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Developments in mycotoxin analysis: an update for 2014-2015

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REVIEW ARTICLE

Abstract

This review summarises developments in the determination of mycotoxins over a period between mid-2014 and mid-2015. In tradition with previous articles of this series, 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 new methods used to analyse botanicals and spices and newly developed LC-MS based multi-mycotoxin methods. The critical review aims to briefly discuss the most important developments and trends in mycotoxin determination as well as to address shortcomings of current methodologies.

Keywords: aflatoxin, *Alternaria* toxins, ergot alkaloids, fumonisin, ochratoxin A, patulin, trichothecene, zearalenone, sampling, multi-mycotoxin, botanicals, method development

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 2013-2014 period (Berthiller *et al.*, 2015). The primary purpose is to raise awareness of the developments and advances in analytical methods for mycotoxins, derived from articles published between mid-2014 to mid-2015. Critical comments on the method, its validation parameters or application are usually added to guide readers in assessing the impact of a method. The observant reader might notice a gradual change in the format of the articles in this series from rather comprehensive reviews to more critical ones. The main reason for this development is the steadily increasing number of articles dealing with mycotoxin methods over the last years. 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. Unchanged, the covered topics 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 (AFs, Section 5), *Alternaria* toxins (Section 6), ergot alkaloids (Section 7), fumonisins (FBs, Section 8), ochratoxins (Section 9), patulin (PAT, Section 10), trichothecenes (Section 11) and zearalenone (ZEA, section 12).

Several reviews regarding mycotoxin determination have been published recently, which the interested reader might refer to for additional information. Advanced mycotoxin

detection techniques along with recent mycotoxin survey data reported from China have been reviewed (Selvaraj *et al.*, 2015). Lippolis and Maragos (2014) summarised fluorescence polarisation immunoassays (FPIAs) for the determination of mycotoxins in food and beverages. Gilbert and Pascale (2014) described analytical methods for mycotoxin determination in wheat in a recent book chapter. Finally, methods for the (semi-)quantitative determination of ergot alkaloids in a variety of matrices were reviewed, describing sampling, extraction, clean-up, detection, quantification and validation techniques (Crews, 2015). Clearly worth reading, the European Food Safety Authority (EFSA) published two articles on mycotoxins lately. First, a scientific statement on the increase of risk for public health related to a possible temporary derogation from the maximum levels (MLs) of deoxynivalenol (DON), FBs and ZEA in maize and maize products was given (EFSA, 2014a). EFSA concluded only a minor impact on exposure levels for DON and FBs by temporary exceeded MLs. As ZEA exposure levels with both current and elevated MLs are around or above the tolerable daily intake (TDI) for infants and toddlers, a health concern was considered. Another scientific opinion evaluated the risks related to the presence of modified mycotoxins in food and feed for human and animal health (EFSA, 2014b). In the absence of specific toxicity data, toxicity equal to the parent compounds was assumed for the modified forms. A potential health risk was identified regarding the presence of free and modified FBs in food, as the exposure of toddlers and other children exceeded the TDI.

It should be mentioned that despite the large number of analytical methods for the determination of mycotoxins that are proposed each year, not all of them also present (sufficient) performance characteristics. Proper in-house validation to verify the limit of detection (LOD), limit of quantification (LOQ), (linear) working range, precision (repeatability and reproducibility), trueness (recovery), selectivity and robustness is a mandatory measure to generate reliable methods. External quality assurance is equally important. There are now a number of proficiency testing schemes (organised e.g. by FAPAS®, BIPEA®, CODA-CERVA, ISPA-CNR, etc.) which include various mycotoxins in several food commodities. The inclusion of food and feed materials contaminated with multiple mycotoxins, which can be assessed by LC-MS/MS multi-mycotoxin methods, are a major improvement over the last years. Finally, there is still a lack of reference materials (RMs) and certified reference materials (CRMs) for mycotoxins. Considering the increasing need and number of mycotoxin analyses which are carried out worldwide, more suitable RMs and CRMs are needed for (multiple) mycotoxins in various commodities.

2. Sampling

Improvements in sampling of food and feed products continue to be a high priority among regulatory agencies, international organisations such as Codex, and commodity industries worldwide. Several articles were published that cover a wide range in mycotoxin/commodity combinations.

Tittlemier *et al.* (2015) determined that the poor quality grade components separated from wheat, called screenings, were contaminated with high levels of DON, ZEA and ochratoxin A (OTA). Of the four poor quality components screened from the whole kernels of wheat, light dockage (dust, stems, etc.) contained the highest levels of the three mycotoxins. Results of the study showed a strong correlation between the DON concentration in the light dockage and the mycotoxin levels in the unscreened wheat samples. Because of this strong correlation, there is a potential to develop a rapid screening technique to predict the DON levels in the entire lot by measuring DON in samples taken from the light dockage. The study also demonstrated which fractions of wheat could be considered high risk components for the above mentioned mycotoxins. Removing these poor quality grade components from wheat lots would definitely reduce mycotoxin contamination levels in wheat lots during processing.

Farkas *et al.* (2014) developed an early warning sampling protocol to be used by milk producers in Italy to detect aflatoxin M_1 (AFM_1) levels in milk consignments that exceed the European ML of 50 ng/kg. The early warning sampling protocol was based upon AFM_1 measurements in 21,969 milk survey samples collected in Italy during 2005-2008 and again in 2010 (Trevisani *et al.*, 2014). The sampling protocol, while specific to certain Italy milk production districts, was designed to take milk samples from individual milk producers and provide verification of compliance with the 50 ng/kg ML in 98% of the milk consignments at a 94% probability level. Probability levels were calculated from equations based upon the binomial distribution. The AFM_1 sampling protocol was designed as a compromise between providing a cost effective sampling protocol with a high certainty of effective control of detecting contaminated milk exceeding the ML.

The Association of Official Analytical Chemists (AOAC) International invited Kim Esbensen (Denmark), Claudia Paoletti (Italy), and Nancy Thiex (USA) to be Special Guest Editors to organise a special section of papers for the Journal of AOAC International that introduce the theory of sampling and the principals governing representative sampling (Esbensen *et al.*, 2015). Five authors prepared 12 papers intended to integrate with each other and provide a comprehensive overview of the criteria that needs to be followed to ensure representative sampling. While only one paper specifically addressed the representative sampling

for mycotoxins (Wagner, 2015), concepts addressed by the other papers can be applied to selecting representative samples to detect mycotoxins in food and feed. The guest editors recommend that the 12 papers, listed below, be read in the order presented in the journal:

1. Food and feed safety assessment: the importance of proper sampling (Kuiper and Paoletti, 2015).
2. Towards a unified sampling terminology: clarifying misperceptions (Thiex *et al.*, 2015).
3. A systematic approach to representative sampling (Wagner and Ramsey, 2015).
4. ample quality criteria (Ramsey and Wagner, 2015).
5. Material properties: heterogeneity and appropriate sampling modes (Esbensen, 2015).
6. Theory of Sampling – Four critical success factors before analysis (Wagner and Esbensen, 2015).
7. Quality control of sampling processes-A first foray: from field to test portion (Esbensen and Ramsey, 2015).
8. Considerations for interference to decision units (Ramsey, 2015a).
9. Distributional assumptions in agricultural commodities (Paoletti and Esbensen, 2015).
10. Critical practicalities in sampling for mycotoxins in feeds (Wagner, 2015).
11. Considerations for sampling contaminants in agricultural soils (Ramsey, 2015b).
12. Considerations for sampling of water (Ramsey, 2015c).

The guest editors and authors hope these 12 papers will initiate a global discussion that will lead to the harmonisation of techniques to select representative samples.

On the Codex front, the 9th session of the Codex Committee on Contaminants in Foods (CCCF) sent forward to the Codex Alimentarius Commission (CAC) draft MLs and sampling plans for DON for: (1) cereal-based foods for infants and young children; (2) flour, meal, semolina, and flakes derived from wheat, maize, and barley; and (3) cereal grains (wheat, maize, and barley) destined for further processing. CCCF agreed to forward sampling plans (including performance criteria for methods of analysis) for the three cereal-based products mentioned above to the Codex Committee on Methods of Analysis and Sampling (CCMAS) for endorsement. A detailed description of the sampling, sample preparation, analytical steps of the draft DON sampling plans for each of the three cereal-based products is described in the March 2015 CAC document distributing the 2015 CCCF report (CAC, 2015). In brief, the laboratory sample size and ML recommended by CCCF for: (1) cereal-based foods for infants and young children; (2) flour, meal, semolina, and flakes derived from wheat, maize, and barley; and (3) cereal grains (wheat, maize, and barley) destined for further processing are 1 kg and 200 µg/kg, 1 kg and 1000 µg/kg, and 1 kg and 2,000 µg/kg, respectively.

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

With respect to the development of LC-MS(/MS) approaches for multi-mycotoxin analysis, literature relevant to the period 2014-2015 reports advances in separation and detection techniques rather than new strategies for analyte extraction and purification. Overall, already established generic protocols, based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) or 'dilute-and-shoot' have been applied for the analysis of challenging or unconventional matrices. This highlights an even broader application range for LC-MS(/MS) techniques, with the perspective of generating new data on mycotoxin co-occurrence in previously uninvestigated food commodities.

For example, an emerging issue is represented by multi-mycotoxin determination in dried seafood and fresh fish. The few available literature studies suggest that mycotoxins, such as AFs or ZEA, can be present in seafoods if fish were exposed to mycotoxin contaminated feed (Huang *et al.,* 2011; Woźny *et al.,* 2013).

Therefore, an LC-MS/MS multi-mycotoxin method (including AFs, T-2 and HT-2 toxin (T-2 and HT-2), OTA, ZEA and DON) for dried seafood and fresh fish has been proposed by Sun *et al.* (2015). Special focus was given to test portion pre-treatment due to the high complexity of the studied matrices (muscle and entrails of fresh crucian carp, and dried fish products). Extraction solvent, time, and temperature as well as clean-up cartridges were optimised. The final procedure included ultrasound-assisted extraction with acetonitrile (ACN):water:acetic acid (79:20:1, v/v/v), defatting with *n*-hexane and purification by Oasis HLB® (Waters, Milford, MA, USA) cartridges. The use of matrix-matched calibration curves was recommended to ensure proper matrix effect compensation. The final method showed the LOQs ≤3 µg/kg, recoveries in the range 72-120% and within laboratory precision ≤18%. The validated method was applied to a set of 27 seafood samples. ZEA was the most frequently encountered contaminant with an incidence of 30%, and the highest concentration (317 µg/kg). OTA (4/27 samples, in the range 0.5-1.9 µg/kg) and traces of AFB_2 (1/27 samples, 1.2 µg/kg) were also detected.

Because of the paucity of information on the occurrence of mycotoxin in sorghum samples, in March 2011, the Codex Committee on Contaminants in Foods opened a discussion paper on 'mycotoxins in sorghum grain', highlighting the need for information on mycotoxin contamination in sorghum. Four sub-Saharan African countries most involved in sorghum agriculture were chosen to generate such data. Therefore, a multi-mycotoxin LC-MS/MS method for the determination of 23 mycotoxins, including AFs, OTA, *Fusarium* and *Alternaria* toxins, in different varieties of sorghum was developed and validated (Njumbe Ediage *et al.,* 2015). Analyte isolation included extraction with

methanol:ethyl acetate:water (70:20:10, v/v/v), defatting with *n*-hexane, and purification by amino solid phase extraction (SPE) cartridges. The method was validated for red, cream and yellow sorghum varieties. Different matrix effects were observed for different sorghum varieties. Consequently, the use dedicated matrix-matched calibration curves was necessary for accurate quantification. For the regulated mycotoxins, the LOQs were lower than the ML of the different analytes except of AFs and OTA in regulated foodstuffs (EC, 2006). The validated method was applied for the analysis of 10 red sorghum samples sourced from markets in Belgium and Germany, of which 9 were positive for at least one mycotoxin.

