported to be higher than 70% whereas inter-day precision was lower than 21%, the LOQs were 0.02 and 0.09 µg/l.

Some papers dealt with attempts for reducing the levels of OTA in foodstuffs. Solfrizzo *et al.* (2010) proposed to reduce OTA levels by repassage of contaminated musts or wines over grape pomaces from the same grape variety having no or little OTA contamination. This treatment was found to remove up to 65% OTA in 24 h. The capacity of *Oenococcus oeni* to eliminate OTA from synthetic media was studied by Mateo *et al.* (2010) recording mycotoxin reductions higher than 60% in 14-day cultures spiked with 2 µg OTA/l. Ponsone *et al.* (2009) reported that OTA levels dropped to an average of about 86.5% in a pilot scale vinification study to obtain red wine using two red grape varieties (Bonarda and Tempranillo); the type of *Saccharomyces* showed no effect on toxin reduction. On the other hand, Iha *et al.* (2009) reported that combination of washing, soaking and cooking was able to eliminate about 50% of the toxin from whole dried beans.

As in former years, there were many papers reporting data on the presence of OTA in a number of commodities from different countries. These data indicate the wide occurrence of OTA in agricultural commodities and some products of animal origin. In a study on OTA in human

blood samples, Märtlbauer *et al*. (2009) estimated on a worldwide basis, that the mean of means for OTA in human serum was 700 pg/ml corresponding to a mean daily OTA intake of 940-1,380 pg/kg bw; the authors stated that this level was relatively stable over the last decades, being well below published tolerable daily intake values. OTA was reported in blood plasma in Spain (Coronel *et al*., 2009), Tunisia (Karima *et al*., 2010) and Turkey (Erkekoğlu *et al*., 2010), in breast milk in Turkey (Gürbay *et al*., 2010), in milk and blood of lactating women in Chile (Muñoz *et al*., 2010), and in urine in Turkey (Akdemir *et al*., 2010). Duarte *et al*. (2010a) monitored the OTA exposure of the Portuguese population through a nationwide urine survey, the prevalence incidence was higher than 95% in samples taken in four out of the five locations studied.

OTA occurrence in cereal, cereal-derived food products and non-conventional exposure sources was reviewed (Duarte *et al*., 2009, 2010b). The presence of OTA has been reported in infant formula milks (Meucci *et al*., 2010), in dry-cured ham (Dall´Asta *et al.,* 2010) in Italy, in spices and dried fruits in Tunisia (Zaied *et al.,* 2010), in cereal products in Chile (Vega *et al.,* 2009), in white and black pepper products in Malaysia (Jalili *et al*., 2010), in coffee in Japan (Noba *et al*., 2009), in wine in Turkey (Altiokka *et al*., 2009), Croatia (Flajs *et al.*, 2009), Italy (Gambelli and D´Addezio, 2009; Spadaro *et al*., 2010) and Spain (Murillo-Arbizu *et al.*, 2010). Zinedine *et al*. (2010) described the use of pressurised liquid extraction and LC with a limit of quantification of 0.25 µg/kg for the analysis of OTA in breakfast and infant cereals in Morocco. The presence of OTA in foodstuffs marketed in Hong Kong was reported by Chung *et al*. (2009). The occurrence of OTA in Moroccan foods was reviewed by Zinedine (2010).

Many papers gave account of the co-occurrence of OTA with other mycotoxins: with aflatoxins in liquorice products (Pietri *et al*., 2010), with aflatoxins and ZEA in paprika and chilli in Spain (Santos *et al.,* 2010), with aflatoxins, ZEA, DON, fumonisins and T-2 in Jordan (Salem and Ahmad, 2010), with $AFB_1$ in maize in Pakistan (Shah *et al.*, 2010), with aflatoxin, ZEA, DON and fumonisins in dog food in Austria (Böhm *et al*., 2010), with DON, T-2, sum of T-2 + HT-2 toxins, fumonisins and total ergot alkaloids in horse feed in Germany (Liesener *et al*., 2010), with aflatoxin in baby food in Portugal (Alvito *et al.*, 2010), with fumonisins in retail foods in Japan (Aoyama *et al*., 2010), with $FB_1$, aflatoxin, DON and ZEA in food in Cameroon (Njobeh *et al*., 2010), with aflatoxins in dairy cattle feed and milk in Sudan (Elzupir *et al*., 2009), with ZEA, DON, aflatoxin, fumonisins, ergovaline and lolitrem B in Australian pasture and silage (Reed and Moore, 2009), with aflatoxins, ZEA, DON, T-2, citrinin and fumonisins in medicinal and aromatic herbs in Spain (Santos *et al*., 2009), with $AFB_1$, $AFM_1$, trichothecenes and fumonisins in 24-hour diets of children in the Netherlands (Bakker *et al.*, 2009), and with aflatoxins in dried fruits (Bircan, 2009b), in maize samples (Giray *et al.*, 2009) and in high-sugar-content traditional Turkish foods (Senyuva *et al.,* 2009).

