

Developments in mycotoxin analysis: an update for 2015-2016

F. Berthiller^{1*}, C. Brera², M.H. Iha³, R. Krska¹, V.M.T. Lattanzio⁴, S. MacDonald⁵, R.J. Malone⁶, C. Maragos⁷, M. Solfrizzo⁴, M. Stranska-Zachariasova⁸, J. Stroka⁹ and S.A. Tittlemier¹⁰

¹Christian Doppler Laboratory for Mycotoxin Metabolism and Center for Analytical Chemistry, Department of Agrobiotechnology (IFA-Tulln), University of Natural Resources and Life Sciences, Vienna, Konrad Lorenz Str. 20, 3430 Tulln, Austria; ²Istituto Superiore di Sanità, Department of Veterinary Public Health and Food Safety – GMO and Mycotoxins Unit, Viale Regina Elena 299, 00161 Rome, Italy; ³Adolfo Lutz Institute of Ribeirão Preto, Nucleous of Chemistry and Bromatology Science, Rua Minas 866, Ribeirão Preto, SP 14085-410, Brazil; ⁴National Research Council, Institute of Sciences of Food Production, Via Amendola 122/o, 700126 Bari, Italy; ⁵Fera Science Ltd., Sand Hutton, York YO41 1LZ, United Kingdom; ⁶Trilogy Analytical Laboratory, 870 Vossbrink Dr, Washington, MO 63090, USA; ⁷USDA-ARS-NCAUR, Mycotoxin Prevention and Applied Microbiology Research Unit, 1815 N. University St, Peoria, IL 61604, USA; ⁸Department of Food Analysis and Nutrition, Faculty of Food and Biochemical Technology, University of Chemistry and Technology, Prague 6, Czech Republic; ⁹European Commission, Joint Research Centre, Retieseweg, 2440 Geel, Belgium; ¹⁰Canadian Grain Commission, Grain Research Laboratory, 1404-303 Main St, Winnipeg, MB R3C 3G8, Canada; franz.berthiller@boku.ac.at

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Abstract

This review summarises developments in the determination of mycotoxins over a period between mid-2015 and mid-2016. Analytical methods to determine aflatoxins, *Alternaria* toxins, ergot alkaloids, fumonisins, ochratoxins, patulin, trichothecenes and zearalenone are covered in individual sections. Advances in proper sampling strategies are discussed in a dedicated section, as are methods used to analyse botanicals and spices and newly developed liquid chromatography mass spectrometry based multi-mycotoxin methods. This critical review aims to briefly discuss the most important recent developments and trends in mycotoxin determination as well as to address limitations of presented 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 2014/15 period (Berthiller *et al.*, 2016). The primary purpose is to raise awareness of the developments and advances in analytical methods for mycotoxins, derived from articles published between mid-2015 to mid-2016. Critical comments on the method, its validation parameters or application are usually added to guide readers in assessing the impact of a method. Rather than to provide an exhaustive list of publications, a selection of the most relevant advances in analytical methodology should render the whole article interesting to read both for

mycotoxin veterans and newcomers in the field. The topics covered in detail are sampling (section 2), multi-mycotoxin liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods (section 3), mycotoxins in botanicals and spices (section 4), aflatoxins (section 5), *Alternaria* toxins (section 6), ergot alkaloids (section 7), fumonisins (section 8), ochratoxins (section 9), patulin (PAT, section 10), trichothecenes (section 11) and zearalenone (ZEA, section 12).

An unusually high number of reviews regarding mycotoxin determination have been published recently, which the interested reader might refer to for additional information. These include some quite general ones, which will give analytical chemists new to the field – among other

information – a quick start and overview about possible options for the detection and quantification of mycotoxins (Aiko and Mehta, 2015; Anfossi *et al.*, 2016; Bueno *et al.*, 2015; Lee and Ryu, 2015; Selvaraj *et al.*, 2015; Turner *et al.*, 2015). Several review articles in particular summarise advances in (nano-) sensor technologies to determine various mycotoxins (Lin and Guo, 2016; Rai *et al.*, 2015; Xu *et al.*, 2016a). Maragos (2016) describes biosensors for mycotoxin determination which can be and have been used in multiplexed formats. Appell and Mueller (2016) discuss recent developments in molecular imprinted polymers (MIPs) for mycotoxin determination, which are alternatives to antibodies or aptamers. Warth *et al.* (2016) describe the current state and discuss future perspectives of human biomonitoring of mycotoxins using breast milk. Stroka and Maragos (2016) review challenges in the analysis of multiple mycotoxins in their article, which is embedded in the World Mycotoxin Journal special issue 'Mycotoxins in a changing world'. Finally, a book covering the formation, determination, occurrence and toxicological relevance of masked mycotoxins in food became available last year (Dall'Asta and Berthiller, 2016).

2. Sampling

The increase in attention and interest in addressing the challenges with sampling various commodities for mycotoxin analysis continued over the past year. Several articles have been published since the previous review that describe sampling methods or guidance to mitigate the effects of heterogeneous distribution of mycotoxins in various matrices, ranging from grain (Whitaker *et al.*, 2015a,b), flour (Armorini *et al.*, 2015), pistachios (Wesolek and Roudot, 2016), to bales of hay and silage (Hägglblom and Nordkvist, 2015; McElhinney *et al.*, 2016) and pit silage (McElhinney *et al.*, 2016).

