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Developments in mycotoxin analysis: an update for 2016-2017

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REVIEW ARTICLE

Abstract

This review summarises developments in the determination of mycotoxins over a period between mid-2016 and mid-2017. Analytical methods to determine aflatoxins, *Alternaria* toxins, ergot alkaloids, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone are covered in individual sections. Advances in proper sampling strategies are discussed in a dedicated section, as are methods used to analyse botanicals and spices and newly developed LC-MS based multi-mycotoxin methods. This critical review aims to briefly discuss the most important recent developments and trends in mycotoxin determination as well as to address limitations of the presented methodologies.

Keywords: analysis, sampling, botanicals, multimycotoxin analysis, aflatoxin, *Alternaria* toxins, ergot alkaloids, fumonisin, ochratoxin A, patulin, trichothecene, zearalenone

1. Introduction

This article is the latest instalment in a series of annual reviews highlighting analytical method developments for mycotoxin determination, continuing from the previous paper covering the 2015/2016 period (Berthiller *et al.*, 2017). The primary purpose is to raise awareness of the developments and advances in analytical methods for mycotoxins, derived from articles published between mid-2016 to mid-2017. Critical comments on the methods, their validation parameters or applications are usually added to guide readers in assessing their impact. Rather than to provide an exhaustive list of publications, a selection of the most relevant advances in analytical methodology should render the whole article interesting to read both for mycotoxin veterans and newcomers in the field. The topics covered in detail are sampling (Section 2), multi-mycotoxin 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).

Several review articles, providing a good overview about the determination of mycotoxins in food and feed, have been published recently. For instance, current methods for the determination of aflatoxins, fumonisins, ochratoxins, PAT, trichothecenes and ZEA, among information about

the occurrence and toxicity of the toxins were summarised (Alshannaq and Yu, 2017). Another review focusses on currently used analytical methods for the determination of aflatoxins in different food matrices, including sampling, extraction, purification, separation and determination methods (Xie *et al.*, 2016). Advances in the development of biosensors for mycotoxin detection were covered recently as well (Chauhan *et al.*, 2016). Available mycotoxin aptamers and the biosensing platforms into which they have been incorporated were compiled and discussed (Ruscito *et al.*, 2016). Finally, a very interesting critical review sheds light on the current status of methods of analysis for mycotoxins and suggests more focus on the development of on-site tests, which can be used to better cope with the mycotoxin problem in developing countries (Shephard, 2016).

Recently, the European Commission fostered the release of two guidance documents, one concerning the identification criteria for mycotoxins (EC, 2016) and the other relating to the estimation of the limit of detection (LOD) and limit of quantification (LOQ) for several classes of contaminants in the frame of food law enforcement (Wenzl *et al.*, 2016). It can be envisaged that these guidance documents will be valuable in the future when the methods are validated, given that they foster a better harmonisation and transparency in the comparison of method performance data for 'official' mycotoxin methods.

2. Sampling

New research on sampling published this past year included work on the development (Elegbede *et al.*, 2017; Kılıç Altun *et al.*, 2017; Ozer *et al.*, 2017a,b) and assessment (Bouzembrak *et al.*, 2017; Lee *et al.*, 2016) of sampling plans. New research has also investigated the effectiveness of certain sampling and sample preparation procedures for the analysis of aflatoxins in groundnuts (Walker *et al.*, 2017).

Ozer *et al.* (2017a) examined the variability associated with sampling, sample preparation, and the analytical procedures used to analyse figs for aflatoxins in Turkey. This work used an experimental method that has already been applied to other mycotoxin/commodity combinations (Whitaker, 2006). In this study, the large and variable size of figs posed some challenges, as sampling variance is dependent on the number of items in a sample. The 10 kg composite lots in the balanced nested experimental design were comprised of anywhere between 350 to 960 individual figs. The authors adjusted the sampling variance measured in all lots to the average lot size of 584 figs in order to obtain models of variance for the sampling, sample preparation, and analytical method as functions of aflatoxin concentration. As observed for other mycotoxin/commodity combinations, sampling made the largest contribution to the total variance. The process of sampling to obtain 10 kg composites contributed over 99% to the total variance of the quantification of aflatoxins, followed by sample preparation using water slurry to obtain a test portion of 55 g (0.6%) and subsequent analysis of one aliquot by high performance liquid chromatography (HPLC) with fluorescence detection (FLD) (0.3%). There was an unexpectedly lower sampling variance for dried figs (coefficient of variation (CV)=69%), when compared to dried almonds (CV=145%) at the same concentration of aflatoxins of 10 µg/kg and laboratory sample size of 10 kg. The authors suggested this could be due to a greater percentage of dried figs containing aflatoxins than has been observed in commodities such as dried almonds. This finding highlights the necessity to consider different analyte/matrix combinations separately, as a 'one size fits all' sampling plan is not necessarily realistic.

Results from Ozer *et al.* (2017a) were used by the same authors to develop a model to estimate the probability of misclassifying lots of figs as compliant/non-compliant with respect to aflatoxin limits (Ozer *et al.*, 2017b). The model was based on the negative binomial distribution and was used to develop operating characteristics curves. The model was used to investigate the effect of changing sample size, accept/reject limits and the number of laboratory samples analysed on the probability of misclassifying lots. The model was incorporated into deliberations of the Codex Committee on Contaminants in Food, and was used to develop the sampling plan that was adopted into the Codex Alimentarius Commission's general standard. In the Codex sampling plan, 3×10 kg laboratory samples must all test less than the limit of 10 µg/kg total aflatoxins to be accepted. Ozer *et al.* compared the Codex sampling plan based upon their research, to the European Union (EU) possibility of testing 1×30 kg laboratory sample of figs subjected to further sorting or treatment. They found that more lots would be incorrectly accepted by the EU plan (1×30 kg) versus the Codex (3×10 kg) plan. They advised exporters to use the 3×10 kg plan if there is uncertainty surrounding the sampling plan to be used at destination, in order to more stringently screen exports.

The work of Elegbede *et al.* (2017) examined the effect of seasonality in consumption and occurrence of deoxynivalenol (DON) and ochratoxin A (OTA) amongst other potential chemical hazards in food samples. This was assessed within the context of the French Individual and National Food Consumption Survey and can influence the sampling plan. They observed that for adults, DON and OTA exposure was significantly higher in the 'warm season' (between April and September) when consumption and concentration data were both used from this time period. The various scenarios they examined suggested that seasonal exposure to DON and OTA were mainly driven by seasonality of concentration, as opposed to variation in the consumption of certain foods with season. For OTA, since this was only detected in bread and rice (for which consumption did not vary significantly with

season), changes with season were attributed in part to changes in the source of the grains used in these food products. The authors proposed guidelines for Total Diet Study sampling based on the objectives of the study: if the main objective is risk assessment then seasonality is not a critical issue, as estimated exposure to DON and OTA did not exceed tolerable intakes. However, if the main objective is exposure assessment, then seasonality must be considered. The sampling plan would therefore need to take into account the expected seasonal variation of food items, and samples obtained from different seasons should not be pooled together. For this research, the mean estimated exposure to DON and OTA were approximately 2-2.5 and 40-50 times lower than tolerable intakes, respectively. The relevance of seasonality when assessing risk can conceivably differ for other scenarios. The seasonality of mycotoxins and implications for sampling plans was also emphasised by the work of Kılıç Altun *et al.* (2017). In their work, the authors state there was a statistically significant difference in the concentrations of aflatoxin M_1 (AFM_1) in human milk collected in December versus June of the following year (15.8±7.3 vs 22.9±16.7 ng/l). This work highlights the importance of considering seasonality when developing sampling plans for exposure or risk assessment.

Lee *et al.* (2016) designed a risk-based sampling plan for surveillance sample assignments of chemical and biological hazards in feed, including aflatoxins and fumonisins. Their model used the binomial probability distribution and grouped feed producers based on their compliance history. They determined the average confidence interval from output of three equations developed for the construction of a binomial confidence interval, and used this average to determine the number of individual products needed to be analysed in order to estimate the violation rate of feed products with a desired accuracy. The violation rates of most feed products from a historical database did not fall within confidence intervals predicted from the validation data set. The authors attributed this to the naturally skewed distribution of mycotoxins in samples, the small sample sets used to develop and validate the model, as well as the heterogeneity of mycotoxins in feed products. However, with the ability to refine the model with additional data, the authors concluded that the risk-based sampling plan could provide a more effective and efficient risk management tool to improve compliance to feed safety standards. Bouzembrak *et al.* (2017) developed a model using Bayesian Networks to predict the presence of mycotoxins (and other hazards such as pesticide residues) in herbs and spices, by product and originating country. This work was undertaken to support a risk-based approach to monitoring, and to provide planners guidance to determine at which level of the supply chain herb and spice products should be sampled for mycotoxin analysis to adequately capture violations. The authors used notifications from the EU Rapid Alert System for Food and Feed and data from the Dutch national monitoring program for chemical contaminants in food and feed spanning a 10 year period to develop and validate their model. The model predicted a correct outcome (above limit, below limit, could not be determined, or unauthorised action) in 85% of the test cases.

Walker *et al.* (2017) investigated the effectiveness of the EU sampling protocol (European Commission, 2006) and UK enforcement sample preparation procedures on the analysis of in-shell groundnuts for aflatoxins. A thorough and very well-described experimental design (utilising two people to sample in-shell groundnuts and three laboratories to prepare laboratory samples plus water slurry sub-samples from the comminuted laboratory samples) was used to minimise biased sampling and biased analyses. Sampling personnel took increments from various positions of bags containing groundnuts, instrumental analysis was completed at one lab, and analytical batches were constructed to balance any effects from the individuals sampling groundnuts and the various laboratories used to prepare sub-samples for analysis. The results demonstrated the most significant effect is from variation amongst 10 kg laboratory samples of whole groundnuts taken from 30 kg aggregate samples, followed by variation within the 10 kg comminuted laboratory samples. The authors stated the smaller variation may be expected from random sampling effects, but may also reflect the heterogeneity of comminuted laboratory sample. It is also possible this variation reflects biased sub-sampling to obtain the test portions, as the tools and procedures used to obtain 1 kg sub-samples and 50 g test portions from the laboratory sample slurry were not described and could not be evaluated. The authors recommended further investigation into improving the slurry process, including addition of a non-foaming surfactant to improve homogenisation and disrupt conglomeration of groundnut particles within the water. Overall, the lack of statistical significance for sampling personnel and sample preparation laboratory indicated that these were not important as compared to the variance amongst laboratory samples in this situation. The results demonstrated that the EU-recommended sampling protocol of preparing 3×10 kg laboratory samples which all must be compliant with the maximum level, and the sample preparation procedures applied in UK enforcement laboratories, are effective in assessing compliance with EU limits for aflatoxins in bulk consignments of in-shell groundnuts.

3. Multi-mycotoxin LC-MS(/MS) methods

In line with previous years, also in the 2016-2017 period the interest in implementation of LC-MS(/MS) methods for routine multi-mycotoxin analysis, risk assessment and occurrence studies continues to be relevant. Most of the published methods describe extensions of previous procedures to further matrix/mycotoxin combination,

rather than development of new strategies. However, studies characterised by efforts in optimisation of sample preparation, extensive validation to understand method performances and evaluation of applicability using large sets of real samples could be found in literature and have been selected for this review. Among multi-contaminant methods, a shift from broad scope methods covering hundreds of contaminants to 'dedicated' methods covering a narrower range of contaminants for a specific scope or matrix could be observed.

An LC-MS/MS multi-mycotoxin method was developed for the major *Fusarium* toxins, including modified mycotoxins (DON, deoxynivalenol-3-glucoside (DON-3G), 3- and 15-acetyldeoxynivalenol (3-ADON and 15-ADON), HT-2 and T-2 toxins (HT-2 and T-2), enniatins B, B_1, A and A_1 (ENN B, ENN B_1, ENN A and ENN A_1), beauvericin (BEA) and ZEA), in beer (Habler *et al.*, 2017). The method was based on two sequential extraction steps with acetonitrile (ACN):water (70:30, v/v) followed by pass through a non-retentive solid phase extraction (SPE) cartridge (Bond Elut Mycotoxin®, Agilent, Santa Clara, CA, USA). The stable isotope dilution assay (SIDA) approach was used to compensate for matrix effects. Method validation resulted in intra-day and inter-day precision, and recoveries of 1-5, 2-8 and 72-117%, respectively. To prove its applicability the method was applied to 61 different organic and conventional beer samples. In summary, DON, DON-3G, 3-ADON and ENN B were quantified in concentrations up to 67 µg/l of DON-3G. Another LC-MS/MS method for the simultaneous determination of BEA, ENNs A, A_1, B and B_1, together with cereulide (CER), a bacterial emetic toxin that can contaminate cereals, was developed and validated for wheat, maize, rice and pasta (Decleer *et al.*, 2016). After testing different strategies, the sample preparation was minimised to a one-step liquid extraction without further clean-up. The validation of the developed method was performed based on Commission Decision 2002/657/EC (EC, 2002a). The obtained LOQs for the targeted toxins in the selected matrices ranged from 0.3 to 2.9 µg/kg. All relative standard deviations for repeatability (intra-day) and intermediate precision (inter-day) were lower than 20%. Trueness, expressed as the apparent recovery, varied from 80 to 107%. As proof of principle, the method was applied to 57 commercially available cereal-based foodstuffs. No CER was detected in any of the samples, whereas BEA, ENN A and ENN A_1, were detected at generally low levels, with the exception of maize where in 74% of the samples BEA was detected up to 209 µg/kg.