## 9. Patulin

Patulin serves as a good quality indicator of fruits used in the processing of apple juice. Gaspar and Lucena (2009) described a simple HPLC method for the simultaneous determination of furfuraldehydes (2-furfural (2-F), 5-methylfurfural (5-MF) and 5-hydroxymethylfurfural (5-HMF) and patulin in food products. The compounds were simultaneously determined by reversed-phase HPLC using a C18 column and photodiode array detection. The method showed recoveries of furfuraldehydes from 6% to 100% and the recovery of patulin was about 90%. The authors applied the method to different commercial food matrices, including honey, white and demerara table sugars, white and red balsamic vinegars, caramel, nutritional supplement, sugar substitute, apple juice (clear and cloudy) and also to a mould infected apple. The LODs for patulin, 5-HMF, 5-MF and 2-F were 0.09, 0.06, 0.23 and 0.73 µg/kg respectively.

Gashlan (2009) determined patulin in apple juice by extraction with ethyl acetate and clean-up using sodium carbonate solution. Patulin was then determined by reversed-phase HPLC using a 25 cm Zobraz XDB C18 column. The occurrence of patulin was investigated in 120 samples of apple juice commercially available in Saudi Arabia with a concentration range from 0.057 to 0.104 µg/ml. A survey was conducted by Murillo-Arbizu *et al.* (2009) to determine levels and dietary intake of patulin from apple juice consumed in Spain. One hundred samples of apple juice were bought from distinct supermarkets and patulin was extracted by liquid-liquid extraction and analysed by micellar electrokinetic chromatography (MEKC). Sixty-six percent of the samples contained patulin over the LOD (0.7 µg/l). The mean and median levels of patulin obtained were 19.4 and 4.8 µg/l, respectively, in a range between 0.7 and 118.7 µg/l. In 11% of the samples, patulin contamination exceeded the maximum permitted level of 50 µg/l established by EU regulation. In Spain, no significant variations were observed with respect to data published 15 years ago. Barreira *et al.* (2010) validated an analytical procedure based on HPLC with SPE clean-up and UV detection (SPE-HPLC-UV) for the analysis of 144 apple-based-foods, including 68 apples juices (32 clear and 36 cloudy) and 76 homogenised apple purees marketed in Portugal. Patulin was detected in 33 samples, with values ranging 1.2 µg/kg to 42 µg/kg. Patulin was not detected in the infant drinks, but its presence was quantified in five homogenised apple puree samples labelled as being intended for consumption by infants and young children. High incidence of positive samples was detected in cloudy juices (67%) when compared with clear ones (13%). With respect to production mode, the incidence of positive

samples was 20% and 24% of organic and conventional origin, respectively.

Several methods for control and/or elimination of patulin have been proposed, as no unifying method has been commercially successful for reducing patulin. Dong *et al.* (2010) evaluated exposure to germicidal UV radiation as a possible commercially viable alternative for the reduction and possible elimination of patulin in fresh apple cider. UV exposure of 14.2 to 99.4 mJ/cm$^2$ resulted in a significant and nearly linear decrease in patulin levels while producing no quantifiable changes in the chemical composition or organoleptic properties of the cider. For the range of UV doses tested, patulin levels decreased by 9.4% to 43.4%.

Jalali *et al.* (2010) analysed patulin in various samples of commercial apple juice from a factory in south-western Iran. The samples were cleaned-up by C18 SPE and analysed by HPLC using UV detection. The mean concentration of patulin in apple juice was 26-92 µg/l. Apples are a seasonable product so it is necessary to apply strategies to enlarge the life of the product. Several post-harvest treatments were discussed to optimise the effect of low temperatures.

Morales *et al.* (2010) reviewed the effects of biocontrol agents and controlled atmosphere on apple decay and patulin accumulation compared to conventional technologies. Welke *et al.* (2009) reviewed the main patulin properties, occurrence, toxicological importance, and methods for patulin detection and control during apple juice production.