An LC-MS/MS method was developed for the simultaneous determination of aflatoxin B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1 and AFG_2), OTA, ZEA, DON, fumonisin B_1 and B_2 (FB_1, FB_2), T-2 and HT-2 in palm kernel cake, a protein source for livestock (Yibadatihan *et al.*, 2014). Mycotoxin contamination in this commodity is generally due to inappropriate handling during production and storage. The method represents a further example of application of a well characterised approach (based on one step extraction by a mixture of ACN:water:formic acid (79:20:1, v/v/v) followed by dilution with water and direct analysis by LC-MS/MS) to a novel food matrix. Suitable method performances were obtained (recoveries ranging from 81 to 112% and LOQ from 0.06 to 58 µg/kg). The results from a limited survey (25 samples of palm kernel cakes) showed that this commodity is prone to be contaminated with ZEA and to a lesser extent with AFs.

The extraction/partitioning QuEChERS procedure, prior to ultra-high-performance liquid chromatography (UHPLC) MS/MS analysis, has been re-proposed for the determination of 10 mycotoxins (sterigmatocystin, OTA, FB_1, FB_2, T-2, HT-2, CIT, ZEA, DON and fusarenone-X) in cereal syrups (Arroyo-Manzaranes *et al.*, 2015b). Matrix-matched calibration was necessary for quantification purposes. Also in this case, testing a relatively new target matrix constitutes the main advance of the study, rather than the developed analytical procedure. However, even though satisfactory method performances were reported in terms of LOD, precision and recovery rates, the applicability of the method to naturally contaminated samples was not proven. Other examples of multi-mycotoxin LC-MS/MS methods based on QuEChERS or 'dilute-and-shoot' approaches can be found e.g. in Hickert *et al.* (2015) or Fernandes *et al.* (2015).

Recent applications to milk and milk powder samples confirm that for trace mycotoxin analysis in milk and derived products careful sample preparation is still needed, due to the complexity of the matrix and, mainly, to the low concentration levels. A potentially interesting application of size exclusion chromatography for SPE purification of four mycotoxins (OTA, ZEA, AFB_1 and AFM_1) in milk and milk powder, prior to UHPLC-MS/MS analysis has been reported (Wang and Li, 2015). A SPE sorbent, based on C_4 and NH_2 mixed macropore silica gel, was proposed to remove high-molecular weight interfering compounds like proteins. However, strong matrix effects (up to 200% signal enhancement) were still observed. Winkler *et al.* (2015) developed a multi-mycotoxin method to investigate the carryover of ZEA, DON and their metabolites into bovine milk by a dose-response study. The sample pre-treatment included fat removal and pH adjustment prior purification through OASIS HLB® columns to achieve the desired sensitivity (LOQ ranged from 0.3 to 1.5 µg/l). ZEA, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), DON and deepoxy-deoxynivalenol (DOM-1) were detected in some milk samples of a dose-response study where three different levels of ZEA and DON were fed to dairy cows. However, on the basis of the detected mycotoxin levels, it was concluded that the carryover of the investigated toxins was negligible when diet concentrations were fed lower than or close to the current guidance values.

Among mycotoxin studies made available last year, strategies to improve the reliability of quantitative determination or to enhance confidence in compound identification in complex matrices can be found. A detailed discussion on the applicability of the stable isotope dilution analysis (SIDA) mass spectrometry approach for multi-mycotoxin determination of challenging matrices, such as animal feed or baby food, has been reported by Zhang *et al.* (2014a). The method required fortification of the test sample with ^{13}C-labelled internal standards prior to extraction with ACN:water. Even though no clean-up was performed, the use of ^{13}C-labelled mycotoxins eliminated the need for matrix-matched calibration achieving satisfactory accuracy and precision for the quantitative determination of the 11 mycotoxins of interest (AFs, DON, FB_1, FB_2, fumonisin B_3 (FB_3), OTA, T-2 and ZEA). However, to reduce internal standard costs, the test sample size was kept quite small (0.5 g), which might not be representative. The paper also reports a critical evaluation of optimal MS/MS parameters, describing the process of identifying suitable selected reaction monitoring (SRM) transitions, including switching from positive to negative ionisation, for each target matrix to minimise interferences due to isobaric matrix compounds. The same approach has been applied to food-grade gums (Zhang *et al.*, 2014b). Reliability of detection approaches based on LC coupled to full-scan high resolution MS (LC-HRMS), with the aim of obtaining quantitative and structural information to enhance confidence in compound identification, has been investigated by Liao *et al.* (2015). The authors described a method based on direct analysis of ACN/water extracts by UHPLC/Q-Orbitrap MS for the simultaneous screening of AFs, ergot alkaloids, FBs, ochratoxins, and trichothecenes in grain and nut samples. A workflow based on full-scan MS

for mycotoxin quantification, and data-dependent MS/MS experiments for structure confirmation was proposed. In Q-HRMS instruments involving data dependent acquisition, a response exceeding a user-defined threshold triggers a product-ion spectrum that can be matched against a library. The article discusses the optimisation of operating conditions in this mode, highlighting the power of the approach to achieve compound identification with a high degree of confidence. The validated method was successfully applied to the analysis of 11 RMs, and for a survey on 70 grain and nut samples, confirming its applicability for mycotoxin screening. Mycotoxin contamination was found in 35/70 food samples with concentrations ranging from 0.3 µg/kg (AFB_1 in peanut and almond) to 1,175 µg/kg (FB_1 in maize flour). However, due to the need for high level skills in establishing proper software settings and careful check of HRMS calibration, the proposed detection approach still appears impractical for routine mycotoxin monitoring. In this regard, a relevant discussion on reliability of data dependent acquisitions can be found in Lehotay *et al.* (2015).

Increasing the number of analytes included in developed methodologies poses the challenge of evaluating new approaches for method validation. In the case of mycotoxins, guidelines set in EU regulations are not easily applicable for methods covering a large number of fungal metabolites. Even though specifically drafted for pesticide residues, the SANCO guidelines (DG SANCO No. 12495/2013 and previous versions) are often taken as reference for validation of multi-analyte methods. An example of multi-mycotoxin method validation according to SANCO guidelines, has been reported by Malachová *et al.* (2014a). The validation procedure included four model matrices chosen as representative commodities, i.e. apple puree (high water content), hazelnuts (high fat content), maize (high starch or protein content, low fat content) and green pepper (complex matrix). Validation data were obtained for 295 analytes. For an additional number of metabolites no sound validation could be realised due to non-availability of analytical standards. For most of the analytes recoveries were in the range of 70-120%. The LOQs of mycotoxins regulated by EU were below the required MLs except for AFs and OTA in baby food and AFM_1 in milk. Finally, method trueness was demonstrated by analysis of various proficiency test (PT) materials. While only regulated mycotoxins could be considered in those trials, a wide range of different matrices were covered. Z-scores <|2| were achieved at all 26 PTs the laboratory participated with the exception of AFG_2 in baby food (z-score = 2.62) and FB_1 in maize (z-score = 2.97). Having such methods covering a large number of mycotoxins allows occurrence studies providing information on both regulated and less investigated mycotoxins. Within this context, particularly important are studies carried out on a large number of samples (e.g. Nathanail *et al.,* 2015a; Sulyok *et al.,* 2015). For example, Sulyok *et al.* (2015) reported the application of LC-MS/MS for a mycotoxin survey of 627 cassava samples collected from different districts across Tanzania and Rwanda. The occurrence of EU regulated mycotoxins was lower than 10% (with the exception of ZEA) and the related limits were exceeded only in few samples, suggesting that cassava is a relatively safe commodity regarding mycotoxin contamination.

With respect to multi-biomarker analysis, no new methods can be found in the last year literature, but applications of previously developed methods for exposure studies have been published. Gerding *et al.* (2014) analysed urine samples of 101 individuals from Germany applying a 'dilute-and-shoot' LC-MS/MS multi-biomarker method to quantify a total of 23 mycotoxins and their urinary metabolites. In this case, no significant correlation to the dietary habits of the participants could be drawn from the food frequency questionnaire, probably due to the low exposure and relatively small group size. Furthermore the study gave evidence for the presence of dihydrocitrinone (DH-CIT) and enniatin B in human urine, that were found in 12 and 20% of analysed samples, respectively, suggesting a need for further research to support these new data on the uptake and excretion of the so-called 'emerging' mycotoxins. DON and its glucuronide were quantified in 11 and 57% of samples, respectively, whereas most of the analysed mycotoxins were not detected or below the LOQ (T-2, ZEA-14-glucuronide, enniatin B and DH-CIT). The results of this study suggested a low everyday exposure of the investigated German population to mycotoxins, but revealed peak exposures above the widely accepted tolerable daily intake to DON in parts of the population.

As highlighted already in previous years, a strong trend in food safety is to integrate various groups of food contaminants into high-throughput analytical methods with a common sample preparation and end determination. Developing multi-class methods based on advanced MS approaches requires the use of a generic sample preparation procedure, while most of the efforts are focused on evaluation and optimisation of the MS detection approach. Despite the high number of methods available from literature, only limited demonstration of method applicability to real samples, confirming the fitness for purpose of multi-class methods, can be found. An LC-HRMS/MS method for determination of 389 pesticide residues, mycotoxins, and pyrrolizidine alkaloids, in extract obtained by optimised QuEChERS protocol has been developed for wheat, leek, and tea (Dzuman *et al.,* 2015). Efforts in evaluation and optimisation of MS parameters resulted in generation of a MS/MS mass spectral library of 323 pesticides, 55 mycotoxins and 11 pyrrolizidine alkaloids, potentially useful also with Q-Orbitrap instruments located in other laboratories. The article also reports interesting considerations on the influence of matrix effects on the quality of generated

spectral data. When comparing relative intensities of fragment ions measured in pure solvent (the MS/MS library) with those measured in matrix, quite significant discrepancies could be observed in some cases (e.g. for OTA). This phenomenon might complicate confirmation of analytes, especially when these occur very close to its LOQ in a complex matrix. Martínez-Domínguez *et al.* (2015a,b) reported two further applications of LC-HRMS based on Orbitrap technology for the determination of more than 250 toxic substances (including pesticides and mycotoxins) in diverse isoflavones supplements obtained from soy and ginkgo biloba samples. Suitable validation parameters in terms of trueness, reproducibility and repeatability, were obtained in both cases, however method application to real samples was limited to 13 soy isoflavone supplements and 9 samples of ginkgo biloba. Pesticides such as flutolanil (12.2 µg/kg) and etofenprox (48.2 µg/kg), and mycotoxins, such AFB_1 (8.2-17.1 µg/kg) and AFG_2 (6.4 µg/kg) were detected in isoflavones nutraceutical products; whereas hymexazol (10 µg/kg) and tebufenozide (55-459 µg/kg), AFB_1 (5-54 µg/kg), AFB_2 (4-300 µg/kg) and T-2 (18-20 µg/kg) were found in ginkgo biloba nutraceutical products.

Finally, it is worth mentioning that the increased number of LC-MS screening methods covering a high number of contaminants, poses relevant challenges in validation aspects including the need to update official method validation criteria to better reflect modern technologies, practices and needs. A critical review discussing aspects dealing with validation of qualitative/screening methods taking into account current needs for monitoring chemical contaminants in foods has been published (Lehotay *et al.,* 2015).

4. Mycotoxins in botanicals and spices

The number of published analytical methods for the determination of mycotoxins pertaining to botanicals and spices has decreased this year over the amount published the previous year. The methods still utilised high performance liquid chromatography with fluorescence (HPLC-FLD) or tandem mass spectrometric detection with one method developed using a fluorometric optosensor. The trend still continues for the analysis of multi-mycotoxins in the development of new analytical methodologies and also market surveys.