The process of optimising a Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) procedure to obtain satisfactory recoveries and acceptable matrix effects for multi-mycotoxin determination in brown rice has been reported by Jettanajit and Nhujak (2016). The influence of the following parameters was evaluated: percentage of acetic acid or formic acid in ACN, the addition of acetate or citrate buffer to the salt mixture ($MgSO_4$ and NaCl), the volume ratio between water and extraction solvent, amount and composition of the sorbent mixture (including C18 materials, graphitised carbon black (GCB), primary secondary amines (PSA) or silica) for dispersive SPE. The main conclusions were that increasing the formic or acetic acid content in ACN provided a better extraction efficiency for the three acidic mycotoxins (fumonisins B_1 and B_2 (FB_1 and FB_2) as well as OTA) with similar extraction efficiencies (recoveries of 79-104%) for the other mycotoxins. $MgSO_4$ and NaCl salts with a citrate buffer improved the overall recoveries and the addition of water to the sample before extraction to hydrate and swell the rice matrix positively affected extraction efficiency. The optimised method was applied to real samples, 6/14 samples of brown rice were found to be contaminated with at least one of the target mycotoxins, ranging from 2.5-5.4 µg/kg of FB_1, 4.3-4.4 µg/kg of FB_2 and 6.1-15 µg/kg of ZEA. The development and validation of a multi-mycotoxin LC-MS/MS method and its application for official control purposes has been reported by the State General Laboratory of Ministry of Health, Cyprus (Kafouris *et al.*, 2017). The method, based on ACN/water extraction followed by the analysis of the diluted crude extracts, was intended for the determination of aflatoxins, OTA, ZEA, DON, fumonisins, T-2 and HT-2 in nuts and cereals. Validation experiments resulted in mean recoveries ranging from 74-132% in spiked nuts and from 53-114% in cereals. Participation in FAPAS proficiency tests (PTs) were included in the study. The analysed maize, dried figs, wheat flour and pistachio PT samples yielded |z-scores| of ≤1.4 for all analytes. LOQs for almonds, peanuts, pistachios and wheat ranged from 0.25 to 50 µg/kg. Only in maize, the LOQ (99 µg/kg) was higherer for a single mycotoxin – FB_1. While matrix effects are likely the cause for this issue, all obtained LOQs are satisfactory to enact official control of the EU maximum levels. The validated multi-mycotoxin method was applied for the analysis of 120 samples collected from import and market during 2014-2015. 81% of the samples were contaminated with at least one of these mycotoxins, but only 3% of the samples exceeded the EU maximum levels.

An LC-MS/MS method for the simultaneous determination of 24 mycotoxins in maize silage using ACN/acidified water extraction with or without clean-up on Mycospin® 400 columns (Romer labs, Tulln, Austria) was developed and validated (Dagnac *et al.,* 2016). In both cases a strong LC-MS signal suppression was observed for most of the targeted mycotoxins (up to -120% relative matrix effects), indicating the need for matrix assisted calibration. The achieved average recoveries from spiked samples at three levels ranged from 60-122% with relative standard deviations below 11%. LOQs were between 0.06-57 µg/kg. The calculated repeatability and within-lab reproducibility ranged from 5.2 to 23% and from 7.2 to 24%, respectively.

However, it was observed that the use of Mycospin® columns for the purification of silage extracts did not allow recoveries of fumonisins, significantly affecting the fitness-for-purpose of this procedure for maize silage. The method based on direct injection of crude extracts was applied to 148 samples collected over two years from 19 dairy farms from Galicia, Spain. Of the analysed samples, 62% contained at least one mycotoxin. An 'environmental friendly' method replacing ACN by ethyl acetate for the extraction of trichothecenes (DON, T-2, HT-2) and ZEA from cereals and feeds has been proposed by Breidbach (2017). The manuscript is based on a previously developed method, which has been validated through a collaborative study (Breidbach *et al.*, 2013). The extraction protocol was based on an ethyl acetate/water mixture, whereas mycotoxin detection was performed by LC-MS/MS using isotope labelled internal standards. The method was validated in-house and through a collaborative study and the performances were fit-for-purpose. Repeatability relative standard deviations (RSD_r) were between 4-16%, reproducibility relative standard deviations (RSD_R) were mostly between 12-32%. The trueness of results for T-2 and ZEA were not different from 100%, for DON and HT-2 they were larger than 89%. As starting point for further method developments, Pesek *et al.* (2017) reported on the use of silica hydride-based stationary phases (C18 and phenyl columns) for the LC-MS analysis of aflatoxins, OTA and fumonisins, providing insights in performances of these new stationary phases compared with commonly used C18 columns. Phenyl and C18 columns showed relatively similar selectivity based on hydrophobicity, but the phenyl phase provided shorter retention times. The latter was selected for the analysis of food samples. Selected contaminated bean, maize, rice, and wheat samples were analysed. A single maize sample was found to be contaminated with 10.3 mg/kg of FB_1 and 48 µg/kg of OTA, thus exceeding Brazilian as well as European maximum limits for both toxins.

Validated official methods for multi-mycotoxin analysis based on LC-MS(/MS) will be issued soon by the European Committee for Standardization (CEN), within the framework of the current mandate M/520 (EC, 2013b). When official methods are not available or interlaboratory validation is not affordable, PTs and quality control through the analysis of reference materials are effective tools for quality assurance and performance verification of analytical methods, ensuring that laboratory validation and within laboratories procedures are working satisfactorily. The trend of performances of LC-MS(/MS) methods for multi-mycotoxin determination in maize together with method-related issues was assessed by comparing three PTs organised over the years 2011-2014 (De Girolamo *et al.,* 2017). The obtained data showed an improvement of laboratory performances with the overall acceptable z-scores that progressively increased from 59% in 2011 to 85% in 2014, while the rate of unacceptable z-scores decreased in the same period. The following major conclusions could be drawn: (1) the evaluation of satisfactory results (|z-score|<2) showed that the majority of laboratory participants had the ability to provide acceptable results for the simultaneous analysis of DON, fumonisins (B_1 and B_2), OTA, ZEA, T-2 and HT-2, aflatoxin B_1 (AFB_1) and aflatoxin G_1 (AFG_1) in maize; (2) a large variability of LOQ values was observed among laboratories, and only few of them were able to analyse aflatoxins B_2 and G_2 (AFB_2 and AFG_2) in maize; (3) the best performing laboratories used acidified ACN/water extraction, dilute and shoot injection without extract clean-up and internal standard calibration. The improved LC-MS method performances can be attributed to an overall improved knowledge and management of factors affecting reliability of LC-MS analysis, related to proper matrix effect compensation (mainly by using isotope labelled mycotoxins as internal standards) and to a general simplification of sample preparation protocols thanks to the increased availability of highly sensitive and selective mass spectrometers.

Among the available validation studies it is worth mentioning the collaborative study organised by the U.S. Food and Drug Administration (FDA) laboratories to evaluate a SIDA-LC-MS/MS method for the determination of aflatoxins (B_1, B_2, G_1, G_2), DON, FB_1, FB_2, fumonisin B_3 (FB_3), T-2 and HT-2, OTA and ZEA (Zhang *et al.*, 2017a). The main aim of the study was to evaluate the SIDA approach to compensate matrix effects and extraction recoveries. The study involved six participating laboratories analysing certified reference materials, and performing recovery studies in maize, peanut butter, and wheat flour. Samples were fortified with ^{13}C uniformly labelled mycotoxin, then extracted with ACN:water (50:50, v/v), followed by centrifugation, filtration and LC-MS/MS analysis. The method performance was evaluated according to the Guidelines for the Validation of Chemical Methods for the FDA Foods Program. Furthermore, the manuscript reports an interesting use of principal component analysis to study the effect of concentration, matrix, laboratory/instrument or analyte-dependent effects on method performances. The overall results of the study demonstrated that the method was fit for FDA compliance testing and surveillance and provided support for the utilisation of SIDA-LC-MS/MS methods for routine regulatory analysis of multiple mycotoxins. The widespread application of LC-MS/MS multi-mycotoxin methods for official control purposes raises an urgent need for multi-mycotoxin reference materials. The process of production and characterisation of a multi-fusariotoxin contaminated wheat flour reference material (including DON, ZEA, T-2, HT-2, BEA, ENNs A, A_1, B and B_1) has been described in detail by Tangni *et al.* (2017). The study included the homogeneity assessment followed by interlaboratory study to determine the assigned values, and provided information on the stability of the fusariotoxins and their shelf life in the

wheat flour. Such metrological tools may play a significant role in the application of LC-MS/MS based multi-mycotoxin methods. Although the material described in this study was not valid anymore, the detailed process might be helpful for future attempts in producing matrix reference materials.

As an example of the use of high resolution mass spectrometry merged with chemometric analysis in metabolomics studies, Rubert *et al.* (2017) proposed a metabolomics-based approach for the early recognition of *Fusarium* disease, based on the detection of infection-related metabolites. The authors developed a workflow to study plant-pathogen cross-talk and applied it to a pilot study involving 86 naturally contaminated wheat samples to investigate *Fusarium* infection related metabolites. The monitoring of the most significant markers identified in this work, including oxylipins, alkylresorcinols and acyl glycerols, was proposed as a tool for the early detection of mycotoxins and *Fusarium* disease prevention. However, it should always be taken into account that whilst the genetic background is stable under any environment, metabolic profiles are strongly impacted by environmental and experimental conditions, resulting in significant constraints that require to be carefully considered for the validation of metabolic markers.

With respect to advances in multi-mycotoxin biomarker detection, a rapid LC-MS/MS approach was developed for biomonitoring and quantification of 27 relevant mycotoxins and metabolites in human blood samples and in particular in dried spots of serum and whole blood (Osteresch *et al.,* 2017). For sample preparation a defined blood or serum volume (100 µl) was spotted on standard filter paper cards, followed by drying, cutting the entire spot out of the paper, and extraction with a mixture containing 35% ACN, 35% acetone, and 30% water. After the extraction, an aliquot of the extract was evaporated to dryness, reconstituted and analysed. Matrix-assisted calibration was necessary due to severe matrix effects observed for some toxins. The developed multi-mycotoxin method was applied for the analysis of 50 German blood samples, showing that besides OTA, all samples were positive for ENN B with mean levels of 0.037 ng/ml.

Among multi-contaminant methods, a step back from broad scope methods covering hundreds of contaminants to 'dedicated' methods covering a narrower range of contaminants for a specific purpose or matrix could be evidenced this year. An LC-MS/MS method for the simultaneous analysis of AFM_1, avermectins, organophosphate pesticides, and milbemycin in milk, based on the QuEChERS approach has been developed (Dos Anjos *et al.,* 2016). It is taking into consideration residues and contaminants to be controlled in milk according to the National Plan for Control of Residues and Contaminants (NPCRC) from the Ministry of Agriculture in Brazil. After a careful optimisation of the QuEChERS protocol, the method was validated according to the requirements of Commission Decision 2002/657/EC (EC, 2002a), and applied to 72 cow milk samples. All of them were found to be contaminated by AFM_1 at levels up to 0.48 µg/l. A further example of the suitability of the QuEChERS protocol coupled with LC-MS/MS for the simultaneous detection of AFM_1 and pesticides in milk can be found in Michlig *et al.* (2016). To evaluate the relationship between fungicide application to wheat grains susceptible to *Fusarium* head blight and mycotoxin production, an LC-HRMS method for simultaneous determination of mycotoxins (DON, ZEA, aflatoxins, FB_1, OTA) and fungicides (trifloxystrobin, prothioconazole, epoxiconazole, pyraclostrobin and mancozeb) has been proposed (Da Luz *et al.,* 2017). Again, sample preparation was based on the QuEChERS protocol including dispersive SPE, enabling matrix effects to be kept between 80-120%. Analysis of field trial samples showed that DON was the prevalent mycotoxin in wheat grains and epoxiconazole was the fungicide residue found in the highest concentration. All fungicidal treatments induced an increase in AFB_2 production when compared to the control (without application). AFB_1 and DON, on the contrary, were reduced in all fungicide treatments compared to the control. Finally, an LC-MS/HRMS methodology for the identification and determination of 492 different toxicants (including pesticides, mycotoxins, veterinary drugs and synthetic dyes) has been described by Amelin *et al.* (2016). Also in this case, rather than a generic, broad scope sample preparation protocol, the authors developed commodity dedicated extraction procedures for different commodity groups (milk, feed and grain, fruits and vegetables), followed by a common dispersive SPE clean-up. The analysis scheme includes the identification of toxicants under positive and negative electrospray (ESI) ion monitoring. In case of detection of a given analyte, standard addition and replicate analysis was carried out. The suitability of the method was demonstrated in comparison with results obtained by using an external calibration curve. The reliability of the proposed approach was proven by analysing a large set of reference materials, covering a wide range of food matrices.

4. Mycotoxins in botanicals and spices

Due to profuse mycotoxin contamination and matrix complexity of botanicals and spices, new methods continue to be developed and market surveys continue to be performed. Following market demand, two distinct types of methods continue to emerge on opposite sides of the analytical spectrum, highly selective multi-mycotoxin LC-MS/MS methods and cost-efficient quick ELISA screening methods.