## 10. Trichothecenes

Trichothecenes are a group of approximately 150 mycotoxins. They are mainly produced by species of *Fusarium*. Type A and B trichothecenes are commonly known as contaminants of food and feed, mainly cereal-based crops. Within the EU maximum levels currently exist for DON in cereals and maize. A review, with 60 references, on advances in analytical methods for trichothecene mycotoxins in cereals and cereal-based food products was published (Lattanzio *et al.,* 2009).

The number of new publications on the use of gas chromatographic (GC) methods is relatively small. A new analytical method for the determination of ZEA, DON, fusarenone-X (FUS-X), 15-acetyl-DON (15-ADON) and nivalenol (NIV) in breakfast cereals and flours by heart-cutting GC-MS has been developed and validated (Cunha and Fernandes, 2010). Extraction was performed with acetonitrile, applying a modified QuEChERS procedure, and the extracts were analysed after silylation. Acceptable recoveries for all mycotoxins at two different spiking levels (20 and 100 µg/kg) were achieved with good repeatability (from 9 to 21%). LOD ranged from 2 to 15 µg/kg and LOQ ranged from 5 to 50 µg/kg. GC with electron capture detection has been applied for the determination of DON in medicinal herbs and related products. Sample was extracted with water followed by IAC clean-up. DON was determined as its heptofluorobuturyl ester. Positive results were further confirmed by GC-MS (YanTao *et al.,* 2010). An LC-ESI-MS/MS method using a $^{13}C_{15}$-DON internal standard was used as a reference procedure for an in-house method comparison study of the determination of DON in wheat materials (Neuhof *et al.,* 2009). Alternative methods included LC-diode array detection (DAD), and GC after trimethylsilylation with electron capture detection (ECD) or a chemical ionisation mass spectrometry detector. This is claimed to be the first report of a GC-MS method for the determination of DON using a fully carbon-labelled $^{13}C_{15}$ -DON as an internal standard (IS) and produced comparable results to the $^{13}$C-IS-LC-MS/MS reference method with a similar sensitivity. ECD was slightly less sensitive, but is also suited for DON analysis in wheat.

HPLC coupled to a variety of detectors is a common technique for trichothecene analysis. Bononi *et al.* (2009) reported a method using HPLC-DAD and purification of the extract with Mycosep #227 (LOD 50 µg/kg). Antonios *et al.* (2010) reported a method for determining DON in crushed wheat (bulgur) using an HPLC-UV. Two papers describe the determination of DON by using IACs prepared by sol-gel method for sample clean-up. Klinglmayr *et al.* (2010) entrapped anti-DON antibodies and quantified DON by HPLC-UV. Brenn-Struckhofova *et al.* (2009) co-immobilised anti-DON and anti-ZEA antibodies. Concentrations of DON and ZEA were determined with HPLC-UV and HPLC-fluorescence detection, respectively. Dombrink-Kurtzman *et al.* (2010) developed an HPLC-UV method to detect DON at low concentrations in infant food. An aqueous extract of infant cereal was cleaned up by IAC. The LOQ was 10 µg/kg for 3 types of infant cereals, the LOD was 5 µg/kg. The European Committee for Standardization (CEN) have approved an IAC HPLC-UV method for determination of DON in cereals, cereal products and cereal based foods for infants and young children (CEN, 2010c). The determination of DON in wheat flour by HPLC-DAD detection was optimised by Moazami and Jinap (2009). The influence of three variables (mobile phase composition, flow rate and wavelength) were evaluated. Efficiencies of four clean-up procedures for wheat flour extract were compared. Recoveries of DON were: Mycosep #225 (99%); Mycosep #227 (65%); IAC (53%) and Oasis(R) HLB column (42%).

The bulk of published methods for trichothecenes continued to use HPLC-MS, some detecting trichothecenes only, while others measured a diverse range of toxins. Bononi *et al.* (2009) reported simplified and optimised operation parameters for routine analysis of DON in pasta. A method based on HPLC, with atmospheric pressure

photoionisation (APPI) MS, has been developed for NIV and DON. Chemical noise and signal suppression of analyte signals due to matrix interferences were investigated for APPI (Tanaka *et al.*, 2009). This method was used to survey the natural occurrence of NIV, DON, FUS-X, 3-acetyl-DON (3-ADON), HT-2, T-2 and ZEA in biscuits in Japan. Further confirmation was performed by LC/TOF-MS) (Tanaka, *et al.,* 2010.)