Three new analytical methods have been published this past year for the analysis of various mycotoxins in complex spices and botanicals. A method for the simultaneous determination of AFs and OTA in single and mixed spices was published (Wan Ainiza *et al.*, 2015). This method utilised a single extraction, purification with a multi-mycotoxin immunoaffinity column and HPLC-FLD with a photochemical derivatisation system. The method was optimised for the analysis of coriander, chili, cumin, fennel, turmeric and then applied to spice mixes including curry, kurma and soup powders. Validation studies were conducted to confirm that the method was suitable for multi-mycotoxin and multi-matrix analysis. The results indicated that the method for the simultaneous determination of AFs and OTA can be applied to both single and mixed spices and is suitable for routine laboratory analysis. Another published method was developed for the determination of OTA in doenjang, a traditional Korean fermented soybean paste (Seonghee *et al.*, 2015). The method utilised SIDA-LC-MS/MS for the analysis of OTA in the samples. The sample preparation conditions and LC-MS/MS measurement parameters were optimised and validated by measuring samples fortified with OTA at various levels. The validation studies demonstrated that the LC-MS/MS method is reliable and reproducible within 2% relative standard deviation. This analytical method was applied to determine OTA in various commercial and home-made doenjang products. A method was developed to determine citrinin (CIT) by using a fluorometric flow-through optosensor using Sephadex® SPC-25 (Sigma-Aldrich, St. Louis, MO, USA) as solid support (Jimenez-Lopez *et al.*, 2014). Multicommutated flow injection analysis was used for the construction of the manifold and for handling solutions. The calibration curve was linear in the concentration range of 35-900 ng/ml and a detection limit of 10.5 ng/ml was obtained for neat standard solutions of CIT. Depending on the used extraction procedure (liquid extraction or QuEChERS), different LOQs are obtained in matrix. An intra-day repeatability <4% was obtained for all recovery experiments with rice and dietary supplements. The developed optosensor was applied to the determination of CIT in rice and dietary supplements containing red yeast rice.

Several market surveys were performed for various mycotoxins in spices and medicinal herbs including HPLC analysis utilising a combination of FLD and LC-MS/MS. A total of 60 Chinese medicinal herbs were examined for contamination of the *Fusarium* mycotoxins enniatins A, A1, B, B1 and beauvericin (Hu and Rychlik, 2014). The herbs, commonly used in China as both medicines and foods, were randomly collected from traditional Chinese medicine stores. Beauvericin was the most frequently detected mycotoxin with a 20% incidence in all samples and each single enniatin was detected in 6.7-12% of samples. Considering the total amounts of five mycotoxins in single samples, values between 2.5 and 751 µg/kg were found. The analysis of AFs and OTA was performed in processed spices marketed in Penang, Malaysia, using immunoaffinity columns and HPLC-FLD (Ali *et al.*, 2015). The mean level, range and incidence of positive samples for total AFs were 1.6 µg/kg, 0.01-9.3 µg/kg and 85%, respectively. The mean level, range and incidence of positive samples for OTA were 2.2 µg/kg, 0.1-20.4 µg/kg, and 79%, respectively. Natural co-occurrence of AFs and OTA was found in 74% of all

samples. Another publication also demonstrated evidence of the co-occurrence of AFs and OTA in spices collected from Northern Italy (Prelle *et al.*, 2014). The analysis of a total of 130 spices was performed for AFs and OTA using LC-MS/MS and HPLC-FLD, respectively. This analysis showed that 15% of the samples were contaminated with AFs and 24% were contaminated with OTA. A review of the natural occurrence of mycotoxins in medicinal plants was published (Ashiq and Hussain, 2014). Numerous natural occurrences of mycotoxins in medicinal plants and traditional herbal medicines have been reported from various countries including Spain, China, Germany, India, Turkey and from the Middle East.

5. Aflatoxins

Similar as in the previous period also in 2014-2015 there are quite a number of publications on demonstrating new (prototype) measurement principles based on recent technologies. However, these contributions seem to have limitations being directly transferable for the analyst focussing on aflatoxin determination in food or feed as such, either for routine test purposes, food law enforcement or exploring new aspects of aflatoxin occurrence. Therefore, this section on AFs focuses on those contributions that appear to be easily transferable and offer a straight benefit to the mycotoxin analyst helping to improve already existing methods or serving as food for thoughts to develop or improve current methods. Furthermore, contributions only involving aspects that have been described in a similar manner before and therefore not demonstrating progress beyond the fact that the authors demonstrated a successful application of existing method principles were omitted as well.

In a special issue of this journal on AFs in maize and other crops, Yao *et al.* (2015a) reviewed more than 60 articles, mainly from the last decade concerning the developments in the detection and determination of AFs. Authors summarise the advantages and disadvantages of different methodological approaches under the aspect of grain food-chain stakeholder needs and compared the progress that has been made with chromatographic, immunochemical and spectroscopic methods in recent years. Another review article of Nardiello *et al.* (2014) focuses more specific on post column derivatisation methods that are used for AFs, FBs, DON and nivalenol (NIV). Authors give well-arranged schematics and reaction mechanisms in their summary in combination with the applications of use in the screened literature. Busman *et al.* (2015) continued with the development of direct analysis in real time (DART) mass spectroscopy and applied this technology. Authors isolated and purified AFM_1 from milk by two different procedures, either by immunoaffinity or a combined SPE and critically discussed the benefit of isotope labelled internal standards. They finally concluded that even levels below 0.5 µg/kg AFM_1 (regulatory limit in many countries incl. the USA) can reliably be monitored with impressive precision using this technology.

Arroyo-Manzanares *et al.* (2015a) proposed a 'dilute-and-shoot' approach for the determination of AFs in animal feed in combination with matrix matched calibration. Aim of the authors was to simplify the method and they compared a simple ACN extraction followed by centrifugation, evaporation and reconstitution prior HPLC-FLD and post column derivatisation. Authors compared the proposed extraction with neat ACN with other extraction approaches by use of spiked samples and validated for precision parameters (repeatability and intermediate precision), LOQ and recovery for a number of feed matrices in the same manner. A more instrumental demanding approach was followed by Campone *et al.* (2015) who determined AFs and OTA by pressurised liquid extraction (PLE) with 30% methanol, followed by SPE and subsequent determination by UHPLC-MS/MS. Authors discussed in detail the optimisation of all relevant parameters by experimental design. Both approaches by Arroyo-Manzanares and Campone cannot be more different in their tactic targeting very similar scenarios, which demonstrates the different perspectives analytical chemistry offers. Similar, Li *et al.* (2014a) used PLE (respectively accelerated solvent extraction, ASE® (Dionex, Sunnyvale, CA, USA) – a synonym for PLE) followed by SPE and UHPLC-MS/MS for the determination of AFs in rice. Two publications describe the use of QuEChERS for determination of AFB_1 and AFM_1 (Karaseva *et al.*2014) and AFB_1, AFB_2, AFG_1, AFG_2, AFM_1 and AFM_2 (Sartori *et al.* 2015). In view that only AFM_1 is known to be relevant in dairy products, the here described scopes in both publications are more of academic nature. However, the clean-up technique used remains an interesting approach. Karaseva *et al.* (2014) coupled the QuEChERS method to a dispersive liquid-liquid (DLL) clean-up and determined the AFs in a variety of dairy products. Like most DLL clean-up methods, they tested and proposed a chlorinated solvent, an aspect critical in some laboratories due to the environmental impact of those solvents. Sartori *et al.* (2015) coupled the QuEChERS method to HPLC-MS/MS and analysed 88 samples of milk powder and UHT milk for the toxins.

Yet another approach was followed by Hashemi and Taherimaslak (2014) who propose magnetic SPE for the determination of AFs from pistachios. Authors obtained satisfactory precision and recovery data, and provide a description on how the self-produced magnetic particles were made. An interesting way to tweak state-of-the-art instrumentation was presented by Breidbach and Ulberth (2015) who showed how two-dimensional heart-cut LC-LC improves accuracy of exact-matching double isotope dilution mass spectrometry measurements of AFB_1 in food. Authors used this approach to further improve

exact matching SIDA mass spectroscopy with the aim to facilitate the best estimate of the true value for the production of RMs.

The reviewed and listed publications should give sufficient food for thoughts for interested readers as authors give rather different arguments as drivers for their approaches, while conclusions also vary in some cases depending on the design of the experiment. Therefore, a critical view into such details is of value for the readers interested in implementing the presented works into their laboratories. An interesting observation is the number of publications that propose the use of rather small test sample intake for analysis, a trend that can be understood taking into account that also financial aspects are a driver for new analytical methods. It has been shown that this is in principle possible but requires a high demand on the sample preparation, in particular milling and mixing to give representative fractions for analysis. This seems however rarely taken into account or demonstrated. Further, readers must consider that analysis of different portions of naturally contaminated samples might result in different performance, especially extraction efficiency and recovery, than validation results achieved by solely use of equally fortified sample fractions. This also holds true when different methods or method steps are directly compared. Certainly, this issue should be more addressed in the future. Reduced test portion intake for aflatoxin determination, but also other heterogeneously occurring mycotoxins in powdered products, is a tendency that has benefits, but also bears some risks in terms of representative sample fractions taken for analysis.

6. *Alternaria* toxins

Almost all papers published in the last year and dealing with the determination of *Alternaria* toxins report the use of LC-MS/MS methods. An inter-laboratory study is ongoing to validate a modified version of the LC-MS/MS method published by Töelgyesi *et al.* (2015). This modified method has been submitted to the European Committee for Standardization (CEN) as proposal for standardisation. Upon demonstrating satisfactory performance criteria, set in section 4.3.1 (h) of the Commission Regulation No 401/2006, the method is foreseen to serve as a good basis for an European Standard for the determination of *Alternaria* toxins in food (sunflower, wheat and tomato products). The toxins considered are altenuene (ALT), alternariol (AOH), tentoxin (TEN), tenuazonic acid (TeA) and alternariol monomethyl ether (AME) and the matrices are wheat, tomato-based foodstuff, and sunflower seeds. The analytical method involves extraction of test sample with methanol:water:acetic acid (80:19:1, v/v/v) mixture. The sample is centrifuged and an aliquot of the upper layer is collected, diluted with an equal volume of 1% aqueous acetic acid solution, and purified on a polymeric based SPE cartridge. The toxins are eluted with methanol from the cartridge and the collected solution is evaporated. The reconstituted extract is filtered through a polytetrafluoroethylene syringe filter and analysed by LC-MS/MS using matrix-matched standards.

To study the toxicokinetic of AOH on mice, an LC-MS/MS and a GC-MS based method were developed for blood and urine, respectively (Schuchardt *et al.,* 2014). Matrix matched standards as well as 7-hydroxy-4-phenylcoumarin or 6,7-dihydroxycoumarin as internal standard were used for quantification of AOH in whole blood and urine. The reason why two different approaches, LC-MS/MS for blood and GC/MS for urine, were used for AOH determination was not reported. Moreover, the analytical protocols did not include enzymatic digestion with β-glucuronidase/sulphatase enzymes, therefore AOH deriving from glucuronide- and sulphate-conjugates could not be measured.

A SIDA-LC-MS/MS method was developed and used for the determination of altertoxin I, altertoxin II, alterperylenol, AOH, AME and TEN at low levels (LODs: 0.09-0.53 µg/kg) in commercial food samples (Liu and Rychlik, 2015). ^{13}C labelled standards of all toxins except TEN were biosynthesised and used as internal standard for calibration. The method was suitable to measure the natural occurrence of these toxins in different food matrices. If the food was contaminated with altertoxins, it was likely to be co-contaminated with the other *Alternaria* toxins, but not necessarily *vice versa*. The type of calibration used for quantitation of TEN was not reported and no explanations were given on why ALT and TeA, two *Alternaria* toxins commonly occurring in food samples, were not included in the panel of analysed toxins.