A highly selective multi-mycotoxin method was developed utilising LC-MS/MS in positive and negative ion mode for the detection of aflatoxins, OTA and ZEA in red pepper

powder with QuEChERS sample extraction and purification (Sunyoung *et al.*, 2017). The method was validated to be linear with correlation coefficients >0.998, LOD of 0.07-0.71 µg/kg, LOQ of 0.20-1.81 µg/kg, reproducibility precision of 1.0-9.0%, and a recovery of 86-95%. This LC-MS/MS method was compared to an ELISA method through the analysis of 56 red pepper powder samples from South Korea and the results of both methods demonstrated consistency. On the opposite end of the spectrum an ELISA method was developed and validated for the detection of fumonisins in dehydrated commercial garlic (Tonti *et al.*, 2017). Results from the newly developed ELISA method were compared against a validated HPLC method showing good correlation between the two methods. Fifty six commercial dehydrated garlics were analysed by the developed ELISA method and the HPLC method. Of the samples analysed by ELISA, two samples were positive at trace levels and one sample was positive at quantifiable levels. The same samples analysed by HPLC confirmed trace FB_1 levels, with the absence of FB_2 and FB_3.

Several market surveys were performed studying the natural occurrence of mycotoxins in spices. In Iran, red pepper, black pepper, turmeric and cinnamon samples were analysed by HPLC (Jalili, 2016). The spices were extracted with aqueous methanol, followed by immunoaffinity column clean-up and FLD. Of the 80 samples analysed, 40% were found positive for aflatoxins ranging from 0.85-25 µg/kg. In Pakistan, 200 samples of spices including fennel, cinnamon, white cumin seeds, black cumin seeds, caraway seeds, coriander, black pepper, white pepper, turmeric and carom seeds were investigated (Naz *et al.*, 2016). Sample clean-up was performed by immunoaffinity columns followed by trifluoroacetic acid derivatisation with HPLC-FLD detection. 62% of the spices were contaminated with aflatoxins and 54% of those contaminated were positive above the EU regulatory limits of 10 µg/kg for total aflatoxins. A total of 312 chili samples from Pakistan were analysed for aflatoxins and OTA utilising immunoaffinity column clean-up with HPLC-FLD detection (Iqbal *et al.*, 2017). 56 and 40% of the samples were found positive for aflatoxins and OTA, respectively. The mean of total aflatoxins contaminations was 15 µg/kg and the mean contamination of OTA was 17 µg/kg. In Iran, 88 samples including dried mulberry, dates, figs and apricot were sourced from the market (Heshmati *et al.*, 2017). Apricots showed the highest incidence of contamination at 82% while mulberry, dates, and fig contamination incidence ranged from 46 to 59% for aflatoxins. In Nigeria, a total of 120 dried ginger samples were analysed according to AOAC Official Method 2008.02 utilising multi-mycotoxin immunoaffinity column clean-up and HPLC quantification (Lippolis *et al.*, 2017). The incidence of contamination for aflatoxin and OTA was higher during rainy season with 81 and 77% compared to 46 and 37% during dry season, respectively. Seven of the samples were found to be contaminated above EU regulatory limits.

A classification system was developed for the analysis of aflatoxins in traditional Chinese medicines (Zhao *et al.*, 2016). Twenty two traditional Chinese medications were sourced and systematically sorted into four categories based on matrix types including volatile oils, proteins, polysaccharides and fatty oils. Based on the individual categories, methods were optimised for extraction and purification. Samples were quantified using ultra-high pressure liquid chromatography (UHPLC) coupled to MS/MS detection. Detection was performed in positive ESI mode with multiple reaction monitoring (MRM). For extraction of the aflatoxins in the protein and volatile oil group ultrasonic extraction was selected as the best method. For polysaccharides, extraction by shaking was the most efficient method. Of the 22 samples analysed, 14 were contaminated with aflatoxins ranging from 0.2 to 7.5 µg/kg. The investigation concluded that different Chinese medicines can be grouped into categories and based on these categories sample preparation for aflatoxin analysis could be optimised.

5. Aflatoxins

Regarding the methods, usually classified as 'confirmatory methods' for aflatoxin contamination, only a few papers were published in the review period. One of the papers concerns the simultaneous determination of AFB_1 and sterigmatocystin (STE) (Zhao *et al.*, 2017). The authors describe the optimisation of the QuEChERS-based sample preparation and LC-MS parameters, while benchmarking their results against the generally accepted validation guideline for pesticides SANCO/12571/2013 (EC, 2013a) in the absence of specific ones for mycotoxins at the time. It is worth noting that such documents are living documents and a new version recently superseded it. The authors selected an ACN-rich extraction solvent and applied ultrasonification during extraction. Unfortunately, these experiments were only carried out on spiked material, which explains why there was no effect of extraction time observed. Consequently, it is rather difficult to estimate the effectiveness of the selected extraction solvent and extraction time for naturally contaminated samples. This is regrettable, especially for AFB_1, given the sufficient access to naturally contaminated reference materials from different sources. Furthermore, an LC-MS method for some pesticides was tailored to be also suitable for AFM_1 determination (Michlig *et al.*, 2016). The authors tested a QuEChERS clean-up in combination with chromatography and investigated various parameters for best AFM_1 recovery by experimental design. In the following, they demonstrated that the resulting method is suitable for multi-class pesticide and AFM_1 determination in raw milk. The performance data for AFM_1 showed satisfactory

recovery and precision at levels far below established legislative limits. Their experiments supported the findings of previous publications that replacing ammonium acetate with ammonium formate as mobile phase additive results in an almost doubled sensitivity (signal yield). However, they abbreviate ammonium formate misleadingly with NH_4F ('ammonium fluoride'). The method was applied to whole (raw) milk and defatted milk with satisfactory results and showed comparable results with a method based on immunoaffinity clean-up that was run in parallel during a limited monitoring.

Chu *et al.* (2017) used hyperspectral spectroscopy to identify individual contaminated maize kernels. The authors concluded reduced spectral data (individual wavelengths) as well as single pixel information as interesting features. The correlation between the measured AFB_1 content in fungi-infested kernels and their spectroscopic signals was characterised by a correlation of (r=0.70-0.77), based on the procedure applied. The authors also stated the need for further progress to make the method sufficiently robust for precise AFB_1 prediction. However, metabolic profiles (including AFB_1, kojic acid or anthraquinone production) are subject to numerous natural factors. Thus, the question remains whether improved spectroscopic methods could allow more precise AFB_1 predictions, or if current systems already provide the best possible results for scenarios useful for practical application in the screening for aflatoxins. Another important factor when testing new methods is the reference method chosen. Such methods must be fully characterised as the classification would be against AFB_1 'according to the method used in each particular study'. This factor cannot be underestimated, especially if samples are not analysed under repeatability conditions. Finally, the authors concluded that hyperspectral spectroscopy is a suitable tool for AFB_1 prediction and provided measurement data for this. However, such applications are prime candidates for improving sorting machine results. Consequently, the speed at which single kernels can be identified at different kernel scanning angles are features of interest, while seldom mentioned. A step towards a spectroscopy-based system intended to be used as the first-line-control method was described by Sieger *et al.* (2017). The authors demonstrated the use of infrared laser spectroscopy to classify peanuts as compliant or non-compliant for levels of AFB_1 (in addition to cereals for DON). The method is based on the measurement of the extract of the product of interest, other than directly measuring the milled commodities. In contrast to Chu *et al.* (2017), the authors observed that '*the evaluation of individual absorption bands* [in infrared spectroscopy] *is of limited utility*' for their purpose as this technique has assets in detecting '*matrix changes due to physical alterations caused by fungal contaminations (i.e. content of fatty acids, proteins) rather than detection of individual metabolites*'. This concept of targeting matrix changes is underpinned by selecting extraction solvents according to the matrix they investigate rather than the mycotoxin extractability alone. As a reference, they used an LC-MS method that had been comprehensively validated. The 17 samples they used for training their system were confirmed to have levels either below LOD or contaminated with 2-8 µg/kg AFB_1. The authors showed that a classification of samples is possible with principal component analysis. However, the authors extracted relatively small mass fractions for their experiments. In the absence of homogeneity data for the sample size used for analysis (200 mg), milling of test materials is crucial for the method to be representative of the (whole) lot under investigation, even though the mycotoxin itself is not the target, but the matrix changes. This is also critical as it seems to be unclear whether the LC-MS analysis was done from an aliquot of the same extract that was used for laser spectroscopy.

Yang *et al.* (2017) reported on an optimised ultrasonic-assisted extraction of AFB_1 from peanuts with response surface methodology. The authors came up with rather unusual optimised parameters for extraction. These are a sample-to-extractant ratio of 1 g per 86 ml of 70% aqueous methanol at 73 °C for 7 min. Such parameters are not common in many currently established conventional methods for aflatoxin testing and would be difficult to implement due to the need of temperature controlled ultrasonification. Also, the authors used a specific mash fraction of milled peanuts, which is unusual as most legislation refers to the edible part, which for peanuts is the whole fruit body. The use of derivatisation with trifluoroacetic acid, as the authors described, is no longer state of the art. This is unfavourable for AFB_1 analysis, as it leads to a rather polar derivative that often elutes with matrix constituents at the beginning of the chromatogram. However, the chromatograms in the paper rather show late eluting peaks at about 15 min, which is unusual for the chromatographic system mentioned. Calculations by the reviewer from the standard solution and the matrix chromatograms indicate theoretical plate numbers for the target peaks that differ significantly. Plate number differences can appear depending on the matrix co-injected with the target analyte. However, users who derivatise AFB_1 with trifluoroacetic acid are strongly advised to confirm the identity of their peaks by co-chromatography.

An interesting and promising approach for the extraction of AFB_1 from wheat samples for further enzyme-linked immunosorbent assay (ELISA) determination has been reported by García-Fonseca *et al.* (2016). The authors propose restricted access supramolecular solvents made from carboxylic acids in tetrahydrofuran for sample treatment. The idea of using such solvents is to restrict larger molecules, which might interfere with consecutive ELISA determination, from dissolution. The proposed method is rather simple and easy to implement in a standard

laboratory. After the supramolecular solvents are prepared through the spontaneous formation in solution, the test material is agitated with the prepared solvent, the blend centrifuged and the supernatant containing the analyte used for further analysis. The organic solvent (tetrahydrofuran) as part of the supramolecular solvent is evaporated in the following step and the residue is reconstituted with phosphate buffered saline (PBS). Doing so, a large fraction of the matrix is discriminated, while AFB_1 is extracted. The authors showed that such solvents are not only useful for AFB_1 determination, but also for other mycotoxins and matrices for simultaneous extraction and clean-ups. The authors optimised their approach and could demonstrate satisfactory performance criteria for the recovery of 85-93% and a repeatability of 2-4% RSD. Like previous authors, the applied milling process was deemed sufficient allowing sample size portions of 300 mg for analysis. This is a rather challenging set-up that requires confirmation of homogeneity of such sample intakes, not only for certified reference materials, but also with samples prepared in common ways as most usually carried out in a routine laboratory.

Proposals for rapid analytical methods for aflatoxins, based on chemical molecular recognition represent still the largest fraction of newly published articles on aflatoxins. However, most papers focus on the measurement principle demonstrated in the authors' laboratory rather than its applicability to the end user. This reduces the number of relevant papers to the targeted readership of this review in combination with a simple discriminator to a few. The discriminator was that those papers with performance figures in mass per volume were excluded for the cases where mass fraction (µg/kg) is the unit of interest, except for AFM_1. Some authors aimed to determine aflatoxins at extremely low levels. Despite the deficiency of such technologies for routine use, they seemingly have the potential for scenarios where not the concentration of aflatoxins is challenging, but rather where available sample amounts are limited. Interesting approaches for determining AFM_1 down to lower ng/kg levels were proposed by Guo *et al.* (2016b) by combining aptasensor technology with polymerase chain reaction (PCR). The method strongly depends on the aptamer selectivity, otherwise false positive findings are likely due to the enormous signal amplification in the PCR. The authors claim a LOD of 30 pg/l AFM_1; however, the usefulness of determining AFM_1 in infant rice cereals is questionable. Another approach used magnetic nano-particle technology with fluorometry to determine AFM_1 using an AFM_1-oxim-fluoresceinamine conjugate for a competitive ELISA (Atanasova *et al.*, 2017). The results were compared with those from a commercial ELISA, while the question that would need to be addressed is how the selection of suitable antibodies can be tailored such that they do not cross-react with other related mycotoxins, for example AFB_1 or STE, while being fully reactive with the mycotoxin conjugate used. Lou *et al.* (2017) employed an optofluidic-based immunosensing assay. The resulting sensor achieved a LOD of 5 ng/l in dairy products and could be used for more than 200 measurements. However, the method requires some sample preparation, but it demonstrates what could be achieved using this version of a fibre optic sensor. Campbell *et al.* (2017) described the results for a fully developed end-user method. The method is assumingly based on some molecular recognition to immobilise aflatoxins called ToxiTrace. However, the authors did not describe the details of its principle and classified the method as 'alternative spectroscopic approach'. The measurements were compared with results obtained from a validated LC-MS method, also described in the article. Performance data were presented for peanuts, maize, rice and dried distillers grain; all matrices for which a rapid analysis at a transfer-site is of interest. The authors presented an unusual parameter, which is of clear interest for every user, beyond precision, recovery and working range. This is the number of wrong classifications into compliant or non-compliant against a decision limit (e.g. legislative limit) when findings are compared with those of a previously validated method. It could be demonstrated that the method is equivalent with LC-MS in classifying samples for making decisions.