Whitaker and co-workers experimentally determined the sampling, sample preparation and analytical contributions to the total variance during the analysis of ochratoxin A (OTA) in oats (Whitaker *et al.*, 2015a) and wheat (Whitaker *et al.*, 2015b). They used an existing experimental framework that has already been applied to study a number of mycotoxin/commodity combinations. The experimental data produced in their studies were used to generate operating characteristics curves that can be used to estimate risks of misclassifying grain as (non-)compliant with specific limits. As with past studies, the bulk of the total variance was associated with sampling (for example, 89.0–99.7% for oats). Interestingly, deviations from the defined study protocol for wheat were noted for the two commercial laboratories that performed the sample preparation and analysis (Whitaker *et al.*, 2015b). The researchers were still able to obtain meaningful results, however, the deviations reported should emphasise to readers the complexity of

performing proper sampling and sample preparation when analysing mycotoxins.

A different approach to modelling mycotoxin concentrations was taken by Wesolek and Roudot (2016). The authors validated a model they had previously developed based on Monte Carlo simulations that described the distribution of aflatoxin B₁ (AFB₁) in pistachios. They compared the accuracy of their model in correctly classifying pistachio lots as (non-)compliant to the ability of a model used in the Codex Alimentarius to design pistachio sampling plans. The 'Codex model' uses the same framework as employed by Whitaker *et al.* (2015a,b) described above. The Monte Carlo simulation method appeared to be more accurate than the Codex model. This apparent advantage was attributed to the fact that the Monte Carlo simulation method incorporated both the distribution of the number of contaminated individual pistachios within a sample, and the distribution of AFB₁ concentrations amongst the individual contaminated nuts. The Codex model is built upon experimental data that do not incorporate the range of variance that may occur at a given AFB₁ concentration.

Results from Armorini and co-workers demonstrated that there can also be significant heterogeneity with respect to the presence of AFB₁ in flour (Armorini *et al.*, 2015). It is difficult to ascertain whether the sub-sampling and homogenisation used in their study mitigated heterogeneity and avoided introducing sampling bias due to a lack of experimental details on equipment and procedures used. The data presented emphasise that care must still be taken to sample a product consisting of very small particles such as flour. The results obtained clearly show an advantage in reducing the variability of AFB₁ concentrations by analysing 20 vs 5 g of flour.

Work by Hägglblom and Nordkvist (2015) described the variability of deoxynivalenol (DON) and ZEA in hay bales from individual fields. From a logistical point of view, they remarked that sampling of hay is challenging due to the heterogeneity from the irregular shape, varying density, and wide range of particle sizes of this material. They noted that drilled core sampling of bales had a particular advantage in that it cuts the material into manageable pieces that could immediately be ground for analysis. Their results indicated that DON concentrations varied up to an order of magnitude in bales of straw from winter wheat produced in one field. ZEA was detected less frequently and at lower concentrations that spanned a smaller range. The uncertainty in DON concentrations in hay due to selection of specific bales versus the number of hay bales sampled from the field was modelled and suggested that for this situation, increasing sampling from 2 to 5 bales decreased the sampling uncertainty by 50%.

The heterogeneity of a variety of mycotoxins in silage bales and pit silage was investigated by McElhinney *et al.* (2016). Mycotoxins produced pre-harvest (enniatins, ENNs) and post-harvest (roquefortine C (ROC) and mycophenolic acid) were both studied in samples taken using two different methods. Silage was sampled using drill coring, as well as grab sampling of further processed bale material laid out in a trough. Variation of mycotoxin concentration within bales was greater than amongst bales, and was especially noticeable for the post-harvest mycotoxins that would form in 'hot spots' during storage. Generally, variation was less in samples taken from pit silage due to the increased homogenisation and comminution of materials prior to pit storage. The authors also used a Monte Carlo simulation to model the effect of sampling frequency on the ability to detect mycotoxins in a silage bale or pit silage. Under the specific conditions of their study, they determined that over 100 core samples or 20 trough samples would be required to maximise the possibility of detecting mycotoxins when they were truly present in the silage.

In addition to the scientific publications, other activities were organised over the past year to help analysts, researchers, and regulators manage challenges due to sampling. The joint meeting of the 9th Conference of the World Mycotoxin Forum and the XIVth IUPAC International Symposium on Mycotoxins that took place in June 2016 held its first dedicated session on sampling (World Mycotoxin Forum, 2016). The half-day session included specific method-related presentations on: (1) an online sampling tool for mycotoxin analysis (FAO, 2016) to visualise effects of sampling plan parameters on the risk of mischaracterising commodity lots; (2) a new grain sampler to minimise distributional heterogeneity of mycotoxins in grains; and (3) sampling plans to balance the number of increments required to produce a 'fit for purpose' plan for the analysis of mycotoxins. As well, another online tool was launched with the aim of providing a pragmatic tool for inspections of imported food and feed products this past year. The Italian Ministry of Health, in collaboration with the Istituto Superiore di Sanità and the Istituto Zooprofilattico Sperimentale Lazio e Toscana, produced and published a video on sampling (Istituto Superiore di Sanità, 2016). The video discusses how to sample commodities stored in various ways, including packages, containers, warehouses, and trucks, and also covers some basic – yet important – aspects of sample preparation. The video presents a high level overview of sampling from a European Union viewpoint; therefore viewers will need to consult guidance and regulations from additional sources for more detailed directions on the selection and use of sampling equipment as well as a more global picture of sampling procedures. While the video is not solely focused on mycotoxins, the theory and general guidance presented are applicable to all analytes that are not homogeneously distributed in food and feed.

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

In the 2015-2016 period, literature relevant to the development of LC-MS(/MS) approaches for multi-mycotoxin analysis reports few advances in separation and detection techniques as well as strategies for sample preparation compared to previous years. The interest in applications of high resolution mass spectrometry (HRMS) techniques for target quantitative analysis and retrospective investigations is confirmed, mainly in the field of multi-class methods. Interestingly, efforts in establishing guidelines and setting rigorous workflows to make these approaches more reliable can be found in recent publications.