There is an increase in the number of publications using the QuEChERS method. An extraction and purification method for the LC-MS determination of five mycotoxins, diacetoxyscirpenol (DAS), T-2, HT-2, DON and NIV, optimised using a modified QuEChERS method, was reported by Sospreda *et al.* (2010). Desmarchelier *et al.* (2010) compared use of a QuEChERS-like method and accelerated solvent extraction (ASE) for LC-MS determination of 17 mycotoxins including T-2, HT-2, NIV, DON, 3-ADON, 15-ADON, FUS-X, DAS, and neosolaneol (NEO) in cereal-based commodities. Both gave similar performances in terms of linearity and precision. The QuEChERS-like method was found to be easier to handle and allowed a higher sample throughput. Two methods focussed on animal feed products. The first was for determination of 27 mycotoxins and other secondary metabolites in maize silage. Extraction was based on the QuEChERS method. The method was successfully validated for determination of eight analytes qualitatively and 19 quantitatively. Matrix-matched calibration standards were used giving recoveries ranging from 37% to 201% with the majority between 60% and 115% (Rasmussen *et al.,* 2010). The second was validated for the detection of 23 mycotoxins including DON, T-2, HT-2, NIV, 3-ADON, 15-ADON, DAS, FUS-X and NEO (Monbaliu *et al.,* 2010).

Methods with no clean-up were reported. An APCI LC-MS/MS (SRM mode) method for several fusariotoxins was reported by Santini *et al.* (2009). Three different methods for sample preparation and for the extractions of NIV, DON, FUS-X, 3-ADON, DAS, HT-2, T-2, NEO, zearalanone (ZAN) and ZEA were tested. One extraction method, (84% (v/v) acetonitrile aqueous solution), was validated without any further clean-up step. Frenich *et al.* (2009) also reported a method to determine 12 mycotoxins (DON, $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $AFM_1$, $FB_1$, $FB_2$, OTA, HT-2, T-2 and ZEA) in maize, walnuts, biscuits and breakfast cereals. The method is based on a single extraction step followed by UHPLC-MS/MS, the selectivity of the MS/MS detection allowed the elimination of further clean-up. Matrix-matched calibration was used for quantification and recoveries of the extraction process ranged from 70.0% and 108.4%. A method for the determination of eleven mycotoxins with little sample manipulation using UHPLC-MS/MS was reported (Beltrán *et al.,* 2009). The mycotoxins were separated by gradient elution in only 4 minutes. Satisfactory recoveries were obtained (70-110%). Quantification of samples was carried out using matrix-matched standards. DON could be only validated at 200 µg/kg, due the poor sensitivity for this mycotoxin.

Gottschalk *et al.* (2009) reported a LC-MS/MS method for the determination of type A, B and D trichothecenes in cereals. The method was applied to 289 samples of wheat, rye and oat products. DON, NIV, T-2 and HT-2 were detected in a range of samples. Another UHPLC-MS/MS method was developed and validated by Romero-Gonzalez *et al.* (2009) for the analysis of mycotoxins ($AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $AFM_1$, $FB_1$, $FB_2$, DON, OTA, HT-2, T-2 and ZEA) in beers. Mycotoxins were extracted by C18 SPE. Di Mavungu *et al.* (2009) reported LC-MS determination of mycotoxins in food supplements. The analytes included NIV, DON, 3-ADON, 15-ADON, NEO, FUS-X, DAS, HT-2 and T-2, as well as 14 other mycotoxins. A more recent development is the use of approaches that enable retrospective data analysis and identification of unknown signals in sample extracts. Two procedures have been validated for the determination of 11 *Fusarium* toxins. In both cases, UHPLC coupled with HRMS were employed. C-13 isotopically labelled surrogates as well as matrix-matched standards were used for quantification. For TOF-MS, the use of modified QuEChERS was required to obtain low detection limits. Direct analysis of crude extracts could be carried out by the use of Orbitrap technology (Zachariasova *et al.*, 2010).

A range of methods based on alternative techniques have been published. Calibration models for durum wheat, common wheat and durum+common wheat samples, were obtained by De Girolamo *et al.* (2009) using Fourier transform near-infrared spectroscopy (FT-NIR) for rapid and non-invasive analysis of DON. The model correctly classified 69% of 65 validation samples with most misclassified samples (16 of 20) showing DON contamination levels quite close to the cut-off level. Another study explored the feasibility of the Raman technique for rapid and non-destructive screening of DON-contaminated wheat and barley meal. The results indicate that Raman spectra can be used to classify low from high DON grain (Liu *et al.,* 2009).