6. *Alternaria* toxins

The detection methods of *Alternaria* toxins, such as thin layer chromatography, gas chromatography (GC) and GC-MS, LC and LC-MS, ELISA and electrochemical methods, have been recently reviewed. Extraction and clean-up strategies as well as the sensitivity of each method for the tested toxins were also reported (Man *et al.*, 2017a). A new report has been published by the European Food Safety Authority (EFSA) on dietary exposure assessment to alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA) and tentoxin (TTX) in the European population, although 70% of 15,563 analytical results originated from Germany. Only 8% of analytical results were >LOD and the values of LODs of the methods used to analyse the 4,249 samples, within the 15 food groups considered, ranged from 0.3 to 25 µg/kg (EFSA, 2016).

A modified QuEChERS method was used to extract TeA from wine grapes and to purify the extract before HPLC-UV determination. Grape samples were slurried with 1% formic acid, $MgSO_4$ and NaCl, shaken with ethyl acetate for extraction and centrifuged. The top layer was collected, evaporated, re-suspended in acidic mobile phase and injected (20 µl, equivalent to 100 mg matrix) in the HPLC-UV system. Matrix-matched calibration was used, probably because interfering peak(s) co-eluted with TeA. However, this type of calibration is unusual for UV detection and its usefulness to compensate for coeluting interfering peak was not shown. The values of LOD (10 µg/

kg), LOQ (50 µg/kg), recovery (82-97%) and RSD_r (5-13%) were considered acceptable and the method was capable to detect and quantify TeA in naturally contaminated grape samples (57-595 µg/kg) (Fontana *et al.*, 2016). An LC-MS/MS method was proposed for the determination of AOH, AME and TTX in strawberries. After milling and centrifugation, the upper liquid phase was extracted with ethyl acetate. The extract was dried, dissolved in aqueous methanol and analysed by LC-MS/MS by using matrix-matched calibration for toxin quantification. The evaluation of matrix effects, conducted at 10 times the LOQs, showed ion suppression for TTX (61%) and ion enhancement for AME (165%) and AOH (117%) (Juan *et al.*, 2016). The same group used another clean-up approach before LC-MS/MS determination of the same toxins (AOH, AME and TTX) in tomato products, i.e. dispersive liquid-liquid microextraction (DLLME). ACN was used as disperser solvent and chloroform as extraction solvent. It was necessary to use matrix-matched calibration to compensate the ion suppression observed for AOH (65%), TTX (78%) and AME (80%) (Rodríguez-Carrasco *et al.*, 2016). A self-made SPE column containing aminopropyl and mixed-mode cationic exchange (MCX) adsorbents was used to clean-up crude extracts of various fruits (apples, oranges, sweet cherries and tomatoes) for consecutive UHPLC-MS/MS determination of AOH, AME, TeA, TTX and altenuene (ALT) in addition to OTA, PAT and citrinin (CIT) (Wang *et al.*, 2016). A homogenised test portion was diluted with water and extracted with acidified ACN by shaking. The sample was then treated with NaCl, centrifuged and the upper ACN phase was passed through the SPE column. The purified extract was dried, re-dissolved in aqueous ACN containing 5 mM NH_4Ac, filtered and analysed by UHPLC-MS/MS. The values of recovery, LOQ and repeatability were 74-102%, 1-5 µg/kg and <5%, respectively. Matrix-matched calibration was used for a correct quantification in order to compensate for matrix effects. The performances of two different UHPLC columns, BEH C18 (1.7 µm, 2.1 mm ×100 mm) and CORTECS C18 (1.6 µm, 2.1 mm ×100 mm), were also compared, showing higher sensitivity for the latter one due to sharper chromatographic peaks.

The same extraction and SPE clean-up procedures developed by Wang *et al.* (2016) were used by Man *et al.* (2017b), who developed a semi-quantitative immunochromatographic strip-test for the detection of AME in cherries and orange fruits. The anti-AME monoclonal antibody (mAb) was prepared and identified as well as the immunogen (AME coupled to bovine serum albumin (BSA) via methyl 4-bromobutanoate). The cross-reactivity of anti-AME mAb with AOH was 2.1%. The cut-off value (disappearance of the line) of the strip for AME was 10 ng/ml, which is equivalent to 5 µg/kg in fruits. The result can be visualised on the strip within 10 min but a much longer time is necessary for toxin extraction, centrifugation, SPE clean-up of sample extract, evaporation and reconstitution of the purified extract (Man *et al.*, 2017b). An immunochromatographic strip-test was developed for the analysis of AOH in cereals and fruit juice. Each test portion was extracted with ACN:water (80:20, v/v) and centrifuged, before the supernatant was diluted with PBS and analysed with the strip. Apple juice and orange juice were analysed directly using the strip. The cross-reactivity of anti-AOH mAb with AME was about 48%, whereas no cross-reactivity was observed for TeA and other mycotoxins (AFB_1, AFM_1, DON, ZEA, OTA). The cut-off value of the strip for AOH was 640 µg/kg in cereals and 100 µg/kg in fruit juice. Lower values were obtained by evaluating the strips with a scan reader (Kong *et al.*, 2017a,b). The high values of cut-off for AOH could be explained by the sample preparation used that was rapid, but without clean-up and concentration of the sample extract. Twenty eight samples of sorghum (n=12) and sorghum-based infant cereals (n=16) naturally contaminated with TeA were analysed by using two methods, a stable isotope dilution LC-MS assay and an ELISA, previously developed for infant food, apple and tomato products, respectively (Gross *et al.*, 2017). TeA was detected in all samples by the LC-MS assay, whereas the ELISA method detected TeA in sorghum grains containing TeA at levels >30-60 µg/kg and in sorghum-based infant cereals containing TeA at levels >200-300 µg/kg. For samples positive with both methods, the coefficient of correlation was good for sorghum grains (r^2=0.977), while for infant cereals it was very low (r^2=0.169). For most samples the ELISA method underestimated the TeA content determined by LC-MS assay. The authors concluded that the ELISA method seems to be suitable to determine TeA in sorghum grains at a level of 500 µg/kg, the action limit established by the Bavarian Health Protection Authority, but further work seems necessary to improve recovery of TeA.

7. Ergot alkaloids

Although not directly related to analysis, a key publication about ergot alkaloids last year was the EFSA Scientific Report on human and animal dietary exposure to ergot alkaloids (EFSA *et al.*, 2017). For the majority of the samples (97%), the analysis included all 12 main *Claviceps purpurea* ergot alkaloids: ergometrine, ergosine, ergocornine, ergotamine, ergocristine, ergocryptine (α- and β-isomers) and their corresponding -inine (S)-epimers. LC was the only separation method reported, mainly coupled to MS/MS, although FLD was also used in a minority of cases – highlighting the current 'state of the art' for ergot alkaloid analysis.

Currently, ergot alkaloids are not regulated in food, but the European Commission has set limits for ergot sclerotia of 1000 mg/kg in feedstuffs containing unground cereals (EC, 2002b) and 500 mg/kg in cereals intended for human consumption (EC, 2015). A detection and quantification method using near-infrared (NIR) hyperspectral imaging

to measure particles of ergot bodies in cereal flour was published by Vermeulen *et al.* (2017). Ground ergot sclerotia and wheat flour samples and mixtures containing 100 mg/kg to (the unrealistically high concentration of) 500,000 mg/kg ergot were analysed. Partial least squares discriminant analysis (PLS-DA) models were developed and applied to spectral images to detect ergot body particles. Ergot particles were detected in 100% of samples spiked at more than 10,000 mg/kg and no false-positives were obtained for non-contaminated samples. For the cereal flours containing less than 10,000 mg/kg ergot, results were more variable at lower levels of incorporation of ergot sclerotia. At the EU limit of 1000 mg/kg one sample (MA6) was predicted to contain a mean value of 897 mg/kg but another (MA7) was only predicted to contain a mean value of 51 mg/kg. It was possible to detect levels as low as 100 mg/kg in spiked samples, however some samples were under- or overestimated. This was explained by lack of homogeneity, sampling issues and the thickness of the sample. Results predicted with the PLS-DA model were referenced against measurements from a chromatographic method (Krska and Crews, 2008) undertaken by five laboratories. A correlation of 0.99 was obtained between measured alkaloid content and the ergot sclerotia weighed during the mixture preparation. This study demonstrated the potential of NIR hyperspectral imaging combined with chemometrics as an alternative solution for determining ergot body particles in cereal flour. More work on establishing appropriate sampling is however required before it could be used to reliably detect ergot contamination at the regulatory limit.

Oellig (2017) reported a screening method using lysergic acid amide (LSA) as a chemical marker for the determination of total ergot alkaloids in rye. An ammonium acetate buffered extraction step was followed by liquid-liquid partition for clean-up. Extracts were incubated with a mixture of methanol/lithium triethylborohydride solution (Superhydride®) to form LSA from ergopeptines. Ergometrine was unaffected, so was determined separately. The two LSA epimers and two ergometrine epimers were analysed by high-performance thin-layer chromatography-FLD on silica gel with a mixture of isopropyl acetate, methanol and aqueous ammonium hydroxide as the mobile phase. The enhanced native fluorescence of LSA and ergometrine were used for quantitation, the sum of the LSA epimers and the ergometrine epimers (converted to LSA based on molar mass ratio) were used to calculate the total ergot alkaloid content. Validation was carried out to establish recoveries and repeatability and a comparison made with two established HPLC-FLD methods. Although the validation was not comprehensive, it was sufficient to demonstrate recoveries close to 100% for different rye flours at two spiking levels, with good (maximum 4.3%) repeatability. The total ergot alkaloid content correlated well with the two HPLC methods, giving comparable, if slightly higher results. Limits of detection and quantitation were 8 and 26 µg LSA/kg rye, which meant the method would be suitable for checking compliance with quality control criteria for rye. The author stated that the method offers fast, efficient screening for total ergot alkaloids in rye as alternative to HPLC analysis of the individual compounds.

An HPLC-FLD method was developed for the determination of five ergot alkaloids: ergometrine, ergotamine, ergocornine, ergocryptine and ergocristine in animal feedingstuffs (Kowalczyk *et al.,* 2016). The method was based on several publications with modifications and used a mixture of QuEChERS salts and solvents for extraction and modified QuEChERS with dispersive SPE for the clean-up step. Alkaloids were separated on a C18, 250 mm×4.6 mm, 5 µm column. The mobile phase contained ammonium carbonate and ACN, the excitation and emission wavelengths used were 330 and 420 nm, respectively. The use of this column resulted in a run time of 25 min and broad peak shapes, although the gradient used ensured good separation of the ergot alkaloids and good selectivity from matrix interferences. The authors reported water/ACN separation alone was not sufficient to clean-up the sample extracts and a dispersive sorbent – PSA, magnesium sulfate and activated carbon – was required to remove impurities. The method was validated according to Commission Decision 2002/657/EC (EC, 2002a) and all performance parameters met the stipulated requirements. Linearity was determined for the range of 25-400 µg/kg, the coefficient of determination for all curves was from 0.985 to 0.996 for all five compounds. LODs were in the range 3.2-6.5 µg/kg and LOQs ranged from 11 to 13 µg/kg. Recovery and repeatability were determined over the range 25-400 µg/kg, recoveries were from 87-108%. The highest CV for repeatability was 14.3% (ergocristine at 400 µg/kg) and for reproducibility 15.4% (ergotamine at 25 µg/kg). The method would be suitable for analysis for official control of feedingstuffs by laboratories without LC-MS instruments. The authors stated the scope could be extended to include the -inine epimers of the alkaloids in the method, although they did not state why these (and ergosine) had not been included in the method initially.

The main method used for ergot alkaloid analysis continued to be LC-MS/MS. Several papers were published that highlighted the use of this technique. Jarmusch *et al.* (2016) carried out a comparison of the use of ESI and atmospheric pressure photoionisation (APPI) mass spectrometry methods for the analysis of ergot alkaloids in grass *Achnatherum robustum* infected with the ergot producing *Epichloë* fungus. This fungus is known to produce the ergot alkaloids LSA, ergonovine and chanoclavine. Samples were extracted using cold methanol and analysed by reversed-phase HPLC-ESI-MS and HPLC-APPI-MS. Method validation was performed to establish regression parameters, LODs, LOQs as well as precision and accuracy. The performance of APPI and