To improve the efficiency of mycotoxin determination, the researchers at the FDA's Center for Food Safety and Applied Nutrition (FDA/CFSCAN) have been evaluating modern LC-MS techniques. These evaluations resulted in a review, describing LC-MS/MS and LC-HRMS methods developed at the FDA/CFSCAN (Zhang *et al.*, 2016a). The authors emphasise advantages of these multi-mycotoxin methods with respect to conventional methods due to the superior specificity, sensitivity and fast data acquisition features, which allow simplified sample preparation. However, reviewing analytical performances of these LC-MS multi-toxin methods revealed that improvements in accuracy and efficiency, as well as in management of matrix effects are still needed. Different methods for managing matrix effects ranging from extract dilution, use of matrix-matched calibration or uniformly ¹³C-labelled mycotoxins as internal standards are discussed. Practical solutions for the future implementation of LC-MS multi-mycotoxin methods are proposed, underlining that researchers performing mycotoxin analyses must be flexible and use the fitness for purpose approach when deciding which approach should be chosen to address matrix effects in mycotoxin analyses. The matrix effects related to LC-MS/MS analysis of 9 major mycotoxins (AFB₁, fumonisins B₁, B₂ and B₃ (FB₁, FB₂ and FB₃), OTA, DON, T-2 and HT-2 toxins (T-2 and HT-2), and ZEA) in various sample extracts giving moderate to strong matrix effects (maize, compound feed, straw, spices) have been studied in detail by Fabregat-Cabello *et al.* (2016) in order to evaluate and compare different strategies for compensation. The authors compared five different calibration approaches: multi-level external calibration using isotopically labelled internal standards, multi-level and single level standard addition, and two ways of single point internal calibration: one point isotopic internal calibration and isotope pattern deconvolution. Experimental results showed that in general, recoveries and precision meeting the European Union requirements could be achieved with all approaches, with the exception of the single level standard addition at levels too close to the concentration in the sample. An interesting outcome of this study is the demonstration of the reliability of single-level standard addition, representing a practical and efficient

solution to be applied when isotopically labelled internal standards are not available.

Among recent developments in terms of analyte isolation protocols, two papers describing the manufacturing and application of multi-antibody immunoaffinity columns (IACs) can be found last year. Zhang *et al.* (2016b) reported on the production of four monoclonal antibodies produced against aflatoxins, OTA, ZEA and T-2, respectively, and their combination as a pool bound to sepharose 4B for affinity chromatography. The columns were used to purify seven mycotoxins after extraction from wheat, maize and peanuts samples by using acetonitrile (ACN):water:acetic acid (80:19:1, v/v/v), prior to LC-MS/MS analysis. Performances of the newly produced columns were compared with those of two commercially available multiple IACs, obtaining similar results in terms of recoveries, repeatability and organic solvent tolerance. In a following study a similar IAC has been proposed, also including an anti-streptomycin antibody in the antibody pool (Hu *et al.*, 2016). Also in this case the new IAC was compared with two commercially available columns showing similar performances. The resulting method, based on ACN/water extraction followed by IAC clean-up and LC-MS/MS detection was applied to 80 feed samples. AFB₁ showed the highest frequency of occurrence (33%) with concentrations ranging up to 32 µg/kg.

On the opposite side in terms of clean-up selectivity, QuEChERS (Quick Easy Cheap Effective Rugged Safe) based approaches were investigated in detail also last year, offering an interesting compromise between increasing the analyte range and removal of matrix compounds. The development and small scale inter-laboratory comparison (two laboratories) of an ultra high performance liquid chromatography (UHPLC)-MS/MS multi-mycotoxin method to simultaneously identify and quantify 20 mycotoxins in grass silages, including EU regulated mycotoxins, has been described by McElhinney *et al.* (2015). A careful optimisation of the sample preparation protocol is reported in the article, including testing of different acidic conditions for the extraction solvent (applied in order to enhance fumonisin recoveries), and testing of a range of different dispersive solid phase extraction (dSPE) clean-up sorbents, including primary secondary amines (PSA), amino-, C18-, C12-, C8- and C2-phases. Due to unacceptable recoveries for ROC and OTA strongly retained on all sorbents, and for the non-polar ENNs, not recovered from the more hydrophobic sorbent materials, a QuEChERS type extraction was finally set up without dSPE clean-up. Analytes were detected using a fast polarity-switching MS/MS method that allowed both positive and negative ions to be analysed from a single injection. The applicability of the method was evaluated through application to six naturally contaminated maize silage samples, and comparison of generated data with results obtained by another laboratory

with an independent method, obtaining satisfactory agreement. Another paper reporting a detailed investigation of sample preparation aspects has been published by Zhao *et al.* (2015), describing a method for multi-mycotoxin analysis in animal feed and animal-derived food (meat, edible animal tissues, and milk) using LC-MS/MS. In this study, three extraction mixtures, as well as various clean-up procedures, were evaluated. Several clean-up cartridges (Oasis HLB[®], an amino cartridge, Oasis MAX[®] and MycoSep[®] 226 multifunctional cartridge) and various adsorbents (C18, chitin, multi-walled carbon nanotubes and florisil) were compared for the analyte isolation of 30 mycotoxins including aflatoxins, OTA, trichothecenes, ZEA, fumonisins and citrinin (CIT). Finally, a C18/MgSO₄ mixture was selected for dSPE clean-up.