Several antibody-based methods have been reported. A rapid lateral flow assay was developed by Yang *et al.* (2010a). A colloidal gold immunochromatographic strip (ICS) was used for detection of DON. The test was completed in 10 minutes and results for DON in wheat and maize were in good agreement with data from ELISA and GC-MS. The ICS test can be used as a qualitative tool for screening DON on-site. Another immunochemical-based test for non-instrumental detection of ZEA and T-2 in feed was developed by Basova *et al.* (2010). The method combined clean-up, pre-concentration by immunoextraction and immunodetection through the enzymatic reaction of

horseradish peroxidase (HRP). The test is housed inside a standard 1-ml SPE column and consists of three layers: two test layers (one each for ZEA and T-2) with immobilised specific antibodies and one control layer with bound anti-HRP antibodies. Total assay time was about 15 minutes for six samples.

Kadota *et al.* (2010) reported a SPR immunoassay to measure NIV and DON in wheat. A highly sensitive and stable DON-immobilised sensor chip was prepared. The assay used a monoclonal antibody that cross-reacts with NIV and DON. It can rapidly screen NIV and DON co-contamination of wheat and is one of a very few immunoassays to detect NIV directly. A second SPR screening assay has been developed for the combined detection of T-2 and HT-2 in cereals using a sensor chip coated with an HT-2 derivative and a monoclonal antibody. The antibody raised against HT-2 displayed high cross-reactivity with T-2, whereas there was no cross-reaction observed with other commonly occurring trichothecenes (Meneely *et al.,* 2010).

The use of a 96-well electrochemical plate for the detection of DON and NIV in wheat was reported by Ricci *et al.* (2009). DON and NIV were hydrolysed using a microwave hydrolysis procedure to give electroactive compounds that can be sensitively detected by the use of cheap screen-printed electrodes. Romanazzo *et al.* (2010) also reported an electrochemical method for the detection of DON in cereals and cereal-based food. The Enzyme-Linked-Immunomagnetic-Electrochemical (ELIME) assay, is based on the use of immunomagnetic beads (IMBs) coupled with eight magnetised screen-printed electrodes as electrochemical transducers.

## 11. Zearalenone

Most of the methods for the quantification of ZEA and its metabolites published last year employ LC-MS or LC-MS/MS. Perez-Torrado *et al.* (2010) utilised pressurised liquid extraction with methanol/acetonitrile to extract cereal flours. The extracted samples were consecutively analysed with LC-ESI-quadrupole-MS in SIM mode without prior clean-up. Both polarities were used for ionisation of ZEA, with higher sensitivity in the negative ion mode. Method recovery, evaluated at a single spiking level, was about 75%. Shin *et al.* (2009) determined ZEA in rat serum. After spiking with ZAN as internal standard the serum was extracted with *t*-butylmethyl ether. Samples were injected without any clean-up and measured with a LC-ESI-triple quadrupole MS/MS in SRM mode. Extraction recovery was determined to be ≥102% for ZEA, while the accuracy was 82-91% depending on the concentration. Kaklamanos *et al.* described two methods for the determination of anabolic steroids, including ZEA, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL), in bovine urine (2009a) and muscle tissue (2009b), respectively. The samples were spiked with deuterated internal standards (including α-ZAL-d4 and β-ZAL-d4), enzymatically hydrolysed, extracted with *t*-butylmethyl ether and defattened with hexane. After a two-step SPE clean-up using polymeric reversed phase and amino cartridges, the extracts were analysed with a LC-APCI-triple quadrupole-MS/MS in SRM mode with positive ionisation. Accuracies of 71-122% were reached in bovine urine, while 73-117% were obtained in spiked meat samples for the five compounds.