ESI methods was comparable. Both methods were subject to very little matrix interference, with recoveries ranging from 82 to 100%. LSA and ergonovine were determined in an *A. robustum* sample infected with the *Epichloë* fungus at concentrations of 1,140 µg/kg and 282 µg/kg, respectively. There was no statistically significant difference between these concentrations obtained after APPI and those determined using ESI. The occurrence of ergot alkaloids in wheat harvested in Albania was investigated by Topi *et al.* (2017). An LC-MS/MS method based on other published methods was used to analyse for the 12 most important ergot alkaloids ('EFSA 12'; EFSA, 2012) in a total of 71 winter wheat samples. The authors used a larger test portion for extraction (20 g) than used in many other papers, although they did not give an explanation why. Mycosep® columns were used to clean-up extracts, before LC-MS/MS analysis. The method was validated over the concentration range of 10 to 500 µg/kg for each alkaloid. Recovery values for the 12 ergot alkaloids were 100-124%, with RSD_r and RSD_R ranges of 5.8-17 and 7-35%, respectively. The method was used to analyse samples in the harvest years of 2014 and 2015. In 2014, 49% of samples were contaminated with ergot alkaloids, whereas in 2015 only 19% of samples were contaminated. In 2014, the concentrations of total ergot alkaloids ranged from 17-975 µg/kg, and in 2015 they ranged from 10-391 µg/kg. The samples contained one to nine ergot alkaloids, ergometrine, ergosine and ergocristine were the most frequently found. This was the first report on the occurrence of ergot alkaloids in wheat from Albania. Shimshoni *et al.* (2017) reported the use of an LC-MS/MS method to investigate sorghum ergot disease, commonly caused by *Claviceps africana*. The method itself was not a new method, but based on a multi-toxin method published by Malachová *et al.* (2014). Ergot species were identified by ergot alkaloid profile analysis and alkaloids were determined in sorghum silages. Dihydroergosine was identified as the major ergot alkaloid, while dihydrolysergol and dihydroergotamine were identified for the first time as significant ergot alkaloid components within the *C. africana* sclerotia. Guo *et al.* (2016a) reported a comprehensive LC-MS/MS method for the simultaneous determination of 25 ergot alkaloids in cereal samples. The method was developed as the authors reported current methods may underestimate total ergot alkaloid content as many compounds that can exert toxic effects, such as agroclavine are not included. The analytes in this expanded method included the main EFSA 12 compounds, as well as erginine, agroclavine, chanoclavine-I, elymoclavine, festuclavine, lysergol, dihydroegocristine, dihydroergocryptine, dihydroergotamine, dihydroergocornine, dihydroergine and dihydrolysergol. The analytes were extracted using an ACN/aqueous ammonium carbonate solution, followed by purification with C18 sorbent. After full separation on a C18 column, the 25 ergot alkaloids were detected by LC-MS/MS using MRM in the positive ion mode. The method was validated by determining matrix effects, LODs, LOQs, linearity, recoveries and repeatabilities. The linear range was 0.05-5.0 µg/kg for the 25 ergot alkaloids. The C18 sorbent was selected as it gave better clean-up results than PSA sorbent, shortened the clean-up process and reduced costs. Matrix effect investigations showed slight ion enhancement or suppression for the 25 compounds in rye flour compared to neat solvent standards, therefore matrix-matched standard curves were used for calibration. The mean recoveries at three spiked concentrations varied from 77-120% with RSD <15%. The method was validated by analysis of a FAPAS PT sample of ergot alkaloids in rye flour. The overall results showed that for the EFSA 12 ergot alkaloids that had been included in the PT, eight z-scores within the satisfactory range (i.e. $|\leq 2|$). Concentration values had not been assigned for three compounds during the original FAPAS PT round, so it was not possible to calculate z-scores for all 12 compounds. This method appeared to have a slight negative bias, as of 9 possible z-scores, 6 were negative and for one compound (ergosinine) the z-score was -2.38, which would be considered questionable in the PT round. The method was applied to rye flours, wheat flours, whole wheat flours, bread and noodles. Interestingly the only ergot alkaloids detected were found in rye and wheat flour samples and were all from the main EFSA 12 group.

In summary, there have been very few advances in the determination of ergot alkaloids in the past year and no reports of novel methods. A review article by De Middeleer *et al.* (2016) discussed the production of molecularly imprinted polymers (MIPs) for ergot alkaloids, however the review was about MIP characterisation methods and did not describe their application. Therefore any possible pros and cons of using MIPs in an analytical method were not covered. All methods summarised here were based on previous work, in some cases with modifications, but were used for other purposes, i.e. to carry out investigations or small surveys. It is good to note all authors carried out in-house validation before using the methods. The major difference was the inclusion of additional ergot alkaloids that have not been considered by EFSA to date (Guo *et al.,* 2016a, Jarmusch *et al.,* 2016; Shimshoni *et al.,* 2017). However, the results presented by Guo *et al.* (2016a) and Shimshoni *et al.* (2017) showed this to be less of an issue for human food as no additional ergot alkaloids were detected in cereals, but it could be important for animal feed ingredients.

8. Fumonisins

In the area of fumonisin analysis, instrumental methods such as LC-FLD or various forms of LC-MS have generally received greater attention from researchers than have screening assays. Perhaps because LC-MS technology has reached a state of maturity and because multi-analyte methods are the trend for toxin detection, the amount of innovation in this area, for fumonisin analysis, has

declined somewhat from previous years. However, new ways to use the established technology continue to be developed. A multi-toxin LC-MS/MS method using stable isotope standards was applied to the determination of 'free' and 'hidden/bound' fumonisins in maize products (Andrade *et al.*, 2017). The free forms were extracted with acidified ACN, then the residue was dried and subjected to alkaline hydrolysis for determination of the bound/hidden fumonisins. Acidification and cooling of the extracts following hydrolysis was used to aid isolation of the hydrolysed fumonisins (HFBs). LOQs for the free fumonisins ranged from 8 (FB_2) to 32 µg/kg (FB_3). Average recoveries from spiked maize meal ranged from 93.7 (FB_1) to 116% (FB_2). Recoveries for HFB_1, HFB_2, and HFB_3 averaged 75.6, 108, and 74.9% respectively. The method, by measuring both free fumonisins and those that were hidden/bound will be able to provide insights into the degree to which hidden/bound fumonisins contribute to the total fumonisin levels in maize. Maize-product samples were not analysed in the context of this study. A single laboratory validation of another LC-MS/MS method was reported for fumonisins in maize, popcorn kernels, white maize kernels, and yellow maize grits (De Oliveira *et al.*, 2017). The method used a matrix solid phase dispersion approach with silica, which permitted simultaneous extraction and clean-up. With good recoveries and precision, the method appears to be a good way to determine fumonisins in maize.

Within the past year there were numerous reports of immunochromatographic or lateral flow devices (LFDs) for fumonisins. However, as with LC-MS, this area has likewise matured. A recent report described the application of a new fumonisin antibody in a competitive indirect ELISA (CI-ELISA) and in an immunochromatographic device (Tang *et al.*, 2017). The immunochromatographic device was very sensitive, with an analytical range of 0.24 to 15 µg/kg. Recoveries from spiked samples (type of sample unreported) ranged from 78.5 to 115%. For several samples of rice, maize, peanut and wheat, agreement was good between the immunoassay and an HPLC-FLD reference method.

Improvements to screening methods remain desirable and this is an area in which there has recently been much activity. While antibody-based screening methods continue to predominate in the literature, there are distinct trends evident in the areas of alternative toxin-binding materials and in multi-analyte screening assays. Commonly reported alternatives to antibodies include DNA or RNA oligonucleotides (aptamers), and MIPs. Recently, several assays using such materials were developed, all of which were based on electrochemical detection. The first of these (Wang *et al.*, 2017a) used a fumonisin aptamer immobilised onto silica particles attached to quantum dots (QDs) containing CdTe. This reagent was used to form a complex (aptasensor) with magnetic beads through DNA complementary to a portion of the aptamer. The aptasensor was incubated with FB_1 for 60 min, during which time the aptamer released from the complex. The silica-QDs remaining within the complex were isolated magnetically. Hydrolysis of the isolated complex permitted electrochemical detection of Cd^{2+} by square wave voltametry. The LOD for FB_1 (S/N=3) was 0.02 ng/ml, with a linear range from 0.05 to 50 ng/ml. Recovery from maize extracts spiked with FB_1 at 5, 10, and 50 ng/ml ranged from 92 to 103%. Unfortunately, how these levels related to FB_1 concentrations in the samples prior to extraction (i.e. µg FB_1/kg maize) was not described. A group from the same institution used the same aptamer in an electrochemical sensor with a different format. In the latter case a disposable carbon electrode was modified with gold nanoparticles (AuNP), to which the aptamer was subsequently attached (Ren *et al.*, 2017). FB_1 was allowed to interact with the sensor for 30 min, after which the sensor was washed and binding assessed by electrochemical impedance spectroscopy. Impedance increased as the concentration of FB_1 increased. The sensor had an LOD (S/N=3) of 0.0034 ng/ml and a linear range of 0.01 to 50 ng/ml. Good recoveries were obtained from maize spiked at levels from 0.2 to 20 ng/ml. Unfortunately, as with the previous article, how these concentrations corresponded to levels in the maize was not described. MIPs have also been used in an electrochemical assay for FB_1 (Zhang *et al.*, 2017c). A carbon electrode was modified with AuNPs, Ru-doped silica nanoparticles ($Ru@SiO_2$) and chitosan. The MIP was formed on the modified electrode using FB_1 as the template. After removing most of the FB_1 by washing, the rebinding of FB_1 to the polymer was measured by electrochemiluminescence. Incorporating AuNP onto the surface of the electrode improved the signal from the device. The LOD of the device was 0.00035 ng/ml, with a linear range from 0.001 to 100 ng/ml. It is unclear whether it was maize or diluted extracts of maize that were spiked. Spiking levels were 0.1, 1, and 10 ng/ml. Recoveries ranged from 92 to 104%. As an alternative to forming MIPs on an electrode, they can be prepared in aqueous solution by solid phase synthesis with the template immobilised. A fumonisin MIP was prepared in this fashion with FB_2 immobilised (Smolinska-Kempisty *et al.*, 2016). The resulting 'nanoMIP' was coated onto a surface and used in an assay analogous to a competitive direct ELISA. The LOD (6.1 pM) and linear range (1 to 10^4 pM) indicated the technique was very sensitive for FB_2. Unfortunately, the method was not applied to food samples, so the potential for matrix interferences was not evaluated.

While every year the number of applications using alternative binding materials continues to increase, antibodies continue to be the 'workhorse' binding materials used in screening assays, and novel immunoassays continue to be developed. This includes the recent development of an electrochemical immunosensor for FB_1 (Lu *et al.*, 2016). Electrodes were prepared by deposition of pyrrole and

graphene oxide (GO), followed by reduction of the GO, deposition of AuNPs, and immobilisation of antibodies onto the AuNPs. Binding of FB_1 to the immobilised antibodies was determined by differential pulse voltametry. The LOD was 4.2 µg/l, with a linear range of 0.2 to 4.5 mg/l. Recoveries from maize spiked at levels from 100 to 4,000 µg/kg ranged from 93 to 103%. Optical immunosensors also continue to be developed. Imaging surface plasmon resonance (iSPR) is an extension of SPR that allows the selection of multiple regions of interest on an SPR sensor surface, which facilitates multiplexing. Recently, SPR assays were developed for 6 groups of toxins in barley. The assays were then transferred to a portable iSPR instrument that was based upon nanoplasmonics, which had the advantage of eliminating the need for optical prisms in the device (Joshi *et al.*, 2016). For fumonisin detection, an FB_1-ovalbumin conjugate was immobilised and the signal resulting from binding of antibody was measured. Significantly, the sensor chips could be re-used for at least 60 cycles, which would help reduce analysis costs. The LOD for FB_1 in barley was 2 µg/kg, with a working range of 10 to 1,200 µg/kg. Intra-day and inter-day precision was generally good: 4.3 and 7.2% in spiked barley. When transferred to the nanoplasmonics-based instrument, the assay was less sensitive, with an LOD of 13 µg/kg and a working range of 48 to 3,800 µg/kg. However, both formats of the assay would be able to detect FB_1 at the maximum level established by the European Commission. Antibodies have also been incorporated into polymers (Li *et al.*, 2017a). Anti-fumonisin antibody was first reacted with a functional monomer to form a conjugate. The antibody-monomer conjugate was then combined with a cross-linker, a porogen, and an initiator with the mixture polymerised on the surface of a modified glass substrate. The embedded antibody was used in a competitive direct ELISA format with toxin-horseradish peroxidase conjugates and chemiluminescent detection. Average recoveries of FB_1 spiked into barley at four levels from 200 to 4,000 µg/kg ranged from 88 to 96.2%. This is an interesting way for immobilising antibodies and potentially reducing matrix interferences through the use of a three dimensional antibody/polymer monolith.

Many screening formats use immobilised toxin-protein conjugates (antigens) and while the toxin-binding elements (antibodies, etc.) are important reagents, construction of the antigens is likewise critical to assay performance. To avoid the use of toxin-protein conjugates in screening assays, peptides that mimic the ability of the toxin to bind to an antibody, known as mimotopes, have been used. Recently a mimotope was combined with a fumonisin antibody in a competitive indirect immunoassay format with FLD (Peltomaa *et al.*, 2017). The assay was constructed as a microarray with the mimotope immobilised onto glass slides. The LOD was 11.1 ng/ml and the dynamic range was from 17.3 to 79.6 ng/ml. Samples of maize or wheat were spiked concomitant with extraction using 60% (v/v) methanol/PBS. Recoveries ranged from 73 to 122%, suggesting this microarray technique may be worth further studies to validate the method.

Biosensors for mycotoxins are slowly finding their way into the marketplace. In the past year, the applications of two commercial antibody-based multi-toxin biosensors were reported (Bánáti *et al.*, 2017; Plotan *et al.*, 2016). The two have very different formats and were used in very different ways. The product 'Fungi-Plex™' uses microbeads in combination with flow cytometry and FLD (Soft Flow Hungary Ltd., Pécs, Hungary). The Fungi-Plex™ was used to follow the production of several mycotoxin by *Fusaria* in genetically-modified maize (Bánáti *et al.*, 2017). While the focus of the article was on the effects of the maize genetics upon toxin production, the article has demonstrated a practical application for the multiplexed bead assay. It is very uncommon to see multi-toxin immunoassays undergo rigorous validation with actual commodities and foods. So the recent report by Plotan *et al.* (2016) was a refreshing reminder that the endpoint for assay development is not proof of concept, but rather functional assays that can be widely used. The device involved was the Evidence Investigator (Randox Food Diagnostics, Crumlin, UK), which uses a miniaturised ELISA on a biochip. The format, which used immobilised antibodies, is frequently referred to as a 'competitive direct' format. Chemiluminescence of the product was measured. The assay validated was a semi-quantitative screen for seven groups of mycotoxins, including the fumonisins in feeds. The time to complete assays, after sample preparation and extraction, was approximately 90 min. Average recoveries with fumonisins spiked at 10, 20, and 40 µg/kg, ranged from 96 to 109%. The relative standard deviations for three experiments performed on three different days by one operator (on the same instrument) ranged from 2.2 to 11.5%. The relative standard deviations within the laboratory, using different operators on different equipment, ranged from 7.0 to 11.0%. For 8 references feed samples that were tested by the method and by LC-MS/MS in an external laboratory, fumonisins were detected in six, with an r=0.98 when compared to the confirmatory method. The results suggest the method is suitable for the intended purpose as a semi-quantitative method for feeds.