A further comparative evaluation of various adsorbents (C18, PSA, HLB[®], mixed-mode cationic exchange (MCX), silica, amino) for the solid phase extraction (SPE) of multiple mycotoxins (*Alternaria* toxins, OTA, PAT, CIT) in fruit matrices, namely apple, sweet cherries, orange and tomato, prior to LC-MS/MS analysis has been reported by Wang *et al.* (2016). The combination of MCX and amino phases in an in-house made SPE column was found to provide the most effective clean-up mainly in terms of minimisation of matrix effects. The optimised protocol included a salting-out liquid-liquid extraction with water and acidified ACN, followed by SPE clean-up. Although satisfactory analytical performances were reported in terms of recoveries and repeatability, application of the proposed method to naturally contaminated samples was not investigated. Nielsen *et al.* (2015) reported on the development of a LC-MS/MS method for simultaneous determination of OTA and fumonisins B₂, B₄ (FB₄), and B₆ (FB₆) in green, roasted, and instant coffee. To maximise recoveries of these toxins containing acidic functional groups, extraction was performed by an acidified water/methanol mixture followed by mixed-mode reversed phase-anion exchange SPE (Oasis MAX[®] cartridges). The method was applied to the screening of 57 roasted coffee samples, 25 instant coffee samples and 18 green coffee samples detecting OTA at levels ranging up to 4.5 µg/kg, and fumonisins at levels ranging up to 134 µg/kg. By comparison of fumonisin contamination in roasted and green coffee samples, the authors hypothesised a degradation of fumonisins during the roasting process. A QuEChERS-like extraction with acidified ACN:water, without any further clean-up, has also been proposed by Zhu *et al.* (2015) for multi-mycotoxin analysis in eggs by LC-MS/MS. The method included 15 mycotoxins, namely aflatoxins (including aflatoxin M₁ (AFM₁) and aflatoxin M₂ (AFM₂)), DON and its acetylated derivatives (3-ADON and 15-ADON), deoxy-deoxynivalenol (DOM-1), ZEA, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL). The method was in-house validated, using 2002/657/EC as guideline, and applied in a pilot study to analyse mycotoxin contamination

in 12 eggs. Trace amounts of DON (up to 11.3 µg/kg), 15-ADON (9.6 µg/kg), ZEA (up to 15.2 µg/kg) and β-ZOL (up to 2.2 µg/kg) were detected in these samples.

With respect to multi-mycotoxin analysis in biological fluids, a new LC-MS/MS method was developed to (semi-) quantitatively measure mycotoxin biomarkers in urine samples of Belgian volunteers (Huybrechts *et al.*, 2015). The method was based on direct LC-MS/MS analysis using two injections with electrospray negative ionisation (ESI-) for glucuronides of DON, DOM-1, ZEA and electrospray positive ionisation (ESI+) for glucuronides of 3- or 15-ADON, α- and β-ZOL and OTA. However to reach suitable sensitivity to confirm the presence of OTA, CIT and AFM₁ at levels of pg/ml, a second method using IAC clean-up was set up resulting in a 50-fold concentration of the analyte compared to the direct method. Analysis of human urine samples (32) revealed that DON glucuronides were the major biomarkers for DON exposure. All urine samples contained DON-15-glucuronide, the major human metabolite of DON, in the range 3-420 ng/ml. Also DON-3-glucuronide and DOM-1-glucuronide were present in 90% and 25% of the samples, at levels up to 550 ng/ml and 16 ng/ml, respectively. DON was detected in 60% of the samples, at lower concentrations. OTA was detected in 70% of the samples in the range 3-33 pg/ml. CIT and/or dihydrocitrinone were detected in 90% of the samples, also at concentrations of pg/ml. This method has also been applied for mycotoxin exposure assessment in Belgian adults and children (overall sample size = 394) within the BIOMYCO study (Heyndrickx *et al.*, 2015). Biomarker analysis showed a clear exposure of a broad segment of the Belgian population to DON, OTA and CIT. Again, DON-15-glucuronide was the main urinary metabolite and was found in 100% of the samples. Also DOM-glucuronide was detected in children urine. In agreement with the previous study, a high prevalence of CIT and OTA was observed suggesting the need for more research concerning the exposure to these two toxins. A LC-MS/MS method for the simultaneous determination of ENNs A, A₁, B and B₁ and beauvericin (BEA), in human urine and plasma samples, has been proposed by Belèn Serrano *et al.* (2015). Analyte isolation was based on SPE by graphitised carbon black cartridges, allowing satisfactory recoveries for the tested mycotoxins. The suitability of the method was tested by analysing 10 samples of human urine and 10 samples of human plasma, however ENNs and BEA were not detected in 9 of the analysed samples of both plasma and urine. Traces of ENNB₁, i.e. lower than the quantification limit of 20 ng/l, were detected in a sample of both biological fluids from the same subject. Finally, Tolosa *et al.* (2016) proposed a LC-MS/MS method for the simultaneous detection of 15 mycotoxins, including aflatoxins, fumonisins, OTA, sterigmatocystin (STE), fusarenone-X (FUS-X) and emerging *Fusarium* mycotoxins, in water and fish plasma samples. Analyte isolation was based on modified dispersive

liquid-liquid microextraction (DLLME), a technique based on a ternary component solvent system where the dispersion of the fine droplets of the extraction solvent is carried out in the aqueous phase. The authors used ACN as disperser solvent and ethyl acetate as extraction solvent added to the aqueous sample. The method was proposed for the analysis of samples from toxicity studies, such as rearing water and fish plasma, however no results of the analysis of naturally contaminated samples were reported.