ZEA is often determined along with other mycotoxins using LC-MS/MS multimethods. Several such methods have been published last year. Romero-Gonzalez *et al.* (2009) developed a method for the determination of 12 mycotoxins, including ZEA, in beer. After C18-SPE clean-up, extracts were analysed by UHPLC-ESI-triple quadrupole-MS/MS in positive ion mode. ZEA was recovered between 80 and 91% with repeatabilities of 9-12%. Similarly, Beltrán *et al.* (2009) presented a method capable of determining 11 mycotoxins, including ZEA, in different cereal based food matrices. After extraction using acidified acetonitrile/water, the samples were diluted and directly injected into a UHPLC-ESI-triple quadrupole-MS/MS system. Ionisation was performed in positive mode for all mycotoxins, except ZEA. The total run time for a sample was 7 minutes. ZEA was recovered between 61-66% in maize kernels, around 88% in pasta and between 79-103% in multi-cereal baby food. A very similar method was presented by Frenich *et al.* (2009). Twelve mycotoxins, including ZEA, were quantified in cereals and cereal based food. Samples were extracted with acetonitrile/water, filtered and injected into UHPLC-ESI-triple quadrupole-MS/MS system. Ionisation was performed solely in positive ion mode and the run time for a single sample was 8.5 minutes. ZEA was recovered between 74 and 103% dependent on the matrix and the spike concentration. Di Mavungu *et al.* (2009) presented a method for the determination of 24 mycotoxins, including ZEA, in food supplements. Samples were extracted with ethyl acetate/formic acid, defatted with hexane and further cleaned-up with polymeric reversed phase SPE. Extracts were analysed using a UHPLC-ESI-triple quadrupole-MS/MS system, which was operated in positive ion mode. ZEA was recovered between 92-98%. Rasmussen *et al.* (2010) described a method for the determination of 27 mycotoxins and other secondary metabolites, including ZEA, in maize silage. Extraction/clean-up was performed by a modified QuEChERS method. The final extract was injected twice in a LC-ESI-triple quadrupole-MS/MS system to allow ionisation in both polarities. ZEA was recovered at 90±12%. Vishwanath *et al.* (2009) expanded a previous method to analyse indoor matrices like house dust for 186 fungal and bacterial metabolites, including ZEA and several of its metabolites. Samples were extracted with acidified acetonitrile/water, diluted and analysed in two runs (covering both ion modes) by LC-ESI-triple

quadrupole-MS/MS. ZEA was extracted well from dust (80% recovery), but showed strong matrix suppression effects, yielding an apparent recovery of just 42%. Finally, also high resolution MS was used for multi-mycotoxin analysis. Zachariasova *et al.* (2010) reported on the analysis of 11 *Fusarium* mycotoxins, including ZEA, in cereals. A modified QuEChERS extraction was followed by addition of $^{13}C$ labelled internal standards and analysis by UHPLC-ESI-TOF-MS. ZEA was recovered between 60-113% in different cereals using external calibration and between 97-98% using internal calibration.

Fluorescence detection of ZEA after HPLC separation is an alternative to mass spectrometry. Majerus *et al.* (2009) presented such a method for edible oils. Oil samples were extracted at pH 9 with methanol/aqueous ammonium carbaminate. The methanol layer was then neutralised, evaporated and re-dissolved in acetonitrile/water in an ultrasonic bath, before injection in a HPLC-FL system. Recovery of ZEA was 87-91%.

Three papers reported on the use of GC-MS to quantify ZEA along with other compounds. Shen *et al.* (2010) developed a method for six resorcylic acid lactones in feed. Samples were extracted with methanol and cleaned-up using liquid-liquid extraction and polymeric reversed phase SPE. Extracts were derivatised with BSTFA and TMCS before analysis with GC-MS. ZEA was recovered between 86-97%. Dickson *et al.* (2009) extended a GC-MS method for the analysis of resorcylic acid lactones and stilbene anabolic steroids in veal liver also for ZEA, α-ZOL and β-ZOL. Tissue was homogenised, acidified and enzymatically hydrolysed. After addition of acetonitrile, hexane and $CH_2Cl_2$ the middle acetonitrile layer was alkalised and further purified on a polymeric SAX column before GC-MS measurement. Recoveries for the 3 analytes were between 29 and 67%. The third method (Cunha and Fernandes, 2010) used a QuEChERS procedure before the GC-MS determination of ZEA and four other mycotoxins in breakfast cereals and flours. ZAN was used as internal standard and total run times of 8 minutes per sample were achieved. ZEA was recovered at 84% from flour and at 96% from breakfast cereals.