9. Ochratoxins

LC-MS and LC-FLD are in most analytical labs the standard and reference methods for the quantification of OTA with chromatographic conditions, fragmentation pathways and fluorescence properties being well known. With purified extracts, these methods can be applied for sensitive detection of OTA at levels of 0.1 ng/ml or below for food or biological matrices. Without clean-up, LC-FLD is usually not applicable due to co-eluting, interfering substances in the LC chromatogram as well as quenching effects in the

FLD. LC-MS/MS or LC-HRMS on the other hand have been shown to work also when dilute and shoot approaches are used, often allowing to meet regulatory limits without laborious clean-up (see Section 3). For more complex matrices such as spices or coffee, immunoaffinity columns (IACs) are the mostly used purification tool, which provides reliable results but is also laborious, time consuming and rather expensive. For screening purposes, ELISAs are widely applied, as they are fast, simple to operate and require less equipment. However, also for this antibody-based method, costs, selectivity as well as ruggedness are an issue. Thus, new developments of (bio)sensors/affinity assays were in focus of most publications of the last year, aiming to reduce the above-mentioned disadvantages.

Several methodological papers have been published describing applications of aptamers, which are artificial single stranded oligonucleotides that have been designed to achieve a high affinity and selectivity for a specific target molecule, in this case OTA. They are easy to produce and possess a defined structure which can be modified or extended for additional functionality. For biosensors, aptamers are used as recognition element and functionalised for various detection modes, namely optical, colorimetric, chemiluminescence-based, SPR-based, fluorescence-based and electrochemical-based detection, as well as various LFDs. The applicability of these sensors with focus on OTA detection has been reviewed by Badie Bostan *et al.* (2017). In the past twelve months, most of the developments in this field were focused on improving the assay sensitivity in buffer solutions, with only little emphasis given to applications in real food samples. However, some matrices, mostly red wine, white wine, rice flour and other cereals have been investigated. For screening in wheat for instance, Liu *et al.* (2017a) developed a method where no significant matrix interferences were observed and OTA could be quantified at levels down to 0.5 µg/kg with RSDs between 4.8 and 6.2%. The reported method is based on the electrochemical reduction of methylene blue, which is released by exonuclease III from a methylene blue DNA probe after hybridisation with a specific DNA section of the OTA aptamer complex. The signal used for OTA quantification is the current measured at the applied reduction potential of methylene blue. As this can be disturbed by food constituents, a specific matrix calibration is mandatory.

Regarding ELISAs, steps towards miniaturised, disposable biosensors were made by Karczmarczyk *et al.* (2017) who developed a competitive ELISA utilising a disposable electrochemical biosensor for the indirect detection of OTA. The sensor applied was a commercially available screen printed gold electrode with BSA-OTA (or BSA-AFM_1, which has also been used in the study for milk samples), being immobilised onto the gold electrode via covalent binding to a self-assembling monolayer of 3-mercaptopropionic acid. For OTA analysis, aliquots of the samples were first incubated with OTA specific antibodies and the mixture applied to the immobilised BSA-OTA to allow binding of the unreacted antibodies to OTA-BSA. After a certain incubation period, a washing step as well as incubation with a secondary antibody labelled with alkaline phosphatase were performed before an enzymatic conversion of 1-naphthyl phosphate to 1-naphthol was done by the alkaline phosphatase. Finally, 1-naphthol was electrochemically detected using differential pulse voltammetry. In their study a LOD of 17 pg/ml, was determined for buffer solution. However, a decrease of sensitivity due to high background signals with large standard deviations was observed when red wine was applied as sample matrix. Despite previous treatment with polyvinylpyrrolidinone for polyphenol removal and pH adjustment, a LOD as high as 15 ng/ml was reported. This huge sensitivity gap might be explained by nonspecific interaction of wine matrix with the immobilised BSA-OTA or partial denaturation of antibodies. Two studies wherein nanobodies have been applied instead of conventional antibodies or aptamers as recognition element for biosensors have also been published in the recent period. Nanobodies are single domain antibodies derived from the antigen-binding variable domain of the heavy chain. They can be expressed in bacteria, fungi and plants, and produced in high yields. Furthermore, nanobodies are readily genetically manipulated for the construction with proteins, such as alkaline phosphatase. The application of nanobodies in ELISA and their resistance to matrix interferences has been studied by Liu *et al.* (2017c) who compared the stability as well as the susceptibility to matrix effects of rice, oats and barley extracts of nanobodies with a commercially available mAb. The authors could show in their study, that nanobodies were more resistant against concentrated matrix extracts and were more temperature stable while leading for most samples to results comparable to those of classical mAb ELISA. For some samples however, assays with nanobodies reported lower OTA levels such as for barley where 1.87±0.15 µg/kg have been found with nanobodies while 2.90±0.21 µg/kg were determined with commercially available antibodies. Unfortunately, the authors did not apply HPLC-FLD or HPLC-MS as reference method to identify whether the nanobodies were just more specific or had lower affinity towards OTA in this matrix.

Only little progress has been made in the field of sample preparation as clean-up or enrichment for OTA analysis. A review discussing the possibilities and limitations of IACs, aptamer-based columns and MIPs for the enrichment and purification of OTA has been published by Pichon and Combès (2016). Furthermore, Sargazi *et al.* (2017) report an extraction method for OTA based on magnetic nanoparticles cationised with dopamine to yield weak anion exchange properties. The dianionic OTA molecule could be bound to these particles and extracted from pure organic

phases such as the lipid containing organic phase of milk samples extracted with ACN/methanol. With this setup, however, only recovery rates in the range of 43-68% were obtained. Furthermore, the authors showed that higher levels of water in the sample treated with the cationized magnetic nanoparticles inhibit any quantitative interaction with OTA, making an application to a broader set of food samples questionable. Miniaturisation of SPE to the size of standard PEEK capillaries was reported as an alternative preconcentration and clean-up step to conventional SPE by Andrade and Lanças (2017). The authors propose that their setup can increase the efficiency of OTA analysis by allowing laborious SPE to be done automatically. The general setup is comparable to well-known online SPE but instead of the SPE cartridge a 50 mm PEEK capillary packed with 10 µm C18 particles between two 2 µm frits was used. Optimisation of loading, washing and chromatographic conditions allowed to minimise carry over to <0.1% and a validation according to the Brazilian ANVISA guideline was successful for concentrations of 2 µg/l and above (Andrade and Lancas, 2017).

Senthilkumar *et al.* (2017) published an initial study for screening of contaminated grain for OTA as well as OTA-producing *Penicillium verrucosum* strains using near-infrared light (NIR). Hyperspectral imaging of 300 individual kernels per sample set allowed to discriminate between wheat kernels inoculated either with two OTA-producing *P. verrucosum* or non-OTA producing *P. verrucosum* strains. Besides the three wavelengths at 1,280, 1,300 and 1,350 nm found to be characteristic for infection with *P. verrucosum* in general, a fourth wavelength of 1,480 nm was found to be specific for infections with the two OTA-producing strains or for OTA. Besides showing presence or absence of *P. verrucosum*, discrimination between different inoculation times and therefore between different OTA concentrations was also possible. The current state of the study seems promising but requires an extension of the data set using a broader variety of *P. verrucosum* strains but also of other fungi. Moreover, it is not clear if NIR allows the detection of OTA or just a specific common feature of the two OTA-producing *P. verrucosum* strains. Thus, additional spiking experiments to characterise the effects of OTA itself on the NIR spectrum might be helpful.

A first approach using matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry for the detection of OTA has been published (Hickert *et al.,* 2016). In this study, the authors used MALDI-mass spectrometry imaging (MALDI-MSI) to investigate the production and distribution of OTA in grapes inoculated with *A. carbonarius*. Therefore, a mouldy grape was embedded in hydroxyethyl cellulose and sectioned on a cryostat at -20 °C into 20 µm sections. The grape tissues were placed on indium-tin-oxide glass slides, dried and coated with 2,5-dihydroxybenzoic acid matrix for ionization. With their method, the authors showed that OTA is not transported into the inside of the grape but retained within the visual fungal mycelium. Identification of OTA was done via monitoring of the different proton, sodium, and potassium adducts, the isotopic pattern as well as via fragmentation spectra. MALDI-MSI does not allow quantification of analytes, and sensitivity of this method is also limited. However, showing that MALDI-MSI of mycotoxins is possible encourages to start further experiments, for instance to investigate the OTA distribution in animal tissues.

OTA biomonitoring has been done mainly in blood due to the high affinity of OTA to blood constituents such as serum albumin, resulting in a long biological half-life. Nevertheless, in recent years OTA biomonitoring in urine became more popular as it employs a non-invasive sampling method and might provide better data on short-term exposure. Most methods for OTA biomonitoring are solely limited to OTA and sometimes extended to its metabolite ochratoxin α. Additional glucuronidase treatment for the indirect detection of phase 2 metabolites is usually not applied in modern dilute and shoot methods because most of the prominent glucuronides are directly detected within the LC-MS run. However, for OTA and ochratoxin α, glucuronidase treatment has been shown to sometimes result in higher values. Muñoz *et al.* (2017) investigated this phenomenon with urine samples from different cohorts by using a synthesised OTA-glucuronide and ochratoxin α-glucuronide reference. While the dilute and shoot approach was not sensitive enough for direct glucuronide detection, glucuronidase treatment that also involves liquid/liquid extraction with chloroform-isopropanol and concentration steps, resulted in values ranging between no effect and approximately six-fold increased OTA levels. Thus, so far only deconjugation using sulphatase and glucuronidase has been successful for phase 2-metabolite detection of OTA in human urine.

10. Patulin

Two interesting review articles regarding PAT were published recently. Li *et al.* (2017b) summarised mainly methods for PAT analysis published over the past decade, including the highly reproducible chromatography and mass-spectrometry-based methods, highly selective sensor-based methods and indirect quantitative PCR methods and promising features of novel materials, such as cyclodextrin-based polymers, magnetic nanocomposites and MIPs used in sample preparation for PAT determination. The safety concern of mitigation of PAT in foods has been further emphasised by a recent article. Ioi *et al.* (2017) gave an in-depth survey review of novel methods to remove and detoxify PAT including non-thermal processing techniques such as high hydrostatic pressure, UV radiation, enzymatic degradation, binding to microorganisms, and chemical

degradation. Since these methods have not been optimised, future development should focus on determining the nature and safety of chemicals produced from the breakdown of PAT in each of these treatment techniques.

Several novel analytical methods have been published as well: two using aptamers, one using GO-based magnetic SPE, two using MIP and one using a combinatory library of organic polymers for the SPE. For instance, Tomita *et al.* (2016) gave a detailed description of a procedure to generate an aptamer-based sensor for PAT detection. PAT was identified by a DNA module platform, comprised of four regions: the aptamer, a joint module, the terminal stem and a DNAzyme conjugated to a horseradish peroxidase that catalyses a redox reaction controlled by a structural change induced by aptamer/target binding. The joint module is capable of sensing a conformational change in the aptamer region, linked to the signal transmission activity of a DNAzyme as the reporter in a concentration-dependent manner. Using the DNA module platform, a microarray containing >10,000 sequences designed by *in silico* secondary structures, a new aptamer against PAT was predicted and identified successfully. However, the PAT aptamer sensor showed low sensitivity to detect PAT in food and beverages. Wu *et al.* (2016) reported the use of a systemic evolution of ligands by exponential enrichment (SELEX) assisted by a GO screening approach for selection of aptamers against PAT. The binding properties of the selected aptamers toward PAT were identified by fluorescence analysis. The aptamer PAT-11 bound to PAT with high affinity and excellent selectivity. PAT-11 had a dissociation constant of 21.8±5.0 nM and was subsequently used as a recognition element to develop a detection method for PAT based on an enzyme-chromogenic substrate system. The colorimetric aptamer sensor exhibited a linear range from 0.05 to 2.5 µg/l, and the LOD was found to be 0.048 µg/l. This method could be used for the detection of PAT in foods.