The use of LC-HRMS continues to be regarded as a powerful tool for multi-class and multi-analyte analysis in food safety control. Among multi-class methods proposed last year for the simultaneous determination of mycotoxins and other classes of contaminants, the article by Leon *et al.* (2016), describing and discussing a workflow for target analysis and retrospective screening of multiple contaminants including pesticides, veterinary drugs, ergot alkaloids and plant toxins, is worth mentioning. A comprehensive strategy for multi-contaminant analysis, based on a generic extraction method (salting-out liquid-liquid extraction with water and ACN) and LC-HRMS analysis is presented. Key aspects such as matrix effect management and the choice of a representative matrix for their compensation, identification and confirmation criteria, settings for retrospective screening, and the procedure of comparison of theoretical and experimental patterns, are discussed with practical examples. The discussed data highlight the analytical potential of the high resolving power (>50.000 (full width at half maximum, FWHM), the accurate mass measurements (1-5 ppm) and the acquisition in full scan (with and without fragmentation), to perform target analysis and retrospective screening over a database of more than 400 compounds of interest. An example of quantitative targeted and retrospective data analysis of relevant pesticides, antibiotics and mycotoxins in bakery products by liquid chromatography-single-stage Orbitrap MS can be found in de Dominicis *et al.* (2015). To prove the applicability of the method for quantitative analysis in finished products, mini-cake samples at two different contamination levels were prepared on an industrial pilot plant. This exercise showed that good quantitative findings could be obtained within the tested analytes, except for OTA and AFB₁, whose sensitivity was not satisfactory with respect to the relevant EU maximum permitted levels.

4. Mycotoxins in botanicals and spices

The published analytical methodologies for the analysis of mycotoxins found in botanicals and spices this past year were based on a variety of different techniques. Two of these published methods utilised high performance liquid chromatography (HPLC) for the mycotoxin analyses including the use of an automated analysis using inline reusable IAC clean-up while the other HPLC method utilised detection by an Orbitrap MS. Other recently

developed analytical methods include a screening test using aptamer based lateral flow and the use of immunosensor technology.

An HPLC method was published for the automated analysis of aflatoxins using an inline reusable IAC clean-up with fluorescence detection (FLD) (Rhemrev *et al.*, 2015). This cartridge, utilised up to 15 times without loss in performance, contains monoclonal antibodies to aflatoxins coupled to a pressure resistant polymer. An inline handling system selects a cartridge from a tray which automatically applies the sample extract, washes the cartridge, and then elutes AFB₁, aflatoxin B₂ (AFB₂), aflatoxin G₁ (AFG₁) and aflatoxin G₂ (AFG₂). The aflatoxins are then detected and quantified using FLD with KOBRA[®] cell postcolumn derivatisation. Paprika and nutmeg were validated with this automated system in two independent laboratories with spiked samples at 5, 20 and 100 µg/kg total aflatoxins. The recoveries exceeded 82% for AFB₁ and 72% for all other aflatoxins, while the between-day repeatability ranged from 2.1 to 9.6% for AFB₁. In addition, satisfactory Z-scores were obtained with this system when used for participation in proficiency testing (FAPAS[®]) for the analysis of aflatoxins in samples of chili powder. This automated system can potentially provide increased sample throughput and reduced costs compared to a manual purification process. Another published HPLC method for the multi-mycotoxin analysis of paprika utilised UHPLC coupled with Orbitrap MS (Reinholds *et al.*, 2016). This new methodology, also used for the simultaneous analysis of pesticides, was compared to the results obtained using a triple quadrupole tandem MS. Both methods obtained similar limits of detection (LODs) and acceptable values of recovery (75-120%) and intraday repeatability (8-15%) from results of spiked (10-500 µg/kg) paprika samples. This method was used for the analysis of eleven mycotoxins (nivalenol (NIV), DON, FUS-X, AFB₁, CIT, HT-2, FB₁, T-2, ZEA, OTA, STE) in ground paprika samples.

An aptamer based lateral flow device (LFD) was developed for on-site rapid detection of OTA in *Astragalus membranaceus*, a herb used in Traditional Chinese Medicine (Zhou *et al.*, 2016). The test procedure is relatively simple with one gram of a test portion extracted with 2.5 ml of methanol:water (80:20, v/v) and diluted with a four-fold working buffer to eliminate the matrix and methanol interferences. After optimisation of the crucial testing parameters, the 15 min qualitative assay demonstrated a visual LOD of 1 ng/ml in buffer with no cross-reactivities to several other mycotoxins. Sample results were compared to LC-MS/MS analysis and in agreement with one out of nine *A. membranaceus* samples found positive for OTA. During sample preparation the test portion was diluted tenfold, resulting in an LOD of around 10 ng/g in solid media. It should be noted that the small 1 g sample size used in this analysis should be

further evaluated for sample homogeneity even for use in a qualitative screening test. Another new method utilising immunosensor technology was published using the optical waveguide lightmode spectroscopy (OWLS) technique for AFB₁ determination in paprika (Majer-Baranyi *et al.*, 2016). This method was applied to the label-free detection of AFB₁ in a competitive immunoassay format. Sample preparation was also evaluated and it was determined that the QuEChERS method was insufficient for the analysis of AFB₁ compared to traditional solvent extraction followed by IAC clean-up. The optimised OWLS immunosensor was used to analyse 60 samples of commercial paprika from different countries and it was found that the results were comparable to HPLC or enzyme-linked immunosorbent assay (ELISA) data with regression coefficients above 0.94. The data comparison demonstrated that the OWLS immunosensor has a potential for the quick determination of AFB₁ in paprika samples.

Two market surveys were published for aflatoxins and OTA in various spices and one review published data on OTA in liquorice products. The occurrence of OTA in commercialised red pepper flakes in Turkey was determined by the analysis of 75 samples collected from different supermarkets and traditional bazaars in Istanbul during 2012-2013 (Tosum and Ozden, 2016). The incidence of OTA found in packed (87%; range: 0.6 to 1.0 µg/kg) and unpacked (100%; range: 1.1 to 32 µg/kg) red pepper flakes was very high, demonstrating the importance of continued monitoring of this mycotoxin. Another survey was conducted to determine aflatoxin contamination in composite spice samples collected from local markets of Pakistan (Asif Asghar *et al.*, 2016). A total of 75 samples of composite spices including biryani, karhai, tikka, nihari and korma masalas were analysed for total aflatoxins by a HPLC method. Aflatoxins were found present in 77% of the samples ranging from 0.7-25.7 µg/kg with a mean of 4.6 µg/kg. It was concluded that a strict and continuous national monitoring plan was necessary to improve the safety and quality of spices in Pakistan. A review of OTA in liquorice products was published which describes analytical techniques, occurrence, information regarding contaminated products, regulatory frameworks and degradation methods (Khalesi, 2015).