Several novel immunoassays for the determination of ZEA were developed. Burmistrova *et al.* (2009) described the application of an anti-ZEA monoclonal antibody in an ELISA, a flow-through gel-based immunoassay column and a flow-through membrane-based immunoassay. Wheat samples were extracted with methanol water. Extracts were diluted and measured with the different assays. $IC_{50}$ values of about 100 µg/kg were reached for the ELISA, while the flow-through immunoassays showed cut-off values at the same concentration. Basova *et al.* (2010) developed an immunochemical test for the detection of ZEA and T-2 in feed. Samples were extracted with acetonitrile/water and diluted. Extracts were pushed through an amino SPE clean-up column as well as the test column, containing a ZEA test layer with immobilised antibodies, a T-2 test layer and a control layer. The test column was washed and a mixture of ZEA protein conjugate and T-2 toxin protein conjugate in buffer was applied. The columns were incubated and washed again before the chromogenic substrate was applied, which itself was removed by air from the columns after incubation. The intensity of the colour was visually evaluated and was inversely proportional to the level of the analytes. Cut-off levels of 100 µg/kg were evaluated for both toxins.

Coupling immunoassays for ZEA determination with magnetic beads results in extremely sensitive methods, as shown in several publications. Mak *et al.* (2010) developed a giant magnetoresistive-based immunoassay for the detection of ZEA, HT-2 and $AFB_1$. A multiplex magnetic nanotag-based detection platform was created by combining a sandwich immunoassay with magnetic detection. The sample is given to an antibody-immobilised spin-valve sensor array before a biotinylated detection antibody is added. Once streptavidin-linked magnetic nanotags are added, the sensor response can be recorded in real time. Hervas *et al.* (2009a) published an electrochemical immunoassay with magnetic beads for the determination of ZEA in baby food. The direct competitive immunoassay employed antibody-coated magnetic beads and horseradish peroxidase as enzymic label. After addition of substrate and mediator, amperometric detection was performed. Recovery rates between 95 and 108% were achieved. A slightly different test format was introduced by the same authors, using electrochemical microfluidic chips coupled to magnetic bead-based ELISA (Hervas *et al.*, 2009b). The main advantage of this method was the fast sample analysis in slightly over 3 minutes. Finally, the same authors presented their electrochemical immunosensor together with magnetic beads on disposable carbon screen-printed electrodes (Hervas *et al.*, 2010). After the immunoassay, the magnetic beads were transferred to the surface of a screen-printed electrode where the electrochemical detection takes place. Recovery rates of 101-111% in baby food samples were evaluated, while the detection limit was in the low ng/l range.

Two different biosensors to detect ZEA have been introduced last year. Lim *et al.* (2009) fabricated a metal-oxide-semiconductor field-effect-transistor-based biosensor. A self-assembled monolayer on a gold surface was used to immobilise anti-ZEA antibody. After addition of buffered sample, a platinum electrode was employed as reference electrode. Vaelimaa *et al.* (2010) developed a bioluminescent whole-cell biosensor for the detection of ZEA, α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL in milk products. A genetically modified *Saccharomyces cerevisiae* strain was used as detection system. This yeast strain produces

luciferase in the presence of estrogenic compounds, which will emit light after addition of the substrate. The detection capability is proportional to the estrogenicity of the analytes and in the low µg/l range for ZEA.

Two different novel approaches for the clean-up of ZEA have been presented. Brenn-Struckhofova *et al.* (2009) developed sol-gel immunoaffinity columns for the co-isolation of ZEA and DON in various matrices. 96-104% of the spiked ZEA were recovered from wheat, 100-106% from cornflakes, 106-112% from chocolate bars, 100-104% from almonds and 82-92% from rusk. Siegel *et al.* (2010c) retained ZEA from edible oils by hydrazone formation on a polymer resin functionalised with hydrazine groups and subsequently released it by hydrolysis. Recovery of ZEA was 89±7%.

A rapid method to determine ZEA (or other mycotoxins) involves fluorescence polarisation. Basic principles of fluorescence polarisation and its application to quantify mycotoxins has been recently reviewed (Maragos, 2009).

ZEA is prone to be metabolised by growing plants and glucosides can occur in naturally infected cereals as masked mycotoxins, contributing to the total mycotoxin load of food and feed. In this context, three masked mycotoxins ZEA-4-glucoside, α-ZOL-4-glucoside and β-ZOL-4O-β-D-glucoside were produced using a genetically modified yeast strain, expressing an *Arabidopsis* UDP-glucosyltransferase (Berthiller *et al.*, 2009b). The analytical standards gained were used to develop a LC-MS/MS based method for ZEA, DON and their major masked metabolites in cereal-based food (Vendl *et al.*, 2009). The final method avoids clean-up and resulted in 93-124% recovery for ZEA, 79-108% for ZEA-4-glucoside, 88-123% for α-ZOL, 78-113% for α-ZOL-4-glucoside, 106-120% for β-ZOL and for 85-119% β-ZOL-4O-β-D-glucoside in various cereal-based matrices.