The interest for modified/masked *Alternaria* mycotoxins is increasing and some papers were recently published which describe their synthesis, development of analytical methods and natural occurrence. Mikula *et al.* (2013) reported the total synthesis of glucosides and sulphates of AOH (AOH-3-O-β-D-glucoside, AOH-9-O-β-D-glucoside, AOH-3-sulphate) and AME (AME-3-O-β-D-glucoside, AME-3-sulphate). These compounds decomposed when dissolved in methanol and stored for several days even at -20 °C, whereas they were stable in dimethylsulfoxide for several weeks. Their stability in ACN was not tested. Hildebrand *et al.* (2015) demonstrated that AOH and AME are efficiently conjugated with glucose in cultured tobacco BY-2 cells. Five conjugates of AOH were isolated and identified by MS and NMR spectroscopy as β-D-glucosides (attached in AOH 3- or 9-position). For AME, conjugation resulted in the β-D-glucoside (mostly attached in the AME 3-position). These compounds can be used as reference standards to develop new analytical methods, however the two studies did not report information on their purity. An LC-MS/MS method for the simultaneous determination

of free (AOH, AME, ALT, TeA, TEN, altertoxin I) and conjugated (3-glucosides and 3-sulphates of AOH and AME) *Alternaria* toxins in rice, oats and barley was developed and used to monitor their natural occurrence in rice and oat flake samples. All samples were negative for conjugated AOH/AME whereas low levels of TeA were found in 71% of rice samples, 31% of oat flakes, and TEN in 35% of rice samples (Walravens *et al.*, 2015).

7. Ergot alkaloids

The major developments in the determination of ergot alkaloids have been the application of extraction and clean-up procedures, particularly the use of the QuEChERS method. Further progress has been made with the use of aptamer based extraction, which has revealed the high specificity of this method, but which might fail to capture some ergot alkaloids. Further developments might be expected in the application of ion trap detection to LC-MS methodology and some preliminary work has been presented that incorporates an improvement of an established clean-up procedure.

Bryla *et al.* (2015) developed a LC/MS method for ergot alkaloids in cereals using ion trap detection. The procedures for extraction and purification were based on a previous method (Müller *et al.*, 2006), which involved extraction with a mixture of ACN with ammonium carbonate solution and clean-up by defatting and activated alumina SPE. The authors increased the polarity of the ACN/ammonium carbonate solution elution solvent, which improved the separation of the alkaloids from the matrix components. After further preparation chromatographic separation was carried out using a reversed phase column. The most effective mobile phase comprised a gradient mixture of aqueous ammonium carbonate buffer at pH 9 and ACN. This enabled separation of the six major ergot alkaloids and their epimers within 35 min although the LC run time was close to double this. The mass spectrometer was operated in positive ion electrospray (ESI) mode as negative ionisation did not give satisfactory results. Product ions were produced by loss of water and protonation for all alkaloids except ergometrine, which lost the $CONHCH(CH_3)CH_2OH$ chain and ergometrinine, which lost $CH_3CH_2CH_2OH$. The method was tested on 65 rye and rye-based cereal samples. The LOQ ranged from 1 to 3 µg/kg, with precision below 18%. Mean recoveries from spiked samples varied as is usual with the spiking level and alkaloid, the lowest being for ergometrine at 25 µg/kg (63%) and the highest (106%) being ergosine at 25 µg/kg and ergocorninine at 12.6 µg/kg. The method was shown to be accurate by comparing the measured and certified values for a certified material of ergot alkaloids in rye flour (BRM 003020).

Walker *et al.* (2015) used a QuEChERS based method with LC-FLD to determine ergovaline, the principal ergot alkaloid in tall fescue grass. The extraction procedure was simpler, more efficient and more environmentally friendly than the usual methods that can involve chlorinated solvent extraction and relatively complex clean-up stages. Fourteen extraction solvent mixtures shown elsewhere to be applicable to mycotoxins were evaluated. They included mixtures of methanol with ACN, with water, or with ethyl acetate and ammonium carbonate or hydroxide; mixtures of ACN with formic or acetic acid or ammonium carbonate; and one system comprising acidified aqueous isopropanol. Test portions were vortexed and shaken with the solvent. QuEChERS salts ($MgSO_4$ and NaCl) were added, vortexed further and centrifuged. An aliquot of the supernatant was dried, redissolved in methanol and filtered for LC-FLD. HPLC analysis was carried out on a C18 phase and a gradient from 30% ACN in aqueous ammonium carbonate to 100% ACN. Ergovaline and ergovalinine were eluted and separated within a total run time of 4.2 min. The FLD was operated with excitation at 250 nm and emission at 420 nm. Method validation used fortified non-infected seed and straw samples and in-house reference seed and straw materials. The highest and most consistent recoveries were obtained with an acidic mixture – water:0.5% acetic acid (1:1, v/v) in ACN, which recovered 98% of ergovaline from seed. However, for the method validation applied to both seed and grass samples the best extraction solvent was a basic mixture of aqueous ammonium carbonate and ACN (1:1). Efficient performance of the QuEChERS stage required immediate vortexing after addition of the salts, and four mixing regimes over 10 min. The LOQ was 100 µg/kg for both seed and straw. Precision and accuracy calculated from the analysis of fescue seed and straw RMs both containing around 200 to 1000 µg/kg ergovaline were good. The performance of the validated method was equivalent to that of an Ergosil SPE based method with LC-FLD detection, however, an important difference was shown in that the signal for ergovalinine was significantly higher with the QuEChERS method in both seed and straw samples. It will be important to determine whether this effect is due to higher extraction efficiency, to limitations in the ability of the Ergosil SPE material to bind ergovalinine, or to epimerisation during the QuEChERS extraction.

DNA aptamer ligands selective for ergot alkaloids were prepared by Rouah-Martin *et al.* (2014), and grafted onto silica gel to build a specific SPE system. The resulting aptamer-functionalised silica gels were used to extract ergot alkaloids from a contaminated rye feed sample. Two aptamers, specific for the ergoline group, were developed by using a smaller fragment of bases containing the predicted binding sites than were used in a previous method (Rouah-Martin *et al.*, 2012). The performance of the aptamer systems was measured using a sample of rye feed contaminated with ergot alkaloids, mainly ergotamine, ergosine, ergocornine and ergocryptine, to a total level of about 1000 µg/kg. A 0.5 g test portion of contaminated feed

was stirred with 0.1 M HCl, centrifuged, and an aliquot of the supernatant reacted with the aptamer-functionalised silica gel. After cleanup, the ergot alkaloids were then eluted with 0.1 M HCl and analysed by LC-QTOF-MS. The QTOF-MS was run in ESI positive mode with extraction of signals for the six major ergot alkaloids and the ergot alkaloid precursor fragment at *m/z* 223.1283. QTOF-MS analysis also allowed matrix components to be detected. In the extract analysed using the aptamer M3.2 functionalised silica gel ergosine and ergocryptine were detected along with the precursor fragment. The extract was free from other compounds. In the extract analysed using the aptamer L5.5 functionalised silica gel ergocornine was found in addition to ergosine and ergocryptine. The ergot alkaloid precursor fragment was present for each compound and no other compounds were seen. The aptamer-based SPE system could specifically extract ergosine, ergocryptine and ergocornine from the ergot contaminated rye feed sample in a relatively simple and rapid one-step procedure. However, other ergot alkaloids known to be present in the sample, including ergotamine, ergometrine and ergocristine were not retained by the aptamer-functionalised gels, or their presence was at a concentration below the LOD of the system. This does suggest that the aptamer-SPE approach does not have a significant concentration effect, and confirms that these particular aptamers cannot be used alone for screening purposes. However several advantages over antibody based approaches are apparent, including higher specificity, robustness and avoidance of the use of animals. Some modification of the extraction process might be required if injection of 0.1 M HCl solution with or without neutralisation might be incompatible with long term use of some LC stationary phases.

8. Fumonisins

Fumonisins are routinely detected in agricultural commodities. However, for purposes of determining exposure, methods have been developed to detect them in human hair, faeces, and bottled water. FB_1, but not FB_2 or FB_3 were regularly found in samples of environmental waters collected in Poland (Waśkiewicz *et al.*, 2015). The analytical method was a version of the commonly used derivatisation with o-phthaldialdehyde (OPA), with HPLC-FLD. Results were confirmed by LC-MS/MS. FB_1 was found in all types of water samples with the highest concentrations in the post-harvest season (48 ng/l). While FBs are often detected together as a group or 'family', increasingly their detection is being integrated into multi-toxin assays. Particularly those involving LC with MS detection, as described elsewhere in this review. Recent novel work in fumonisin detection includes new technologies, existing technologies that probe into issues of 'hidden' or 'masked' FBs, and methods that have undergone multi-laboratory validation. An example of the former is the application of corona charged aerosol detection (Corona-CAD). FBs were isolated from extracts of maize using strong anion exchange SPE then injected onto an HPLC with a Corona-CAD detector (Szekeres *et al.*, 2014). The LOD was 20 µg/kg and the LOQ was 40 µg/kg. The RSDs for intraday measurements of peak area were 3.7, 4.9 and 4.8% for FB_1, FB_2, and FB_3, respectively. Mean recoveries of FB_1 from spiked maize ranged from 82-95% over the concentration range from 0.35 to 1.2 mg/kg, suggesting this method, which does not require derivatisation, may be useful for monitoring FBs in maize. Instrumental methods, such as LC-MS, are also valuable for characterising the extent to which commodities contain matrix associated FBs (i.e. 'hidden' FBs) and structurally modified FBs. In this regard, the extent of contamination of Brazilian maize with 'hidden' FB_1 and FB_2 was determined by using LC-MS/MS to measure the total amount of FB_1 and FB_2 (found after hydrolysis to HFB_1 and HFB_2) and subtracting away the extractable or 'free' FBs (Oliveira *et al.*, 2015). The levels of hidden FBs were found to be 0.5 to 2.0 fold higher than the levels of free FBs, suggesting the techniques that measure solely the free forms may be underestimating potential exposures to FBs.

In past years, there have been significant numbers of papers reporting MS-based methods for fumonisin detection, and such methods continue to be widely used. However, this year much of the innovation in analysis of fumonsins pertained to novel materials for use in screening methods (antibodies and aptamers), and novel detection technologies. The desire to improve the performance characteristics of antibodies has previously led to the development of single-chain fragment variable (scFv) antibodies against FB_1. A phage-display library was used to isolate an scFv with greater affinity than the 'parent' monoclonal antibody (mAb) from which it was derived (Hu *et al.*, 2015). In competitive ELISAs the IC_{50}s with FB_1, FB_2, and FB_3 were 0.11, 0.04, and 0.10 µM (equal to 79, 28 and 71 ng/ml) respectively, indicating a preferential binding to FB_2. Recoveries from maize spiked during extraction were good, with averages ranging from 82-104%, depending upon the toxin and the spiking level (100 to 500 ng/ml extract). For 16 naturally contaminated samples (7 maize, 9 rice) the correlation with an LC-MS/MS method was also good, with an r^2 of 0.89.

Two additional materials useful in immunoassays are anti-idiotype antibodies and mimotopes. Anti-idiotype antibodies bind to primary antibodies (e.g. fumonisin antibodies) within or near the toxin binding site (paratope). Mimotopes do likewise, but are not necessarily antibodies themselves. Both can mimic some of the attributes of the FBs and be useful as substitutes for the toxins or toxin-protein conjugates in immunoassays. A type of anti-idiotype antibody derived from alpacas was isolated from a phage-display library and tested in surface plasmon resonance (SPR) immunoassays and in ELISA (Shu *et al.*, 2015). With the 'nanobody', the ELISA assay demonstrated remarkable

sensitivity, with an IC_{50} of 0.95 ng FB_1/ml, and a linear range of 0.3-5.9 ng/ml. This was an improvement over an assay using a FB_1-bovine serum albumine (BSA) conjugate. The good sensitivity, and recovery rates of 72-112% from spiked commodities, suggests the reagent may make a good replacement for FB_1-protein conjugates in ELISA kits. A fumonisin mimotope derived from a 12-mer peptide library was also reported (Xu *et al.*, 2014). The mimotope was expressed as a fusion protein and used as the immobilised antigen in an immunoassay. In ELISAs, the IC_{50}s for FB_1 using two such proteins were 1.3 and 2.2 ng/ml, which were better than an assay with immobilised FB_1-BSA (21 ng/ml). Cross-reactivity (CR) with FB_2 was 9.2%. Recoveries from maize spiked over the range of 10 to 1000 µg FB_1/kg were from 83-115%. The assay was also applied to 60 samples of maize, feedstuffs, and rice, with generally good agreement for maize between the ELISA and a commercial ELISA kit. These two reports suggest that fumonisin mimotopes and anti-idiotype antibodies have excellent potential to replace toxin-protein conjugates in immunoassay test kits.