5. Aflatoxins

The trend that methods based on LC-MS nowadays target a variety of different mycotoxins continues, and render such methods of lesser interest for the determination of aflatoxins only. This is emphasised taking into account the measurement capacity of dedicated aflatoxin methods based on FLD with outstanding performance at lower µg/kg ranges, which has been demonstrated in the past.

The trend of the last years in the development of various sensor prototypes is unbroken and three complementary review papers on this subject appeared putting the overall progress in some relation to the determination of aflatoxins. Taking into account that aflatoxins are the most important mycotoxins in the field of compliance testing and that Commission Regulation 519/2014 (EC, 2014) has set the legislative basis for the use of screening methods in official food control for mycotoxins, the general and aflatoxin specific developments in this field ought to be of interest for the aflatoxin analyst. Rotariu *et al.* (2016) cited four aflatoxin related publications using electrochemical biosensors in their review, however, the number of citations clearly show that the target analytes for this technology are very miscellaneous. Roda *et al.* (2016) reviewed luminescence biosensors in a similar manner. Both reviews, even though not exclusively focussing on aflatoxins give a rather good overview on the state of the art of such devices, helping putting original papers for aflatoxin identification in an overall context to these technologies. The trend that LFDs evolve from visual detection to instrumentally read tools is reviewed and summarised by Mak *et al.* (2016). Authors describe the development of instrumental reading possibilities for LFDs. In their outlook authors address electrochemical applications, which appears to be promising compared to currently used optical (colorimetric) approaches. The drawbacks mentioned for optical readers (e.g. rather cumbersome and relatively expensive strategies), however, do not appear as an issue for aflatoxin determinations (or mycotoxins in general). Recently available devices (e.g. smart phones) seem to allow fast and reliable measurements of LFDs for mycotoxin determination after proper calibration.

A portable fibre-optic spectrometer was described by Arduini *et al.* (2016) which allowed the determination of AFB₁ without the need for derivatisation or labelling by means of a specific enzyme inhibition. The method makes use of an IAC clean-up after extracting AFB₁ from the test matrix. Authors stressed that this simple approach targets the use as screening method and discussed the use in combination with a confirmatory method for suspect results, an approach that is explicitly promoted in Commission Regulation 519/2014 (EC, 2014). In a different manner, but with a similar intention, Rasooly *et al.* (2016) described the use of a commercial charged coupled device (CCD) camera to quantify fluorescence intensities from cell bioassays as a cheap alternative to a commercial fluorimeter for AFB₁ quantification. This application seems to have little use for the determination of AFB₁ in food as its intention is to monitor AFB₁ activity in cell bioassays, however, it indicates that simplifications of instrumentation originally designed for other purposes can offer new analytical perspectives. Unfortunately, authors refer to 'biologically active AFB₁' and 'detoxified AFB₁' without further specification. Seok *et al.* (2015) described

an interesting test for aflatoxins based on a structure switchable aptasensor for AFB₁. Despite the advantages the authors claim, such as selectivity, they unfortunately lack demonstration that the system does not cross react to some relevant close structural analogues of AFB₁, such as AFB₂ and STC, while they do for a number of other mycotoxins including AFG₁ and AFG₂.

In the period for this review, the number of interesting AFM₁ publications was rather high compared to the years before. While nowadays most LFDs are designed for instrumental evaluation with readers, Wu *et al.* (2016) developed a one-step immunoassay with two different cut-offs for the legislative limit for AFM₁ in milk in the EU (0.05 µg/kg) as well as in North America and China (0.5 µg/kg). The interpretation of the test result is therefore reduced as positive (+) or negative (-) based on the visually observed occurrence of lines. Despite the fact that such binary readings are difficult to evaluate statistically for a validation, these tests seem to have their benefits when operating outside a laboratory. Huang *et al.* (2015) as well as Campone *et al.* (2016) report LC-MS methods for the determination of AFM₁ in milk. With respect to the level of interest for legislative enforcement both author groups focus on an effective clean-up and extract concentration. While Huang *et al.* (2016) used a hollow-fibre micro-extraction, Campone *et al.* (2016) applied salt induced liquid-liquid separation of ACN containing solvents. While the hollow-fibre approach appears to be a good step towards miniaturisation, it also seems to be still at an early stage of being fit-for-purpose for routine applications. Despite the fact that authors make a number of claims, the question remains to what alternatives the claims compare to. The salt induced liquid-liquid separation was tested for a number of liquid dairy products and authors made extensive efforts to demonstrate the influence of operation parameters. The obtained precision and trueness parameters met generally accepted criteria for method performance and were demonstrated for a number of different matrices in the range of 10-100 ng/kg. Yet another focus on the determination of AFM₁ was made by Pietri *et al.* (2016) who focused on the improvement of methods for the analysis of cheese. Authors compared a number of different extraction methods, especially for ripe hard cheese and came to the conclusion that an enzyme-assisted extraction is a good alternative to other established methods. Despite the fact that the digestion takes 16 h this should not be a disqualifier as, unlike the early-stage-of-production product milk, ripened cheese does not require immediate processing. A rather unusual approach for the determination of AFM₁ (and AFB₁) was made by Tessmer Scaglioni *et al.* (2016), who proposed rice-husk as an adsorbent for extract clean-up. While unusual, this approach is innovative as well. The method still has room for further improvement in the use of the organic solvent. Chloroform, an in many laboratories unwanted solvent for routine processes, was still employed,

which has been shown to be replaceable in some cases with less controversial mixtures of higher alcohols with t-butylmethylether.