## 12. Mycotoxins in botanicals and spices

There were several HPLC methods published that validated the analysis of aflatoxins in botanicals and OTA in spices. One publication modified the current AOAC Official Method 991.31 for aflatoxins in maize, raw peanuts, and peanut butter and applied the method to aflatoxins in botanical roots (Weaver and Truchsess, 2010). The samples were extracted with acetonitrile/water (84:16, v/v) instead of methanol/water used in the AOAC 991.31 method. The extracts were then centrifuged, diluted with phosphate-buffered saline, filtered, and added to an IAC for purification. The column was then washed with water, eluted with methanol and the mycotoxins quantified by HPLC using florescence detection. This method was found applicable to the analysis of black cohosh, echinacea, ginger, ginseng, kava kava, and valerian. Another method was validated for the determination of OTA in red paprika and black pepper (Bononi *et al.*, 2010). This method used a SPE column prior to HPLC analysis with fluorescence detection. The relative standard deviation for repeatability of the method was 11.8% for red paprika and 9.9% for black pepper. A method was published that performed a comparison of analytical methods for aflatoxin determination in chilli spice (O'Riordan and Wilkinson, 2009). This method compared extraction solvents, IAC purifications, derivatisation techniques, and ELISA versus HPLC analyses.

This past year there been several surveys of mycotoxins in botanicals and spices published from China, Tunisia, Spain, Brazil, and Malaysia. A total of 126 samples of white and black pepper in Malaysia were analysed for aflatoxins by HPLC with florescence detection using immunoaffinity column purification (Jalili *et al.*, 2009). Seventy of the samples (55.5%) were found contaminated with aflatoxins ranging from 0.1 to 4.9 µg/kg. $AFB_1$ had the highest incidence and was found in all the contaminated samples, with black peppers being more contaminated than white peppers. A total of 112 samples of spices purchased from markets in 24 regions of Tunisia were analysed for OTA by HPLC using fluorescence detection with IAC purification (Bouaziz *et al.*, 2010). The average levels of contamination for OTA found in the spice samples were 224, 206, 290, 274 and 203 µg/kg, respectively for caraway, coriander, curcuma, black pepper and red pepper. This was the first survey to be carried out on the natural occurrence of OTA in the main spices consumed by the Tunisian population. Another survey involved analysing for OTA in traditional Chinese medicinal plants, commonly used as spices, additives or foods, and used in China to prevent and cure human disease (Yang *et al.*, 2010b). The samples were extracted with methanol/water, purified using IAC, and analysed by HPLC with fluorescence detection. The positive results were further confirmed by LC-ESI-MS/MS. A total of 57 botanicals were collected from 6 different areas with approximately half the samples visibly mouldy. The results showed that 23 of the visibly mouldy samples and 2 of the not visibly mouldy samples were contaminated with OTA ranging 1.2-158.7 and 2.5-5.6 µg/kg, respectively. This was the first report of the natural occurrence of OTA in traditional Chinese medicinal plants.

Seventy paprika samples collected in Sao Paulo, Brazil were analysed for aflatoxins and OTA using IAC purification and HPLC with fluorescence detection (Shundo *et al.*, 2009). Aflatoxins were found in 82.9% of the samples ranging from 0.5 to 7.3 µg/kg with a mean of 3.4 µg/kg. OTA was found in 85.7% of the samples at concentrations ranging from 0.24 to 97.2 µg/kg with a mean of 7.0 µg/kg. Sixty-four paprika and 35 chilli samples were purchased in Spain and analysed for the co-occurrence of aflatoxins, OTA and ZEA (Santos *et al.*, 2010). The occurrence of these mycotoxins in paprika was 59% for aflatoxin, 98% for OTA and 39% for ZEA, whereas in the chilli samples

the contamination was 40% for aflatoxins, 100% for OTA and 46% ZEA. None of the samples had aflatoxin levels higher than the legally allowable limits. There was co-occurrence of the mycotoxins reported with 75% of the paprika samples and 65% of the chilli samples containing more than one mycotoxin.

The migration of aflatoxin from botanicals to tea has been reported for the first time by Iha and Trucksess (2010). At a 100 °C steeping temperature, approximately 30-40% of aflatoxins and 20-30% of OTA in a contaminated ginger sealed in a tea bag were found in the ginger tea.

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