While the first aptamers recognising FBs were described already in 2010, in the past year there was notable activity in the application of aptamers in novel sensor platforms (Chen *et al.*, 2015a,b; Shi *et al.*, 2015, Zhao *et al.*, 2014). A microcantilever sensor (Chen *et al.*, 2015b) was developed that measured the extent to which cantilevers were bent when immobilised aptamer bound FB_1. Aptamer-free cantilevers provided a reference signal. The sensor has the potential to be incorporated into 'lab-on-a-chip' devices. The LOD was 33 ng FB_1/ml, with a range of 0.1-40 µg/ml in buffer. The same aptamer was used in a multi-toxin assay based upon silica photonic crystal microspheres (SPCM) (Yue *et al.*, 2014). DNA complementary to a portion of the aptamer was labelled with a fluorophore. The aptamer was immobilised onto SPCM and the fluorophore labelled complementary DNA was hybridised to it. FB_1 displaced the labelled DNA, decreasing the fluorescence attached to the SPCM. The LOD for FB_1 was quite low (0.16 pg/ml), with a linear detection range of 0.001 to 1 ng/ml in buffer. Recoveries from spiked cereal samples ranged from 77 to 115%. This format is amenable to multiplexing, as was demonstrated by a similar assay for OTA (Yue *et al.*, 2014). Both articles described assay sensitivities (LODs) in buffer solutions (i.e. µg/ml) rather than those corresponding to maize (i.e. µg/kg maize).

Several of the reports incorporated aptamers into electrochemically-based sensors. Zhao *et al.* (2014) produced gold nanoparticles that incorporated an iridium complex and an aptamer. DNA partially complementary to the aptamer (PC-DNA) was immobilised on the sensor surface (the working electrode of a 3 electrode electrochemiluminescence (ECL) analyser). In the absence of FB_1, the gold/Ir/aptamer complex was hybridised with the PC-DNA. However, in the presence of FB_1 the hybridisation was prevented due to the interaction of the aptamer with FB_1. The LOD was 0.27 ng/ml, with a working range of 0.5 to 50 ng/ml. Recoveries from spiked wheat (2 g flour extracted with 2 ml of methanol/water) ranged from 98-108%. Aptamers attached to gold nanoparticles were also used in an electrochemical impedance spectroscopy (EIS) sensor (Chen *et al.*, 2015a). Gold was deposited onto a glassy carbon electrode (GCE), and aptamers were then immobilised to it. Binding of FB_1 to the aptamer was detected as an increase in resistance over the concentration range of 0.1 nM (0.07 ng/ml) to 100 µM (72 µg/ml). The spiking and recovery studies were very limited, however the reported recoveries ranged from 91 to 105%. While promising, the 30 min incubation time may limit application of the aptasensor. In a third electrochemical sensor publication, gold nanoparticles were also combined with graphene/thionine nanocomposites (GS-TH) for fumonisin detection (Shi *et al.*, 2015). This report has certain aspects in common with the two previous reports, but has a different format. The sensor surface was a GCE with immobilised aptamer having a sequence identical to that of Zhao *et al.* (2014), without the sulfhydryl linker at the 5' end. The GS-TH served as an electrochemical probe that bound to the aptamer yet could be displaced by FB_1, with a resulting decrease in current as measured by cyclic voltammetry. The LOD was 1 pg FB_1/ml, with an upper end of the linear range of 1 µg/ml. Spiking and recovery from wheat were also reported, although there are insufficient details to determine whether it was wheat or wheat extract which was spiked, or even what the wheat was extracted with. However, with the indicated sensitivities and reported recoveries, the technique warrants further investigation.

As with aptamers, antibodies can be incorporated into electrochemical sensors. Two such sensors were recently reported (Ezquerra *et al.*, 2015; Jodra *et al.*, 2015b). Each was similar to ELISA, with the difference that the fumonisin antibodies were attached to magnetic beads (MBs). Competition was between free FB_1 and FB_1-horseradish peroxidase (HRP) for binding to the antibody. In each case the MBs were transferred to screen printed carbon electrodes (SPCE), where the product of the enzymatic reaction was measured by amperometry. In one report, the device used 8 channels, so that multiple samples could be measured simultaneously (Ezquerra *et al.*, 2015). The LOD was 0.58 ng FB_1/ml and the IC_{50} was 4.3 ng/ml. At the 15 ng/ml level the within day repeatability was 8.2% and between-day reproducibility was 13%. The LOQ in maize, based on a solution LOQ of 0.60 ng/ml, was estimated to be 95 µg/kg. In the other report (Jodra *et al.*, 2015b) the LOD was 0.33 ng FB_1/ml and the IC_{50} was 2.9 ng/ml. The dynamic range was reported to be 0.73 to 11 ng/ml. Within-day repeatability and between-day reproducibility (at 5 ng/ml) were reported to be 2.9% and 6.0% respectively. Good agreement was observed with a maize CRM. Both

sensors show promise, however, both took longer to perform than most commercial test kits.

FPIA is an immunoassay format with several potential advantages that result from having a homogeneous, as opposed to heterogeneous, format. While FPIA was first reported for FBs in 2001, the technology has not become widespread. In an effort to improve FPIA for FBs a recent paper studied combinations of seven antibodies and three tracers in a microplate format (Li *et al.*, 2015). With a conjugate of FB_1 with fluorescein isothiocyanate as the tracer, two antibodies in particular were promising. One of these (4F5) had better sensitivity for FB_1 while the other (4B9) had poorer sensitivity for FB_1, but was more cross-reactive to FB_2. Using matrix matched calibration the LOD in maize for the 4F5-based assay was 54 µg/kg FB_1, with a dynamic range of 108 to 1,201 µg/kg. This was slightly better sensitivity than previous fumonisin FPIA and, in combination with the use of microtiter plates, should improve the utility of the method.

For all assays the eventual goal is to achieve multi-laboratory validation. A multi-toxin immunochromatographic ('dipstick') test that included FBs was the subject of a collaborative study in 12 laboratories (Lattanzio *et al.*, 2014). The assay evaluated was a qualitative test for the presence or absence of toxins at a target level. For FBs the target mixture contained 3,000 µg FB_1 and 1000 µg FB_2 per kg maize. The intensity of the test line ('T' inversely proportional to toxin content) was compared to the intensity a control test line ('C'). Cut-off values for the test were calculated from estimates of precision and assumed an acceptable false negative rate of 5%. The calculated cut-off value for FBs was a ratio (T/C) of 0.6. A ratio below this value classified a sample as 'positive'. With the test samples, the T/C ratio averaged 3.0 for blank maize and 0.4 for maize with FB_1 + FB_2 at the target level. False positive rates were very low for FBs (<0.01%) and, in combination with the other results, suggests the multi-toxin test strip was acceptable for detecting FB_1 + FB_2 at the target level. An inter-laboratory study was also conducted of an LC-MS method for FB_1, FB_2, and FB_3 in pet foods (Nomura *et al.*, 2015). FBs were extracted with aqueous ACN and cleaned-up with a multifunctional column. The LODs were 0.1 mg/kg for wet pet food and 0.2 mg/kg for dry and semi-dry foods. Recoveries over the range of 0.1 to 1.0 mg/kg averaged 93-107% for FB_1 (RSD 7.9%), 87-102% for FB_2 (8.6%) and 91-102% for FB_3 (RSD 8.6%). From the inter-laboratory study (11 laboratories) the Horwitz ratios were 0.41-0.54 (FB_1), 0.33-0.55 (FB_2), and 0.44-0.57 (FB_3). From this it is evident that the method is suitable for detecting FB_1, FB_2, and FB_3 in various types of pet foods.

9. Ochratoxins

Typically, spices, raisins, figs, coffee, cocoa, wine as well as wheat and barley based products are tested for OTA presence. While for official control activities solely HPLC-FLD, LC-MS/MS or conventional screening methods (e.g. ELISA) are used, sophisticated methodologies – such as aptasensors or electrochemical platforms built up on nano-platforms – are currently developed. 77% of the labs participating in FAPAS proficiency tests (PTs, 2015) used HPLC-FLD, 18% used LC-MS/MS and only 5% used ELISA quantitative tests. As far as OTA in beer, insufficient interest caused the cancellation of the PT. The extraction methods based on aqueous mixtures of methanol or ACN continued to be the most widely used. Performed clean-up procedures consisted mainly of immunoaffinity columns.

A SIDA-LC-MS/MS method was used by Ahn *et al.* (2016), for OTA detection in doenjang, a Korean traditional fermented soybean paste, using $^{13}C_{20}$-OTA as internal standard. After addition of internal standard, samples were extracted with aqueous methanol and centrifuged. The supernatant was evaporated, taken up in buffer and purified with immunoaffinity columns. Reversed phase LC separation was performed at pH 3.0 for optimal retention of OTA. Repeatability and reproducibility were in the range of 2%. The LOQs of OTA in coffee and doenjang were about 0.10 µg/kg. It should be mentioned that the method was designed a higher-order reference method to be used for SI-traceable value-assignment of OTA in CRMs. The use of both isotope labelled internal standards and selective clean-up would render the method too expensive for routine analysis.

Regarding OTA clean-up, five peptide-based affinity media differently functionalised with a hexapeptide sequence binding OTA, were investigated by Giovannoli *et al.* (2015), for their thermodynamic and kinetic binding properties. The highest values of the equilibrium binding constant and binding site concentration were obtained for Lewatit® CNP105 (Lanxess AG, Cologne, Germany), followed by Toyopearl® CM-650 M (Tosoh Bioscience LCC, King of Prussia, PA, USA) and micrometric glass beads. These results confirm that the chemical nature of the surface has a key role in the binding properties of solid supports. Different SPE surfaces were compared to recover OTA from spiked wine samples. The extracts were analysed by HPLC-FLD, with a LOQ of 1.5 µg/l. Recoveries of 71-108% for Amberlite® IRC-50 (Rohm and Haas, Philadelphia, PA, USA) and 91-101% for Lewatit® CNP105 were obtained. While both supports showed good recoveries, a statistically significant difference was found in terms of precision.

Going through new noteworthy methodologies, Wei *et al.* (2015) presented a novel, cost-effective approach using a nano-graphite-aptamer hybrid and DNase I as

a fluorescent sensing platform for OTA detection. The presence of the nano-graphite can quench fluorescence of a carboxyfluorescein labelled OTA specific aptamer. In the presence of OTA, that induces conformational change of the aptamer, it will trigger OTA-aptamer binding. The resulting OTA-antiparallel aptamer G-quadruplex does not adsorb on the surface of nano-graphite and thus enhances fluorescence. Meanwhile, the G-quadruplex structure can be cleaved by DNaseI, and in such case OTA is delivered from the complex. With the high affinity of the aptamer against OTA and the signal enhancement strategy, a LOD of 20 nM was achieved, representing about 100-fold improvement when compared to traditional unamplified strategies. The aptasensor might be extended to the detection of other toxins by replacing the sequence of the recognition aptamer. Analogously, Lu *et al.* (2015) synthesised six luminescent iridium complexes for testing their capacity to function as probes for G-quadruplex DNA. A novel Ir(III) complex was discovered to be selective for G-quadruplex structures and was subsequently used for the construction of a label-free G-quadruplex-based OTA sensing platform in aqueous solution. The assay exhibited linearity for OTA in the range of 0 to 60 nM, and the LOD for OTA was 5 nM. Furthermore, this assay was highly selective for OTA over its structurally related analogues. Sanzani *et al.* (2015) developed a quantitative assay to detect OTA in wine. An available DNA aptamer was used as recognition probe with a fluorescence-quenching pair at the stem ends. The aptabeacon could adopt a conformation allowing OTA binding, causing a fluorescence rise due to the increased distance between fluorophore and quencher. Real-time polymerase chain reaction (PCR) was used for signal detection. The proposed system exhibited a good selectivity for OTA. A linear detection range of 0.2-2,000 µM was achieved. The aptabeacon was also applied to detect OTA in red wine. A LOD of 19 nM (7.7 µg/l) and recoveries from 63-105% were observed.