Wang *et al.* (2017b) reported the synthesis and characterisation of a GO-based magnetic nanocomposite (MGO) for purification of PAT in apple juice after extraction. PAT was separated and determined by LC-UV. The authors investigated critical experimental parameters that could affect the extraction efficiencies, such as the amount of GO-based magnetic nanocomposite, the pH, ionic strength of the sample solution, the extraction time and the desorption conditions. The LOD of the method was 2.3 µg/kg, the recoveries ranged from 68.7-83.6%, with relative standard deviations ranging between 1.8-8.7%. The MGO appeared to be a reliable material for PAT determination in juice. Lucci *et al.* (2017) evaluated the use of a MIP (Affinisep SPE PAT) as a selective SPE sorbent for the clean-up and pre-concentration of PAT from apple juice, puree and jam samples. Empty SPE cartridges of 3 ml, capped with fritted polypropylene disks at the bottom and on the top, were packed with 100 mg of the MIP and conditioned with ACN and water prior to each use. For jam and puree samples the method involved adding pectinase and water placing the samples in an oven for 2 h at 40 °C, centrifuging the resulting mixture and filtering over regenerated cellulose and then passing through the MIP column. The column was washed with 1% aqueous acetic acid, 1% $NaHCO_3$ solution and water. PAT was eluted from the column with diethyl ether. UHPLC-UV was used for the analysis of PAT, yielding recoveries of >77%. The method was validated and found to be linear in the range of 2-100 µg/kg with RSDr values below 11% in all cases.

Zhang *et al.* (2017b) presented an optical method for determination of PAT in apple juice based on MIPs capped Mn-doped ZnS QDs. The nanosensor was fabricated by the sol-gel method using 6-hydroxynicotinic acid as the template, 3-aminopropyltriethoxysilane as the functional monomer and tetraethoxysilane as the cross-linker through the surface imprinting process. A photo-induced electron transfer mechanism is implicated by the phosphorescence quenching generated by high polarity PAT binding with the imprinted cavities of the sensor. Sensing tests demonstrated that the MIPs capped Mn-doped ZnS QDs provide better binding performance than non-imprinted polymer QDs with higher selectivity and sensitivity for PAT over its analogues. This method, which utilised the phosphorescence properties of Mn-doped ZnS and the selectivity of MIPs, presented potential advantages in comparison with antibody-based analytical methods for PAT in apple juice samples or other complex matrices. Because the LOD of the method is inferior to LC and other antibody-based immunoassays, it is necessary to improve the sensitivity of this phosphorescent nanosensor in the future. Giovannoli *et al.* (2017) described a screening of a combinatorial library of organic polymers for the SPE of PAT from apple juice. The authors described the preparation of a 256-member combinatorial polymeric library based on the use of 16 functional monomers, 4 cross-linkers and 4 different porogenic solvents. The first part of the experimental work was focused on the screening of the polymer libraries in order to find out the polymerisation mixtures with the strongest binding properties towards PAT. They identified 25 polymers with desired properties, from which the authors selected methacrylic acid-pentaerithrytol tetraacrylate prepared in chloroform to use as the SPE material in a clean-up and extraction procedure for PAT in apple juice. Clean chromatographic patterns and acceptable recoveries were obtained for juice spiked with PAT at concentration levels of 25 (64±12%), 50 (83±5.6%) and 100 µg/l (76±4.5%). The within-day and between-day reproducibility evaluated at a concentration level of 25 µg/l were 5.6 and 7.6%, respectively.

11. Trichothecenes

From the papers being issued between mid-2016 and mid-2017, the most interesting ones have been selected to be included in this review. Publications presenting methods developed primarily for the purpose of analysis a specific set of samples and lacking novelty were omitted here. A publication demonstrating analysis of trichothecenes in blood spots as a part of a multi-mycotoxin biomonitoring method (Osteresch *et al.*, 2017) was also not included in this section, as it is discussed already in Section 3.

Three papers discussed novel methods where LC-MS was employed for separation and detection. Fan *et al.* (2016) demonstrated development and validation of a UHPLC-MS/MS approach utilising an Acquity UPLC®HSS T3 (2.1×150 mm, 1.8 µm, Waters, Milford, MA, USA) column and XEVO TQ-S (Waters) mass analyser for simultaneous determination of four type B trichothecenes (fusarenone X (FUS-X), DON, 3-ADON and 15-ADON) together with the masked DON-3G in various feed products. The study introduced an improved sample pre-treatment exploiting the commercially available GPD HLB cartridges. As reported earlier, adsorbents used in these cartridges were shown to be unsuitable to effectively recover DON-3G. This study presented the optimisation of sample preparation, including selection of extraction solvents and tuning the composition and volumes of SPE solutions, to achieve an efficient purification procedure with recoveries of all analytes (including DON-3G) between approx. 79-108%. The optimised procedure comprised ACN:water (50:50, v/v) as an extraction mixture, water as a loading and washing solvent, and methanol as an elution agent. LOQs for the analytes were 0.10-4.85 µg/kg. Two other LC-MS based papers introduced a special type of magnetic SPE for efficient clean-up of type A trichothecenes (diacetoxyscirpenol (DAS), neosolaniol (NEO), T-2 and HT-2) in coix seed (Dong *et al.*, 2016), and T-2, HT-2, DAS, NEO and ZEA in danshen root extracts (Jiang *et al.*, 2017). The Fe_3O_4/multi-walled carbon nanotube (MWCNT) composite was synthetised as a sorbent, and several key parameters affecting the performance of the clean-up procedure, such as composition of solvents, amount of sorbent, as well as arrangement of the whole purification process, were thoroughly investigated. In the study of Jiang *et al.* (2017), the classical SPE column arrangement with 20 mg of Fe_3O_4/MWCNT sorbent was evaluated as the best option in terms of recoveries and purification efficiency obtained. It should be noted that beside the above mentioned composite sorbent, Fe_3O_4 and MWCNTs were tested also individually, but unsatisfactorily low recoveries of mycotoxins were achieved. In addition to the column SPE, also the dispersive SPE clean-up arrangement with Fe_3O_4/MWCNT composite was examined, however, to get satisfactory recoveries comparable with those obtained by the column SPE, more than 40 mg of the sorbent would have to be used. As concerns the sample preparation, ACN:water (84:16, v/v) was taken for extraction, 2% aqueous ACN was used as the loading solution, 5% aqueous methanol as the washing solvent and acetone containing 0.5% formic acid for analyte elution. The LOQs for all five mycotoxins were 1.2-4.8 µg/kg. In the study of Dong *et al.* (2016), the sample preparation procedure was the same as in the previously discussed study (i.e. extraction with ACN:water (84:16, v/v), evaporation of an aliquot, and re-dissolving and dilution to obtain 2% aqueous ACN), but the dispersive SPE arrangement with 100 mg of Fe_3O_4/MWCNTs was utilised this time. Collection of the sorbent with adsorbed analytes was carried out by applying a strong magnetic field at the bottom of the tube, and desorption was realised by vortexing and ultrasonication in methanol. The LOQs achieved in this study were 0.3-1.5 µg/kg. In both of the studies (Dong *et al.*, 2016; Jiang *et al.*, 2017), separation was achieved on a Poroshell EC-C18 column (100 mm×3.0 mm, i.d., 2.7 µm, Agilent), and MS detection was realised on XEVO TQ-S MS (Waters).

In two other publications, methods employing separation by LC coupled with 'conventional' detectors were introduced. Xu *et al.* (2016) published the simultaneous determination of DON and its acetylated derivatives in wheat flour and rice by UHPLC chromatography coupled with photo diode array detection detection (200-500 nm). The QuEChERS extraction followed by subsequent SPE clean-up was employed. From several SPE cartridges tested, Oasis® MAX columns (Waters) were selected as the best option, with a previous defatting step of the sample extract by hexane. When testing 5% aqueous ammonia (pH >12.0) as the SPE washing solution (in accordance with producer's recommendations), decreased recovery of 3- and 15-ADON caused by partial hydrolysis of acetyl groups was observed. The optimised clean-up procedure used a pH value of 10.5 for the washing buffer. Efforts were made to optimise the chromatographic separation with focus on the problematic couple 3- and 15-ADON. For this purpose, ACQUITY UPLC HSS CYANO (3.0×150 mm, 1.8 µm; Waters), ACQUITY UPLC BEH amide (3.0×100 mm, 1.7 µm; Waters) and Hypersil GOLD PFP (2.1×150 mm, 1.9 µm; Thermo Scientific, Waltham, MA, USA) columns were examined. The results revealed that the amide column had no retention for DON, while the PFP column could separate 15-ADON and 3-ADON without retention of DON. The best separation was obtained on a HSS CYANO column, so this was selected for the final validation. LOQs achieved by this method were 72-191 µg/kg (Xu *et al.*, 2016). Rahmani *et al.* (2017) introduced an interesting method of analysis for the determination of DON in rice samples, based on realisation of air-assisted DLLME and HPLC-UV separation and detection. In this simple pre-concentration method, the extractant solvent (chloroform) is dispersed by a glass syringe in the sample extract (ACN:water, 84:16, v/v), and after centrifugation, mycotoxins are concentrated

in the bottom chloroform layer. To find out the optimised conditions for the proposed method, response surface methodology – a statistical technique for simultaneous investigation of interactive effects between the factors tested – was applied. Under the optimised conditions, the LOD of DON was 23.6 µg/l (authors did not present LOD in solid matrices, but considering the described sample preparation procedure, after recalculation it is 1.9 µg/kg).

Four papers introduced approaches based on non-chromatographic techniques. The first of them described a chemometric classification of mid-infrared Fourier-transform infrared spectroscopy (FTIR) spectra of maize contaminated by DON and peanuts containing AFB_1 (Kos *et al.*, 2016). The great advantage of this method realised with a portable FTIR device (Bruker Alpha, Billerica, MA, USA) was the little sample preparation (direct analysis of defined sieve fraction of milled maize), followed by simple data processing, without the need for expensive laboratory facilities and highly qualified personnel. This is especially important for traders and food processors, which usually need immediate 'on-site' response to make decisions. Spectral data were classified by using a bagged decision tree method, evaluating the absorption regions of indirect markers of fungal contamination, proteins and carbohydrates. The method was able to classify 79% of 110 maize samples at the European Union regulatory limit for DON (1,750 µg/kg) and 77% of 92 peanut samples at the regulatory limit for AFB_1 (8 µg/kg before sorting). As compared with previous works, the important step forward of this study, especially in terms of practical application, was the rather high number of samples with a suitable toxin concentration, which was used for building of a representative database. Once the robust model already exists, the application of the method is very straightforward and easily feasible. A follow-up study (Sieger *et al.*, 2017) is described in Section 5 of this review.

The last two 'non-chromatographic' studies for analysis of trichothecenes were based on immuno-chemical analysis. Righetti *et al.* (2016) presented a work focused on assessment of accuracy of DON results generated by a commercial ELISA kit (Celer® DON v3; Tecna, Trieste, Italy). For this purpose, authors used a set of naturally incurred soft wheat and maize samples which were analysed by both ELISA and LC-MS/MS as reference method. Matrix-matched calibration was performed to compensate matrix effects in ELISA. The ratio of DON measured by ELISA compared to LC-MS/MS was determined. The obtained data showed that the ELISA kit returned an apparent overestimation, when 3-ADON and DON-3G were present in the samples. When the 'total DON', i.e. including contributions from 3-ADON, 15-ADON, DON-3G and nivalenol (NIV) was calculated, the accuracy of the ELISA analysis increased. The reason for this behaviour were the cross-reactivities with DON3-G (60%) and 3-ADON (100%), while cross-reactivities with 15-ADON and NIV were less than 4%. Based on the obtained results, authors concluded that the ELISA kit validated in this study must be regarded as a group detection tool, i.e. measuring the sum of DON and 3-modified parent compounds. Since no cross-reactivity was found for 15-ADON and NIV, this test kit cannot be considered as a total B-trichothecene detection tool. McNamee *et al.* (2017) developed an assay based on a novel multiplex nanoarray capable of the rapid simultaneous semi-quantitative determination of T-2, ZEA and FB_1. Additionally, the assay was shown to be able to detect HT-2, FB_2 and FB_3 due to the cross-reactivity profiles of the antibodies used. Individual mycotoxin-BSA conjugates were nano-spotted onto wells of a microtiter plate, and the assay was realised in a classical competitive arrangement. Briefly, after addition of mycotoxin-specific antibodies, competition for binding sites on these antibodies between the immobilised toxins and those in free solution occurs. The secondary antibody, anti-rabbit IgG conjugated with alkaline phosphatase, reacts with the antibody that is bound to the immobilised toxin, and after addition of the 5-bromo-4-chloro-3-indolyl-phosphate conjugated with nitro blue tetrazolium substrate, colour is generated. The sensitivity of the method was determined as the concentration causing 50% inhibition, and was 197, 0.7 and 217 ng/ml in wheat and 44, 0.5 and 26 ng/ml in maize for ZEA, T-2 and FB_1, respectively. Taking into account the 50% inhibition concentration and the threefold extraction dilution this renders the method sensitive enough for the analysis of at least T-2 and FB_1 in feed, but the European guidance values for ZEA in animal feed (0.25 mg/kg) cannot be monitored with this method.

12. Zearalenone

The vast majority of newly published analytical methods for the determination of ZEA in food and feed continue to be based on LC-MS(/MS) multi-toxin methods, covered in Section 3 of this article. However, also dedicated chromatographic methods to determine ZEA and its metabolites (and/or other oestrogenic compounds) were developed for several foodstuffs.