In the field of vibrational spectroscopy Lee *et al.* (2015) compared Raman, Fourier transform (FT) near infrared (NIR) and FT infrared (FTIR) spectroscopy as tools to predict AFB₁ levels in maize in their laboratory. On the basis of measurements of 230 Texan maize samples over a contamination range from not detectable to 1,206 µg/kg, they aimed for suitable methods to classify samples based on their AFB₁ content. Authors investigated next to the spectroscopic technique also different evaluation methods and models and concluded that further improvement of these techniques has the potential to provide a fast and powerful tool for real-time monitoring of AFB₁. Wang *et al.* (2015) discussed the use of hyperspatial imaging to predict AFB₁ levels and distribution in whole single kernels of different maize. Authors discussed spectroscopy data beyond the scope of this review. In summary, the method allowed authors predicting the spacial AFB₁ distribution at single kernel level. In addition it allowed a classification of kernels contaminated <10 µg/kg from those contaminated above 100 µg/kg similarly to the results cited for sequential single measurements with transmittance and reflectance spectroscopy.

6. *Alternaria* toxins

Several methods were published during the last year for single or multiple determination of free and modified/conjugated *Alternaria* toxins in different types of foods as well as in human urine and animal plasma samples. Most of the methods were based on LC-MS/MS using different approaches for clean-up; one method was based on HPLC diode array detection (DAD) using countercurrent chromatography for clean-up and another method was based on HPLC-FLD using a MIP column for clean-up.

Hickert *et al.* (2016) developed a rapid HPLC-MS/MS method for the simultaneous determination of alternariol (AOH), tenuazonic acid (TeA), alternariol monomethyl ether (AME), altenuene (ALT), isoaltenuene (isoALT), tentoxin (TTX), altertoxin I (ALT-X-I), *Alternaria alternata* f. sp. *lycopersici* toxins (AAL-TA₁, AAL-TA₂) in commercial tomato products, fruit and vegetable juices, bakery products, vegetable oils and sunflowers seeds. Matrix matched calibration was used to quantitate the analytes. Acceptable recoveries were obtained for most toxins with the exception of AME in bakery products (49%), TeA in sunflower seeds (44%), AAL-TA₁ and AAL-TA₂ and AOH in vegetable oils (>120%). Commercial analytical standard solutions of TeA can be a racemic mixture of TeA and *allo*-TeA, if it is obtained by chemical synthesis. Moreover, TeA epimerises to a mixture of TeA and *allo*-TeA, when treated with bases and under acidic conditions. The spiked

sample extracts of sunflower seeds showed two partially separated broad and tailing peaks, presumably TeA and *allo*-TeA, when analysed by LC-MS/MS probably because the standard solution used for the spiking experiments contained both isomers. Naturally contaminated samples showed only one peak, which suggest that only one isomer occurs naturally in sunflower seeds.

Walravens *et al.* (2016) developed an LC-MS/MS method for the determination of free and modified/conjugated *Alternaria* toxins (AOH, AME, TeA, TTX, ALT, ALT-X-I, sulphates and glucosides of AOH and AME) in commercial tomato products and fruit and vegetable juices. Some of the standards (AOH, AME, ALT, TTX and TeA) were commercially available or provided by other research institutions (ALT-X-I, AOH-3-glucoside, AME-3-glucoside, AOH-3-sulphate and AME-3-sulphate, [²H₄]-AME and [¹³C₆,¹⁵N]-TeA). [²H₄]-AME was used as internal standard of AME and [¹³C₆,¹⁵N]-TeA was used as internal standard of AOH, ALT, TTX, TeA, ALT-X-I, AOH-3-glucoside, AME-3-glucoside, AOH-3-sulphate and AME-3-sulphate. This study reported the novel detection of AOH-3-sulphate and AME-3-sulphate in 26% and 78% of samples of tomato concentrate at levels up to 8.7 and 9.9 µg/kg, respectively. Zwickel *et al.* (2016) developed a multi-analyte LC-MS/MS method for the determination of AOH, AME, TeA, TTX, ALT, isoALT, ALT-X-I, ALT-X-II, altenuisol (ATL), altenuic acid III (AA-III) and AAL-TB₁ and AAL-TB₂ in wine, vegetable juices and fruit juices. The liquid samples were diluted with 10% NaHCO₃ aqueous solution (pH 8) and 1 ml was purified by Extrelut[®] SPE, consisting of diatomaceous earth. The non-acidic compounds were eluted from the column by passing ethyl acetate, while TeA, AA-III and AAL toxins were successively eluted by passing 10% formic acid in ethyl acetate. The two eluates were either combined before drying under nitrogen at 40 °C, or dried separately, re-dissolved in appropriate solvents and analysed by LC-MS/MS. Different HPLC conditions (mobile phase composition, pH, and modifiers) and columns were tested and optimised to obtain suitable chromatographic retention and optimal ionisation efficiency with different ionisation techniques (atmospheric pressure chemical ionisation (APCI), atmospheric pressure photoionisation (APPI) and ESI), while avoiding derivatisation steps especially for TeA and AA-III. Weak ion suppression (>70% of signal recovered) was observed for AME, ALT, AOH, TTX, isoALT, AAL toxins and TeA. Stronger ion suppression was observed for AOH-3-glucoside, AME, AME-3-glucoside and ALT-X-I (<25-34% of signal recovered) in some of the tested juices. The type of calibration (external or using internal standard method with labelled standards) used to quantitate each single toxin was not clear. The method was used for a survey of the German market. Red wines and grape juices were most frequently contaminated with multiple *Alternaria* toxins, albeit at levels generally lower

than those reported in the literature for tomato-based products.