Rivas *et al.* (2015) carried out a novel aptasensor for OTA detection based on a SPCE. The electrotransducer surface was modified with an electropolymerised film of polythionine followed by the assembly of iridium oxide nanoparticles. The aminated aptamer selective to OTA is exchanged with the citrate ions surrounding nanoparticles via electrostatic interactions with the same surface. EIS in the presence of a redox probe is employed to characterise each step in the aptasensor assay and also for label-free detection of OTA in a range between 0.01 and 100 nM. LODs of 5.65 ng/l (14 pM) were reported. The system also exhibited a high reproducibility if applied to the analysis of a white wine sample, and a good specificity against other toxins. A novel multi-functional aptasensor was introduced by Wang *et al.* (2015a) for highly sensitive detection of OTA. This device used aptamer-conjugated MBs as the recognition and concentration element and heavy CdTe quantum dots (QDs) as the label. Initially, the thiolated aptamer was conjugated on the Fe_3O_4/Au MBs through covalent binding. Subsequently, multiple CdTe QDs were loaded both in and on a versatile SiO_2 nanocarrier to produce a large amplification factor of hybrid fluorescent nanoparticles (HFNPs) labelled complementary DNA (cDNA). The magnetic-fluorescent-targeting multifunctional aptasensor was thus built by immobilizing the HFNPs onto MBs' surface through the hybrid reaction between the aptamer and cDNA. The presence of OTA would trigger aptamer-OTA binding, resulting in the partial release of the HFNPs into bulk solution. After magnetic separation, the supernatant liquid containing a great number of CdTe QDs produced an intense fluorescence emission. The fluorescence intensity of the released HFNPs was proportional to the concentration of OTA in a wide range of 15 pg/ml – 100 ng/ml with a LOD of 5.4 pg/ml. While peanuts, spiked with OTA, were tested in this study as well, the description in the article allows no relation to the actual spiking levels in matrix. MBs were also employed by Jodra *et al.* (2015a) presenting a reliable disposable electrochemical magnetoimmunosensor for monitoring of coffee samples for OTA contamination. The electrochemical detection performed on carbon screen-printed electrodes used MBs as antibody immobilisation support and enzyme horseradish peroxidase as tracer. A LOD of 0.32 µg/l was achieved for OTA in coffee.

With the aim to overcome the possible loss of the biological activity and/or the appropriate orientation during the localisation and other regenerating steps of some aptamers, a novel reusable aptamer-based biosensing strategy based on an evanescent wave all-fiber (EWA) platform was proposed by Wang *et al.* (2015c). In a target-capturing step using aptamer-functionalised MBs, signal probes conjugated with streptavidin are released and further detected by a EWA biosensor via a facial dethiobiotin – streptavidin recognition. Apart from the inherent advantages of aptamer-based evanescent wave bio-sensors (e.g. target versatility, sensitivity, selectivity and portability), the proposed strategy exhibits a high stability and remarkable reusability over other aptasensors. Under the optimised conditions, the calibration curve obtained for OTA had a linear response ranging from 6-500 nM (2.4-200 µg/l). The dethiobiotin–streptavidin sensing surface can be reused for over 300 times without losing sensitivity.

In a study published by Yang *et al.* (2015), a ECL biosensor combining the characteristics of high efficiency of hyperbranched rolling circle amplification (HRCA) and high selectivity of aptamer was developed for OTA determination. The cDNA was firstly immobilised on the gold electrode surface, then the OTA aptamer was modified on the electrode surface through hybridisation with cDNA. Since OTA can competitively bind with the aptamer due to their high affinity, the releasing of aptamer from the electrode surface could be induced. Subsequently,

the free cDNA on the electrode surface can hybridise with the padlock probe and induce HRCA reactions. Thus, the HRCA products which contain large amounts of double-stranded DNA fragments can be accumulated on the electrode surface. Due to its capacity of intercalating into the groove of DNA, dichlorotris(1,10-phenanthroline) ruthenium(II)hydrate was used as ECL indicator. The enhanced ECL intensity that can be detected from the electrode surface, has a linear relationship with OTA in the range of 0.05-500 pg/ml with an LOD of 0.02 pg/ml. The developed biosensor has been applied to determine OTA in maize samples, but again dilution/concentration factors were not provided. Zhang *et al.* (2015b) used the same approach as the previous one, but utilising SYBR Green I to dye and produce a fluorescence signal. The fluorescence intensity of the system had a linear relationship with the logarithm of the OTA concentration in the range of 4 fg/ml to 400 pg/ml with a LOD of 1.2 fg/ml. The fluorescent aptasensor was applied to detect OTA in maize and oat samples with satisfying results. While extraction conditions were not provided, the lowest detected concentration of OTA in maize was 23 ng/kg. Yao *et al.* (2015b) carried out a competitive sensing protocol for ultrasensitive detection of OTA in about 80 min, by combining the magnetic nanoparticles and *in vitro* amplification of rolling circular amplification (RCA). The magnetic separation enabled avoidance of background fluorescent noise and the QD labelled probes lead to a good response. Concentration of RCA components and RCA reaction time were optimised for the best performance. The final assay was linear in a range from 10^{-3} to 10 µg/kg, and a LOD of 0.13 ng/kg was achieved. Again, the universal signal amplification protocol might be used for other toxins.

A SPR biosensor using an anti-OTA aptamer immobilised sensor chip was developed by Zhu *et al.* (2015). Streptavidin was immobilised onto the surface of a sensor chip and the biotin-aptamer was captured. The biosensor exhibited a linear range from 0.1-10 ng/ml of OTA with a LOD of 0.005 ng/ml. Detection of OTA in wine and peanut oil was further performed after liquid–liquid extraction. The lowest evaluated spiked levels in both matrices which gave a repeatable response were 3 ng/ml. Recoveries of OTA from spiked samples ranged from 87-117% with a repeatability <7%. Another biosensor was developed by Dridi *et al.* (2015) for the direct conductometric detection of OTA in olive oil samples. The biosensor is based on thermolysin immobilisation into a polyvinyl alcohol (PVA)/polyethylenimine (PEI) matrix containing gold nanoparticles (AuNPs) and cross-linked at the surface of gold interdigitated microelectrodes. Under optimal conditions, the biosensor response was linear up to 60 nM OTA, with a LOD of 1 nM in solution. The PVA/PEI hydrogel creates a favourable aqueous environment for the enzyme. Interactions between protonated amino groups of PEI and negative charges of both citrated AuNPs and thermolysin improved enzyme stability and accessibility of the substrate. The proposed OTA biosensor showed repeatabilities below 15% and was stable over 30 days at 4 °C between measurements. OTA spiked in olive oil samples was recovered at 96-101%. For olive oil the LOD was 0.25 µM (100 µg/l). A chemiluminescence immunoassay using magnetic nanoparticles with amine-targeted inhibition using aldehyde, i.e. specifically capping the amine with an alkyl group, targeted inhibition was developed by Kim and Lim (2015) for the determination of OTA. The toxin was determined in rice using a lab-built drop-type chemiluminescence system with luminol/H_2O_2 reagent. The obtained LOD was 1.4 pg/ml, which was about seven times better than that of a comparative ELISA test (MyBioSource, San Diego, USA). Recovery was verified to be 87-99% and lowest evaluated levels in rice were 0.5 ng/g for both the developed assay and the (less sensitive) ELISA test.

In a paper by Norouzi *et al.* (2015), a new electrochemical technique based on combination of coulometric admittance voltammetry and a nanocomposite electrode was developed for the determination of OTA in a flow-injection system. The sensor was designed by deposition of gold nanoparticles on reduced graphene nanosheets mixed with a carbon multiwall nanotube and an ionic liquid casted on a glassy carbon electrode. The sensor response was calculated in form of charge changes under the peak by integrating admittance in selected potential range, after subtracting the background admittance. It was determined that the electrode response was proportional to concentrations of OTA in a range from 0.1-200 nM, with a detection limit of 3.7 pM. The electrode showed good reproducibility and a usage stability for up to 90 days. Pacheco *et al.* (2015) developed a novel electrochemical sensor for OTA detection, through the modification of a glassy carbon electrode with multi-walled carbon nanotubes and a molecularly imprinted polymer (MIP). The nanotubes promoted the sensitivity of the developed sensor and the MIP served as selective recognition element. The electrochemical oxidation of OTA showed a linear relationship between peak current intensity (measured by differential pulse voltammetry) and OTA concentration from 0.05-1.0 µM. A LOQ of 0.014 µM (5.7 µg/l) was achieved with a repeatability <5% and <10% in matrices. The sensor was applied to determine OTA in spiked beer and wine samples, with recoveries between 84 and 104%, without any sample pre-treatment.

10. Patulin

Last year, only few methods for determining PAT were published, of which, three methods used UHPLC. Maragos *et al.* (2015) developed a method using UHPLC-UV for detecting PAT in fruit leathers. Investigations were made to determine the suitability of direct analysis in real time-mass spectrometry (DART-MS) for detecting PAT at the

surface of fruit leathers. Results indicated that DART-MS was insufficiently sensitive for quantifying PAT at the surface of home-style apple leathers, although PAT spiked onto the surface of leather or peel could be detected. The UHPLC-PDA method was used to determine the fate of PAT during the preparation of homemade fruit leathers and was used to screen for PAT in commercial fruit leathers. Each variety was spiked to obtain concentrations of 5-100 µg/kg. Recoveries ranged over a mean of between 77-111%, depending upon the variety and the spiking level. The LOD was 3.5 and the LOQ was 12 µg/kg. The method of Marsol-Vall *et al.* (2014) also used UHPLC-UV, wherein sample preparation was based on QuEChERS. Clean-up was performed using dispersive SPE mixed with magnesium sulphate and primary secondary amines (PSA). The method was validated for cloudy apple juice, apple puree, apple yoghurt, beer with apple juice and cider. The LOQ was ≥2 µg/kg. Recoveries for all the matrices were between 78-95%, while relative standard deviations were between 3.8 and 10%. Another method to analyse PAT using UHPLC with QuEChERS sample preparation was described by Vaclavikova *et al.* (2015), using MS/MS detection. The LOQ ranged from 1 to 2.5 µg/kg, depending on the fruit type. The quantification of PAT was based on SIDA using $^{13}C_7$-PAT as internal standard. The method was validated for apples, apple baby food, apple juice, peaches, strawberries and blueberries. Recovery values ranged from 92 to 109%. Repeatability of the method was below 10% for all tested matrices.

Seo *et al.* (2015) developed a method based on SIDA-LC-MS/MS as a higher-order reference method for the accurate value-assignment of CRMs. $^{13}C_7$-PAT was used as the internal standard. Samples were extracted with ethyl acetate and the sample clean-up was performed with HLB-SPE (Oasis®). Liquid chromatography was performed on a multimode column for proper retention and separation of highly polar and water-soluble PAT from sample interferences. Sample extracts were analysed by LC-MS/MS with ESI in negative ion mode with SRM of PAT and $^{13}C_7$-PAT at *m/z* 153>109 and *m/z* 160>115, respectively. The validity of the method was tested in various apple products. The method was shown to provide accurate measurements in the 3-40 µg/kg range with a relative expanded uncertainty of around 1%.