Regarding clean-up, magnetic SPE gained a lot of popularity recently, with at least four publications reporting its development and application. For instance, a magnetic SPE clean-up using hybrid MWCNTs was developed and employed for the purification of ZEA, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) from maize (Moreno *et al.*, 2016). The authors first synthesised C18 silica nanoparticles. Those were then further used for a high temperature reaction with MWCNTs and Fe^{3+} ions in ethylene glycol. The resulting magnetic particles were used to purify the analytes from maize extracts, prior to HPLC-ESI-MS determination. Interestingly, also the non-C18

nanotubes absorbed the analytes, although not as good as the hybrid particles. Overall, recoveries of 92-98% were achieved for all analytes with RSD_r values <4%. Authors reported a 25 fold concentration factor and a re-usability of the magnetic SPE material for up to six times. This method was further refined using different magnetic nanotubes without the C18 layers, which were also easier to synthesise (Han *et al.*, 2017). Test portions were weighed into a 50 ml centrifuge tube, extracted with ACN:water (84:16, v/v), ultrasonicated and centrifuged. Aliquots of the supernatants were dried down and re-dissolved in ACN:water (5:95, v/v). After addition of the magnetic MWCNTs, the mixtures were vortexed to allow adsorption of the mycotoxins. The nanotubes were collected by adding a magnet under the centrifuge tube, while the supernatants were discarded. Afterwards, the mycotoxins were desorbed by sonification in acidified acetone. The nanotubes were removed magnetically, while the extracts were dried down, taken up in ACN and analysed by UHPLC-ESI-MS/MS in negative ion mode. Only minimal matrix effects (92-104%; no matrix effects=100%) were evaluated for the purified extracts. Recovery for ZEA was 102%, while 94% ZAN and around 80% for all other derivatives were recovered on average. RSDs for inter- and intra-day precision were all lower than 14%, and the LOQs were in the range of 0.07-0.10 µg/kg for all analytes.

The same six analytes were cleaned-up from cow milk using magnetic GCB as SPE material and quantified using UHPLC-ESI-MS/MS (Capriotti *et al.*, 2016). The GCB nanoparticles were decorated with magnetite (Fe_3O_4) after acidic activation. Whole milk was skimmed by centrifugation, diluted and buffered to pH 7 and treated with the SPE material for 30 min under agitation. A permanent magnetic disk was used to sediment the nanoparticles and the analytes were subsequently eluted from them using CH_2Cl_2:methanol (80:20, v/v). The extract was evaporated, reconstituted in aqueous methanol and injected into the UHPLC system. Unfortunately, authors skipped any enzymatic pre-treatment to cleave glucuronides and none of the analytes were found in the used milk samples. Nonetheless, method validation resulted in recovery values from 52-102% depending on the analyte, with acceptable values for both extraction recoveries and matrix effects. RSD_R values were below 22% for all analytes and very reasonable LOQs in the range of 8-15 ng/l were obtained. Finally, magnetic core-shell poly(dopamine) nanoparticles were used for the extraction of the same six oestrogenic analytes from milk and yogurt, followed by LC-MS analysis (Gonzalez-Salamo *et al.*, 2017). Here, Fe_3O_4 nanoparticles were coated with dopamine at slightly alkaline pH to induce the polymerisation process. Samples were first treated with acidified ACN to precipitate proteins. The supernatant was diluted, buffered to pH 7 and shaken with the SPE material for 30 s. Again no glucuronidase treatment was included in this method. After magnetic separation, analytes were eluted from the nanoparticles with methanol. The eluate was dried, taken up in aqueous ACN and injected into the HPLC system. Matrix-matched calibration was used to evaluate the data. In different kinds of milk (and an unsweetened natural yogurt) samples the recovery values of all analytes ranged from 77-120% with RSD_r values below 15%. Compared to the method mentioned above (Capriotti *et al.*, 2016), LOQ values were about 1000 fold worse and ranged from 0.7 to 16 µg/l for the milk and from 1-15 µg/kg for the yogurt samples. This is partly explained by the used ion trap mass spectrometer, which was used in full scan mode.

Three more publications describe the quantification of estrogenic compounds, including ZEA and its metabolites, in milk products using chromatographic methods. In total 22 analytes (eight phytoestrogens, four synthetic and four natural estrogens, as well as ZEA, α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL) were extracted using QuEChERS from different dairy products and measured by UHPLC-ESI-MS/MS (Socas-Rodriguez, 2017a). Matrix-matched calibration was needed to cope with the matrix effects encountered in skimmed and whole cheese as well as in cow and goat kefir. In addition, β-ZAL-D_5 was used as internal standard for all mycotoxins. Using that setup, recovery values ranged from 55-119%, depending on the analyte and spiking level for the six mycotoxins. RSD_r values were below 15%, while LOQs were in the range 0.05-2.5 µg/kg, depending on analyte and matrix. This method was further refined using the same LC-MS/MS setup, but including an important enzymatic treatment of samples to cleave glucuronides and sulfates (Socas-Rodriguez, 2017b). Milk and yogurt samples were analysed for basically the same set of analytes as described above. Samples were weighted in, mixed with acetate buffer (pH 5) and β-glucuronidase was added for overnight incubation at 37 °C. QuEChERS included a dispersive SPE step with C18 material after liquid partitioning. UHPLC-ESI-MS/MS on a triple quadrupole instrument, operated in negative ion mode followed and ^{13}C-ZEA was used as internal standard for the mycoestrogens, which was in particular important for yogurt. 76-119% of the mycoestrogens were recovered in milk and 70-120% in yogurt, depending on the analyte. RSD_r values in both cases were lower than 20%. LOQs were 0.02 µg/l milk or 0.02 µg/kg yogurt for all analytes. No mycoestrogens were found in 11 milk and 13 yogurt samples from a Czech retail market. The importance of the enzymatic sample treatment was underlined as the found bound estrogens occurred in twenty-fold higher amounts, than their free forms. Another method for the determination of oestrogenic compounds in milk and yogurt samples used hollow-fibre liquid-phase microextraction (HFLPME) GC-MS/MS (D'Orazio *et al.*, 2016). Besides four sexual hormones, two exoestrogens, two synthetic oestrogens, also ZEA, α-ZOL, β-ZOL, α-ZAL and β-ZAL were included in the method. Proteins were first precipitated by addition of

ACN and centrifugation. The supernatant was evaporated until an aqueous residue remained, which was diluted with brine, sonicated and used for the HFLPME procedure. No enzymatic treatment to cleave glucuronides was performed. The hollow fibre was impregnated with n-octanol and placed into the aqueous extract (adjusted to pH 8). After 60 min of extraction under agitation, the fibre was put in ACN and sonicated for 10 min for back-extraction. The solvent was evaporated under nitrogen, leaving a drop of n-octanol which was used for derivatisation with N,O-bis(trimethylsilyl)trifluoroacetamide. The formed trimethylsilyl products of the analytes were then detected on a GC triple quadrupole MS with an electron impact interface. For the mycoestrogens, recovery values ranged from 81-116%, with RSD_r values of below 20% for all matrices. LOQ values were 1-6 µg/l for milk and 2-10 µg/kg for natural yogurt.

Also immunoassays to determine ZEA remain very popular. Interestingly, the development and application of at least five such assays were published within the last 12 months by authors from China. For instance, an electrochemical indirect competitive immunoassay based on a glassy carbon electrode modified with carboxylated MWCNTs and chitosan was developed (Xu *et al.*, 2017). Therefore, ZEA-BSA was covalently bound onto the activated nanocomposite film. Indirect competition for (anti-ZEA) antibodies took place between ZEA-BSA and free ZEA and the mixture was incubated on the nanocomposite surface. A secondary antibody, labelled with alkaline phosphatase, captured the bound primary antibody. The substrate 1-naphthylphosphate was used to produce 1-naphthol, which gives an anodic electrochemical signal at 0.3 V, which can be measured on the modified electrode. While the assay showed good sensitivity, it has to be mentioned that immunoaffinity columns were used to purify and enrich the analyte from raw extracts of maize samples. In solution, a working range over five orders of magnitude from 10 ng/l to 1 mg/l was obtained. As the used sample extract dilutions were not specified, the LOQ for cereals cannot be calculated, but maize samples spiked from 10 µg/kg to 10 mg/kg yielded recovery values of 94-106%. RSD_r values (from triplicates) ranged from 8-27%, depending on the spiking level. Another electrochemical immunosensor, based on antibody immobilisation on a glassy carbon electrode – modified with MWCNTs and AuPt nanoparticles – was presented by Liu *et al.* (2017b). The glassy carbon electrode was modified with polyethyleneimine-functionalised MWCNTs. Afterwards, gold and platinum nanoparticles were electro-deposited, thus enhancing the surface area for capturing antibodies as well as the electrochemical performance. Finally, monoclonal (anti-ZEA) antibodies were immobilised on the electrode and the surface blocked with BSA. The biosensor detected ZEA by voltammetry at a working potential of 0.18 V. A wide working range in solution from 0.005 to 50 µg/l was determined. Maize samples were extracted in a 5-fold excess of methanol:water (70:30, v/v) and further diluted five times. The overall dilution factor of 25, therefore results in a working range of about 0.125-1250 µg/kg, which is very suitable for ZEA determination in cereals. A mini-validation yielded 88-109% recovery with RSD_r values of less than 10%.

The synthesis of highly fluorescent upconversion nanoparticles, which were conjugated with the complementary oligonucleotide of a ZEA aptamer, was reported (Wu *et al.*, 2017). Magnetic nanoparticles immobilised with the ZEA aptamer were assigned as capture probes. After aptamer hybridisation and magnetic separation, the luminescence intensity was recorded at an emission wavelength of 543 nm following excitation with a continuous wave laser at 980 nm. With the addition of ZEA, the aptamer dissociated from its complementary DNA and was preferentially bound to ZEA, resulting in a decrease of fluorescence. A working range from 0.05-100 µg/l was established for the final assay and recovery values ranged from 94-104%. This was also the working range for beer samples, which were just degassed before analysis. Maize samples were extracted with a 5 fold volume of methanol:water (60:40, v/v) and further diluted 1+1 with buffer before analysis. The corresponding working range for cereals samples therefore is 0.5-1000 µg/kg. The repeatability of the assay was not tested specifically. Another immunoassay was developed exploring fluorescence polarisation (Zhang *et al.*, 2017d). While that technique is not new for ZEA determination in maize, two new monoclonal antibodies with affinity to the ZEA class and four fluorescein-labelled tracers were prepared and compared. Finally an antibody with cross-reactivities ranging from 78-96% for α-ZOL, β-ZOL, α-ZAL, β-ZAL and ZAN was selected, making the assay a suitable tool to determine the sum of ZEA and its main derivatives. IC_{50} values were around 2 µg/l for all six analytes, yielding an LOD of about 12 µg/kg for ZEA in maize. Recoveries for spiked maize samples ranged from 85 to 114%, with RSD_r values <15%. Finally, an immunochromatographic strip test was developed to test for ZEA occurrence in wheat (Ji *et al.*, 2017). A newly developed monoclonal antibody against ZEA was labelled with 30 nm colloidal gold particles and used as probe for the assay. In the absence of ZEA from the sample extract, the antibody-gold conjugate binds immobilised ZEA-ovalbumin on the membrane to form a red coloured immune complex. After extraction and two-fold dilution in buffer, the assay can be completed within 5 min. A detection limit of 15 µg/l was evaluated for standards, and wheat samples contaminated with more than 50 µg/kg did not produce false positive or negative results. While the strip tests cross-reacted with α-ZOL, β-ZOL, α-ZAL and β-ZAL, the rate was not verified. This assay can be used as a qualitative tool only.

An upcoming trend appears to be the development of immunoassays which are able to determine several mycotoxins. For instance, a membrane-based flow-through test was presented, able to screen for ZEA, DON, AFB_1 and OTA in a variety of cereal-based feedstuffs (Beloglazova *et al.*, 2017). Cereal samples were extracted with varying volumes of methanol/water (80/20, v/v), depending on the exact sample type. After dilution with buffer, the raw extracts were treated with C18 silica sorbent and filtered to remove interfering (e.g. coloured) matrix compounds. The assay consisted of a nylon membrane on which five circles of anti-mouse IgG were spotted. After drying and blocking, four test zones with the individual monoclonal antibodies were spotted, while for the control the fifth spot remained untouched. 3 ml of diluted sample extracts were passed through the prepared membrane. After washing, 10 µl of the respective mycotoxin horseradish peroxidase conjugates were added onto the matching detection zones. After another washing step, a drop of the chromogenic substrate mixture (3,3',5,5'-tetramethylbenzidine/H_2O_2) was added on each spot. The colour intensity of the spots was visually evaluated after 5 min. The cut-off levels for ZEA, DON, AFB_1 and OTA were 50, 200, 1 and 10 µg/kg, respectively, with false positive and false negative rates below 5%. Also, a novel chemiluminescence immune-affinity 96 spots monolith array was developed to detect ZEA, DON, T-2 and FB_1 in maize samples (Li *et al.*, 2016). The monolith array was prepared by UV-initiated copolymerisation with glycidyl methacrylate as monomer, polyethylene glycol diacrylate as cross-linker and polyethylene glycol 200 as porogen. Monoclonal antibodies against the four toxins were immobilised into the monolithic copolymers through an epoxy-amino group reaction. Maize samples were extracted with methanol:water (70:30, v/v). The five-fold diluted extract was mixed with a solution containing all four mycotoxin horseradish peroxidase conjugates and added to the test spots. After 30 min, the spots were washed and the chemiluminescent substrate (luminol/p-iodophenol/H_2O_2) was added. Signals were recorded by a photomultiplier. While excellent LODs of 4.8 ng/l (ZEA), 3.6 ng/l (DON), 3.9 ng/l (T-2) and 1.7 ng/l (FB_1) were evaluated for standard solutions, the linear response was just one order of magnitude. Considering the dilution factor, this would result in an LOD of about 25 ng/kg for ZEA, which cannot be verified with the given data as seemingly also maize spiked at lower concentrations gave a quantitative signal. RSD_r values were below 5% for all four toxins.

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