Pennacchio *et al.* (2015) presented a novel FPIA approach based on the use of emergent near-infrared fluorescence probes. The use of fluorophores coupled to anti-PAT antibodies enables the detection of PAT directly in apple juice without any sample pre-treatment. The method is based on the increase in fluorescence polarisation emission of a fluorescence-labelled PAT derivative upon binding to a specific antibody. Competition between PAT and the fluorescence-labelled PAT derivative allowed detection of PAT. The experiments were performed directly in diluted apple juice. A fixed concentration of anti-PAT antibodies was incubated with increasing amounts of PAT. Each sample was mixed offline and incubated for 30 min before the FPIA measurements. The LOD of the method was 0.06 µg/l.

Tannous *et al.* (2015) described the development of a real-time PCR assay incorporating an internal amplification control to specifically detect and quantify *Penicillium expansum*. A specific primer pair was designed from the patF gene, involved in PAT biosynthesis. The selected primer set showed high specificity for *P. expansum* and was successfully employed in a standardised real-time PCR for the direct quantification of this fungus in apples. Using the developed system, apple samples were analysed for their DNA content. Apples were also analysed for PAT content by LC. A positive correlation (r^2=0.70) was found between *P. expansum* DNA and PAT concentrations. This work offers an alternative to conventional methods for PAT quantification and mycological detection of *P. expansum*. While the method was shown to work for (single) fruits, confirmation of results by measurement of PAT in juices made thereof is still required.

11. Trichothecenes

Following the publication of the scientific opinion from the EFSA CONTAM panel on the health risks of nivalenol (NIV) in food (EFSA, 2013), a review article giving an update on methods for determination of NIV was published (Malachová *et al.*, 2014b). An overview of the chemistry of NIV, analyte isolation and chromatographic methods were presented. The article highlighted the move from GC and HPLC-UV methods to LC-MS. A table summarised a number of methods published since 2006 that have been validated in house, as currently no interlaboratory validated method exists. There are many common factors among the methods, most used aqueous ACN extraction, and were for multitoxin analysis. Method performances in terms of recovery were acceptable. Information on LOQ, repeatability or reproducibility were not given, so readers would need to source the original papers cited in the text to find out full method performance. The authors stated that rapid immunochemical methods are not yet available as antibodies are difficult to produce, but also noted that an immunoaffinity column for both DON and NIV is available commercially. They also mentioned that while a certified calibrant is available no CRMs are available to assist with analysis.

The first CRM for T-2 and HT-2 in ground oat flakes (ERM® BC720) was produced within the framework of a European Reference Material (ERM®) project and according to ISO Guide 35 (Köppen *et al.*, 2015). The whole procedure involved in the production of the material including preparation, homogeneity and stability testing, as well as the certified value assignment were reported. The stability

tests showed that the material has an acceptable shelf life for both toxins when stored at -21 °C. The shelf life at a temperature of 4 °C, while considerable, was somewhat short of the desirable target of 5 years. However, these were estimates and the stability data will be updated following post-certification monitoring over the period of availability of the material. The certified value was assigned based on in-house analysis using SIDA with LC-MS/MS. Twentyfour laboratories took part in an interlaboratory comparison to provide data to support the certified value. A variety of methods were used, while most used acetonitrile extraction, different clean-up methods including SPE and immunoaffinity columns were used. HPLC was most commonly used for determination but 4 laboratories used GC-MS and one used ELISA. The certified values and their corresponding expanded uncertainties for T-2 and HT-2, traceable to the international system of units, were 82±4 µg/kg and 81±4 µg /kg, respectively. The availability of this material is an important development to allow improved quality control and improved measurement of these toxins in cereals. McCormick *et al.* (2015) published a comprehensive description of the production and study of T-2-glucoside. While this study assumes that T-2-glucoside occurs naturally as the T-2-α-glucoside, more recent findings about T-2/HT-2 metabolism in barley showed that the glucosides of T-2 and HT-2 are actually of β-configuration (Meng-Reiterer *et al.*, in press). Two ELISA kits were tested for CR with T-2-α-glucoside. It was determined that an ELISA using an antibody developed with T-2-α-glucoside recognised T-2-α-glucoside better than T-2 and T-2-β-glucoside whereas a commercially available ELISA for T-2/HT-2 worked well for T-2 and cross-reacted with T-2-β-glucoside, but poorly with T-2-α-glucoside. The authors have made T-2-α-glucoside available to other research groups to promote research efforts in modified mycotoxins.

A monoclonal antibody was produced and developed into an ELISA method for the determination of T-2 (Li *et al.*, 2014b). The CR of the antibody was tested for several other mycotoxins, the highest CR observed was for HT-2, which was 3.1%, while for other tested trichothecenes the CR was <0.1%. CR was not assessed for T-2-glucoside. The mAb was used to prepare a competitive indirect ELISA and an extraction protocol that used ethyl acetate was developed. Results were compared with UHPLC-MS/MS and good correlation was obtained. The method was applied to test rice samples. The authors stated the importance of the development was the fact that the method could be applied for T-2 only rather than sum of T-2 and HT-2. However, what the advantage of this would be is not clear as, certainly within Europe, it is the sum of T-2 and HT-2 that is considered for control and safety assessment. Zhang *et al.* (2015a) reported a chemiluminescence enzyme immunoassay (CLEIA) that was capable of detecting very low levels of DON in wheat. A commercially available antibody was used to develop the assay, which was optimised by assessing three parameters (organic solvent, ionic strength and pH) to improve the sensitivity, using the relative light units, the correlation coefficient, and the IC_{50} value as evaluation criteria. The authors claim this is the first report of a CLEIA method for DON, and that the assay was three times more sensitive than colorimetric ELISA using the same antibody. Wheat samples were tested and a DON level below 50 µg/kg could be accurately determined. The high throughput and low LOQ would render this method suitable for rapid screening of cereals.

Different clean-up methods for trichothecenes in baby food were assessed by Pereira *et al.* (2015). The compared methods were QuEChERS with 2 different dispersive SPE phases, the MultiSep®226 column and a immunoaffinity column. The end determination step used was GC-MS. The methods assessed, including different derivatisation methods, were all established methods. Despite the fact the recoveries ranged from 44-135%, the authors opted to use QuEChERS extraction and dispersive SPE for analysis of baby food as the advantages of speed and lower cost and range of analytes that could be determined outweighed this. Commercially available baby foods were screened using this method, however the total number of samples tested (9) and the number found to contain DON (4) was very small and does not provide much information on the occurrence of trichothecenes in baby food in Portugal.

A comparison between several different sample preparation methods for the determination of DON and deoxynivalenol-3-glucoside (DON-3G) was conducted (Nathanail *et al.*, 2014). The methods assessed were 'dilute-and-shoot' (no clean-up), MycoSep® 227 columns, MycoSep® 227 with additional acetonitrile elution and centrifugal filtration followed by analysis by LC-MS/MS. Method performance for these four methods showed that the 'dilute-and-shoot' approach gave the highest recoveries across different concentrations for DON and DON-3G, with MycoSep® giving recovery at levels below 30% for DON-3G. The 'dilute-and-shoot' approach followed by UHPLC-ESI-MS/MS was compared to an on-line automated sample clean-up system. This used a Transcend TLX-1 system coupled to LC and a TurboFlow MCX-2 column (ThermoScientific) for clean-up with a Hypersil Gold C18 column for analytical separation. The comparison of the methods was carried out in two laboratories by analysis of spiked samples of wheat and barley at high and low concentrations. Sample preparation efficiency was assessed by measuring recovery and intra-day precision, recovery was calculated using matrix matched calibration. Both methods were validated in the inter-laboratory study. The study showed that DON and DON-3G could be determined with satisfactory precision and recovery following on-line clean-up using Turboflow chromatography. The other method that gave method performance that gave acceptable performance was the

'dilute-and-shoot' method. However, the Turboflow method had the advantage of a lower LOQ than this method, while maintaining similar recovery. In addition the use of clean-up reduced the need for system maintenance. The use of high resolution mass spectrometry had the additional advantage of allowing retrospective data analysis, which will be useful to assess samples without further extraction and analysis if new modified forms of other mycotoxins are discovered in the future. This method does require significant capital investment, however it is a real development in analytical terms as the use of automation brings advantages in terms of time for sample preparation and reduced system maintenance, and the improved LOQ compared to other methods make this an attractive option going forward.

High resolution mass spectrometry was used in a completely different way by Kluger *et al.* (2014). Rather than carry out targeted analysis for identified compounds, the LC-HRMS was used to detect metabolites in an untargeted metabolomics workflow. A labelled tracer substance was included in wheat cell suspension cultures in the presence of DON. In this system native and ^{13}C-enriched tracer are metabolised to the same degree, and so metabolites of native compounds and their ^{13}C-labelled tracer isotopologs coelute, the MS allows them to be detected separately due to their different *m/z* values. The authors have developed workflows that automatically search for unique isotope patterns and returns a list of metabolic features, corresponding to an ion of a metabolite from the tracer used. The procedure was shown to have a low number of false positives that confirmed the selectivity of the stable isotope labelling assisted method. The approach was applied in two studies, verifying the metabolism of T-2 and HT-2 in barley (Meng-Reiterer *et al.*, in press) and wheat (Nathanail *et al.*, 2015b). A variety of metabolites, partly described for the first time, were detected in both plants. The metabolism routes included hydrolysis of acetyl and isovaleryl groups, hydroxylation as well as glucose, malonic acid, acetic acid and ferulic acid conjugates. Additionally, putative isomers of 15-acetyl-T-2-tetraol-malonyl glucoside, hydroxy-HT-2-glucoside, hydroxy-HT-2-malonylglucoside, HT-2-diglucoside, HT-2-malonylglucoside and feruloyl-T-2 were revealed.

Analytical testing in the field using dust analysis was reported by Reichel *et al.* (2014). Special dust sampling equipment, designed to selectively collect dust particles with a particle size of 0.1-1.0 mm was devised to collect dust on-site during unloading of trucks. To compare the results of dust analysis, grain samples were also collected and both dust and grain were analysed for DON. The results for the grain and dust correlated well for DON and ZEA for wheat (r^2=0.85 and r^2=0.82, respectively). DON results for rye did not correlate so well (r^2=0.73). Dust samples were extracted directly and analysed for DON by lateral flow device as the simplicity of matrix meant few interferences. A regression line was used to predict DON concentrations in grain from the dust analysis results, and these were compared to the results of the grain analysis. No false negative results were observed and a contaminated lot was clearly identified. The described procedure shows promise to allow for cheaper and faster on-site testing of grains. As results can be obtained in a very short time frame, decisions on acceptance and rejection can be made in real time. The authors stated that dust sampling is more representative for a whole lot than current sampling practice where a number of grain samples are selected. The method would also be suitable for on-site laboratories as it does not require expensive laboratory equipment.

12. Zearalenone

The majority of newly published analytical methods for the determination of ZEA in food and feed continue to be LC-MS based multitoxin methods, covered in Section 3 of this article.

Dedicated liquid chromatographic methods to determine ZEA in cereals and edible oils were developed as well. For instance, ultrasound-assisted QuEChERS extraction and clean-up was employed prior to UHPLC-FLD determination of ZEA in different cereals samples (Porto-Figueira *et al.*, 2015). A mixture of $MgSO_4$, NaCl, trisodium citrate dihydrate and disodium hydrogen citrate sesquihydrate (4:1:1:0.5) was added to milled cereals in ACN. After ultrasound agitation for several minutes and centrifugation, the organic phase was subjected to clean-up by dispersive SPE with $MgSO_4$, C18 material and PSA. The purified extracts were further concentrated by evaporation and take-up in the mobile phase. While the miniaturised clean-up procedure uses up to 32 times lower amounts of partitioning salts and extraction solvents, the extremely low sample amount (0.3 g) demands highly homogenous samples, which might be tricky to obtain in routine analysis. Still, QuEChERS clean-up proved to be well-suited to remove matrix interferences in subsequent HPLC-FLD measurements of maize samples. Sufficiently low LOQs of around 5 µg/kg, suitable recoveries of 80-110% and excellent intra- and inter-day repeatabilities (<4% RSD) were achieved. Slightly worrying, the recoveries seem to be concentration-dependent. Due to the acceptable recovery range, correction for recovery is not mandatory though. Worth of notice, isocratic eluent conditions of 70% ACN resulted in quick run times of just 5 min. Still, a wash step with a higher amount of ACN might improve the method further, removing apolar matrix which might be carried over to following injections. A different strategy to extract ZEA from maize was evaluated using ionic-liquid-based DLL microextraction (Wang *et al.*, 2015b). 10 g milled maize samples were extracted with 50 ml aqueous methanol in an ultrasonic bath for 30 min. Afterwards, 100 µl 1-hexyl-3-methylimidazolium hexafluorophosphate as

ionic liquid was added to 1 ml of the filtered extracts. The mixture was poured onto 5 ml acidified water, vortexed and centrifuged. The ZEA containing phase was dried down and dissolved in 100 µl ACN. 10 µl thereof were injected into a HPLC-FLD system. An LOQ of 1.0 µg/kg was achieved, while recoveries ranged from 84-95%, and the intraday repeatability was <5%. Similar to the previous study, isocratic elution from the C18 reversed phase column was performed using 70% acidified ACN. Chromatograms from maize samples prove the efficacy of the clean-up in removing matrix interferences. Drzymala *et al.* (2015) used an automated online-SPE-HPLC-FLD system for the determination of ZEA in maize germ oils. ZEA was retained in the SPE column due to a reversible hydrazone formation on a hydrazine moiety covalently attached to silica gel. The heptane diluted oil samples were pumped over the conditioned SPE column for about 1 h to allow complete coupling. Decoupling takes about as long and is achieved by flushing the SPE column with 20% acetone, displacing and releasing ZEA. The analyte then is trapped on a C18 SPE column and finally 'injected' to the analytical column from there. In the meantime the hydrazine SPE column is recycled for the next injection, a process which can be repeated 15 times before the column has to be exchanged due to lower recoveries. The obtained results were compared to those gained by liquid-liquid extraction prior to SIDA-LC-MS/MS and were in good agreement. An LOQ of 30 µg/kg was obtained with an average recovery of 78% and repeatabilities <8%. While the whole procedure is time-consuming and needs a full day to process 10 samples, it is also fully automated and might prove interesting for routine analysis if the coupling/decoupling times can be further reduced.

Also dedicated LC-MS(/MS) methods to determine ZEA (often with its metabolites or other related compounds) in a variety of matrices were developed and published within the last 12 months. Rempelaki *et al.* (2015) developed a dispersive suspended microextraction procedure, followed by LC-MS to determine ZEA in beer. Beer was degassed, diluted with buffer and extracted with toluene. After 30 s of rapid stirring, a restoration step for about 15 min at slow stirring allowed the toluene droplets, containing ZEA, to coalesce and sink to the bottom of the vial. 20 µl of the toluene phase were directly injected into the HPLC-MS system. The isocratic elution used a mixture of methanol with ACN as a very strong eluent under reversed phase conditions. ESI negative mode was used to charge ZEA and the SIM mode was used to detect the deprotonated ion. Chromatograms reveal a strong clean-up effect of the method. Not unexpected, they also reveal very early elution of ZEA (void time of the column 3.1 min; retention time of ZEA 3.6 min). Such a low retention factor (k=0.16) is typically unsuitable due to elution of polar matrix compounds with or after the dead time, resulting in high matrix effects in LC-MS. In this case however, polar matrix was not extracted at all and only marginal matrix effects were reported. Validation of the methods yielded an LOQ of 1.5 µg/kg and an average recovery of 91%, while both inter-day and intra-day precisions were around 5%. Masked forms of ZEA, DON and HT-2 were determined along with the native toxins in cereals using a 'dilute-and-shoot' LC-MS/MS method (Nathanail *et al.*, 2015a). After milling, wheat, oats or barley samples were extracted with acidified aqueous ACN, diluted and injected into the LC-MS/MS system. The majority of the ZEA metabolites (ZEA, α-ZOL, β-ZOL, α-ZOL-14-glucoside, β-ZOL-14-glucoside, ZEA-14-sulphate) were measured as deprotonated ions in negative ESI mode. Interestingly, both ZEA-14-glucoside and ZEA-16-glucoside gave good signals in positive ESI mode as ammonium adducts. Wheat was validated as sample matrix and yielded excellent recoveries (94-112%) for all analytes. LOQs were in the low or sub-µg/kg range, with ZEA showing an LOQ of 6 µg/kg. All ZEA metabolites were detected in naturally contaminated cereal samples from Finland. Besides ZEA, the highest concentrations were found for ZEA-14-sulphate – sometimes even exceeding the concentration of the native toxin. Qian *et al.* (2015) developed a GC-MS/MS method to determine ZEA, α-ZOL, β-ZOL, zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) in vegetable oils. Oil samples were diluted with ethyl acetate/cyclohexane, mixed and injected onto a gel permeation chromatography system. Fractions were collected each minute and all analytes eluted between 17-24 min. The combined eluent was evaporated and silylated with bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane for 40 min. One µl of the derivatised sample was analysed by GC triple quadrupole MS/MS in SRM mode. Excellent LOQs in the sub-µg/kg range were achieved for all analytes, with the one for ZEA at 0.2 µg/kg. Recoveries ranged from 80-97% and the intra- and interday repeatabilities were <15%. ZEA concentrations up to 185 µg/kg were detected in maize germ oil. Sample preparation times (ca. 30 min for gel permeation chromatography and 45 min for derivatisation) were in the same range as GC run times (about 1 h), allowing an effective sample throughput of about 20 samples per day.

ZEA and metabolites are not only measured in cereal samples. A method based on analyte isolation by DLL microextraction and consecutive LC-ESI-MS/MS determination to analyse environmental water samples for ZEA, α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL was proposed (Emidio *et al.*, 2015). 10 ml of an acidified water sample was spiked with deuterated ZEA as internal standard and extracted with 100 µl bromo-cyclohexane. The sample was vigorously shaken and centrifuged to allow mixture and sedimentation of the organic solvent, which was then dried down and re-dissolved in 80 µl acidified aqueous methanol. The concentration factor therefore was 125. Isocratic elution from a C18 reversed phase column was achieved using a – rather unusual – mixture of 48% acidified

water, 25% ACN and 27% methanol within 17 min. This ternary mixture allowed separation of all analytes. The average recoveries were 81-118% for all analytes, while the precision was <13%. LOQs ranged from 8 (ZEA) to 40 (β-ZOL) ng/l. Compared to other methods, the usage of organic solvents for extraction was very low. Evaluation of dietary mycotoxin exposure can also be assessed by measurement of urine, as far example shown by Belhassen *et al.* (2014) for ZEA, α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL. Human urine samples were enzymatically hydrolysed with β-glucuronidase/sulphatase, extracted with acidified ethyl acetate, defatted with hexane and measured by UHPLC-ESI-MS/MS in negative ion mode. The use of sub-2 µm particle size UHPLC columns combined with gradient elution resulted in fast run times of <7 min with adequate resolution between the stereoisomers. Deuterated ZEA was used as surrogate, seemingly for all analytes, which eluted between 3.3 and 4.0 min. LOQs ranged from 0.1 (ZEA) to 1.0 µg/l. Recovery rates ranged from 96% to 104%, with relative standard deviations lower than 9%. In a study with 42 healthy Tunisian women, only α-ZAL – but no ZEA or α-ZOL could be detected. Considering the human metabolism of ZEA, it is rather unlikely that this compound arises after ingestion of ZEA contaminated cereals. More likely, carry-over of the growth promoter α-ZAL was detected in urine samples, although the authors did not investigate this issue any further.

Also novel immuno-analytical methods for the determination of ZEA have been published recently. Sun *et al.* (2014) developed a rapid strip test using a specific anti-ZEA mAb. ZEA coupled to BSA was used as conjugate to immunise mice. After cell fusion and hybridoma screening, the obtained mAb showed marginal response against α-ZOL, β-ZOL, α-ZAL and β-ZAL, but about 50% CR against ZAN – which is not naturally occurring in maize. The anti-ZEA mAb was further coupled to colloidal gold to give a colour reaction, visible as a red band in the absence of ZEA. In the presence of ZEA, the toxin is bound by the coloured antibody, which in turn then no longer can bind to the test line. The suitable range of the test was quite narrow with about 1-20 µg/kg in cereals using a reader, while the visual range appears to be lower. Recoveries of ZEA from spiked samples were above 90% in all cases and the comparison with HPLC and ELISA yielded similar results for naturally contaminated samples. Still, the narrow concentration range and the low (undefined) cut-off value will result in several dilutions and measurements of mid- to high-contaminated samples, until a suitable signal is obtained which can be read out semi-quantitatively. Liu *et al.* (2015) developed biotin-streptavidin amplified ELISA and fluorescence-linked immunosorbent assays (FLISA) for quantitative determination of ZEA and its metabolites. Contrary to the work described above, the used mAb showed rather high CRs (25-61%) towards α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL. Also contrary to Sun *et al.* (2014) the extracts were diluted in this study, resulting in an overall dilution factor of 25. Again, only narrow working ranges were obtained with about 1-15 µg/kg for the ELISA and about 8-70 µg/kg for the FLISA. Good recoveries (77-115%) and repeatabilities (≤15%) were obtained after spiking experiments with maize flour and maize based baby food for both assay types. Duan *et al.* (2015) developed a QD submicrobead-based immunochromatographic assay for ZEA determination. After encapsulation of CdSe/ZnS QDs, the resulting submicrobeads – showing highly increased luminescence – were labelled with mAbs and used as fluorescent probes in a lateral flow device. CR against other types of mycotoxins was marginal, but ZEA related compounds were not tested. After a 1:30 overall extraction and dilution factor of cereals a suitable working range of about 4-300 µg/kg was obtained. Naturally contaminated maize samples were assayed using the new assay, showing good correlation of results to those obtained by a commercial ELISA. A competitive surface-enhanced Raman scattering immunoassay was developed by Liu *et al.* (2014). A gold nanoparticle was labelled with 4,4'-dipyridyl as Raman reporter and the anti-ZEA mAb. ZEA conjugated to BSA was coated to a surface and can bind to the labelled mAb in absence of ZEA. The mycotoxin was extracted using a saline-methanol mixture. The extract was mixed with $CHCl_3$ and shaken, before the ZEA containing chloroform phase was evaporated to dryness and taken up in buffer containing 10% methanol. Several dilutions were tested, suggesting that an overall 1:50 dilution resulted in little matrix effects. Using this dilution, a wide dynamic range from 0.25-250 µg/kg was observed, covering three orders of magnitude. Again, mAb CRs were only verified to be marginal against AFB_1 and the estrogenic diethylstilbestrol, but not against ZEA metabolites.

Finally, aptamers show great promise to mimic antigen-antibody reactions. A single-stranded DNA (ssDNA) aptamer based enzyme-linked oligonucleotide assay was developed for the determination of ZEA in maize (Wang *et al.*, 2015d). Aptamers were identified using the commonly used systematic evolution of ligands by exponential enrichment technique. Therefore, the random oligonucleotide ssDNA library was exposed to coated anti-ZEA mAbs. After multiple rounds of screening a aptamer with high affinity to the antibody was used to establish an indirect competition enzyme-linked oligonucleotide assay to quantify ZEA in maize. After aqueous methanol extraction and an overall dilution of 1:20, a working range of about 0.5-50 µg/kg was found. Spiked maize samples were used to determine recovery rates, which were in the range of 95-105% for the assay. While the used mAb did not cross-react to structurally diverse mycotoxins, the selectivity of the aptamer to structurally related compounds was not shown in the study. Overall, it remains to be seen if aptamers can (gradually) displace traditional antibody-

based assays for the detection of mycotoxins and other organic contaminants in food.

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