Within the *Alternaria* toxins, TeA occurs in foods at the highest levels and frequencies. The Bavarian Health and Food Safety Authority stipulated a warning limit of TeA (500 µg/kg) in sorghum-based/millet baby foods because infants consuming these products are likely to exceed the threshold of toxicological concern (TTC) of TeA (1,500 ng/kg body weight per day) established by EFSA in 2011 (Asam and Rychlik, 2015). Hickert *et al.* (2015) developed a method for the determination of TeA and *allo*-TeA in tomato products with the objective to chromatographically separate the two analytes. The synthetic racemic mixture of both compounds was separated by preparative HPLC and pure reference standards of TeA and *allo*-TeA were obtained and used to test separately their cytotoxicity and to demonstrate that during acidic extraction, purification and chromatography no epimerisation of TeA into *allo*-TeA or vice versa occurred. In the 20 tomato products analysed, both isomers were detected and the percentage of *allo*-TeA ranged between 7-44%. However, in another study the same authors found only one isomer (TeA) in the 11 samples of naturally contaminated sunflower seeds (Hickert *et al.*, 2016).

Two new methods became available using HPLC with UV or FLD for the determination of *Alternaria* toxins (Abou-Hany *et al.*, 2015; Fan *et al.*, 2016). Fan *et al.* (2016) developed a LC-UV method for AOH, AME and TeA determination in wine and apple juice, which is based on countercurrent chromatography for enrichment and clean-up. This technique is based on the separation of compounds between two immiscible liquid phases as they interact in a thin tube under a centrifugal force. Ethyl acetate and water containing an ionic liquid were used as phases for the enrichment of toxins from wine and apple juice. Basically, ionic liquids are salts which are liquid (at temperatures below 100 °C) without being dissolved in water. The good values for LODs of 0.03-0.14 µg/l make the method suitable for the analysis of naturally contaminated samples. Abou-Hany *et al.* (2015) synthesised MIP microspheres for selective binding of AOH. Four surrogate templates of AOH were synthesised to carry out the polymerisation in multi-gram scale. Home-packed MIP columns were prepared and used to clean-up extracts of spiked (33-110 µg/kg) tomatoes that were analysed by HPLC-FLD. Good recoveries (81-103%) and relative standard deviation for repeatability (RSD_r) values (1-4%) were obtained but the values of LOD and limit of quantification (LOQ) and the applicability of the method to naturally contaminated tomatoes were not reported.

The presence of *Alternaria* toxins in human urine has been recently reported for few samples. For human exposure studies, sensitive methods are necessary to measure the low toxin concentrations usually occurring in urine, especially

in those of people living in the developed world. Hövelmann *et al.* (2016) developed a sensitive LC-MS³ method (LOD 0.04 ng/ml, LOQ 0.11 ng/ml) for the determination of TeA and *allo*-TeA in urine of German people. Acidified urine (4 ml) was spiked with an equimolar mixture of internal standards (¹³C₂-TeA and ¹³C₂-*allo*-TeA) and purified with a polystyrene-divinylbenzene column. The application of a second fragmentation step (MRM³) and the extension of the linear ion trap fill time to 200 ms reduced background signals and increased 3 times the signal-to-noise of TeA and *allo*-TeA. The two isomers were partially separated by using an appropriate HPLC column with acidic mobile phases and individually identified and quantitated in urine extracts. The presence of conjugated forms of TeA and *allo*-TeA in urine was not investigated and urine samples were not hydrolysed with β-glucuronidase/sulphatase before sample clean-up. All of the 48 urines were positive for TeA/*allo*-TeA in the range of 0.16-44.4 ng/ml of TeA and 0.11-5.72 ng/ml of *allo*-TeA and 96-98% of the samples contained TeA and *allo*-TeA at concentrations above the LOQ. Based on the human excretion rate of TeA (90%) the probable daily intake (PDI) of both isomers was calculated (mean 0.21 µg/kg body weight). For one individual the value of the PDI (1.58 µg/kg body weight) slightly exceeded the assumed TTC (1.5 µg/kg body weight per day). Finally, an LC-MS/MS method was developed for the determination of TeA in pig and chicken plasma (Fraeyman *et al.*, 2015). Plasma samples were spiked with internal standard ([¹³C,¹⁵N]-TeA), submitted to protein precipitation by adding ACN, mixed, centrifuged and the supernatant was dried, re-constituted in an appropriate solvent mixture and analysed by LC-MS/MS. The LOD (5 ng/ml), precision (RSD_r 4-15%) and accuracy (<9%) were adequate to measure plasma levels in animals administered with TeA orally or intravenously at 50 µg/kg body weight (single dose). No matrix effects were observed for chicken plasma, whereas a signal suppression (38% of signal recovered) was observed for pig plasma.

7. Ergot alkaloids

In the last year the trend to use LC-MS/MS for the determination of ergot alkaloids continued, but there were also publications highlighting the use of high-performance thin-layer chromatography (HPTLC) to screen for total ergot alkaloids and the use of LC-HRMS for both target analysis and retrospective screening.

Tittlemier *et al.* (2015) developed a method to analyse 10 ergot alkaloids in cereal grains. The method was based on the method of Krska *et al.* (2008), however, only included 10 compounds compared to the twelve in the original paper due to lack of available analytical standards. Analytes included both '-ine' and '-inine' type ergot alkaloids. The method was validated by use of fortified samples, despite a lack of true blank samples. Adaptations to the original method were the use of dihydroergotamine as an internal

standard and not including dSPE material before filtration. In-house validation of the method showed it performed with good accuracy and precision, the overall mean recovery \pm standard deviation for all ergot alkaloids over all fortification levels and days was $83\pm 12\%$ for wheat and $88\pm 15\%$ for rye. Minor enhancement due to matrix effects was present during LC-MS/MS analysis, but this was mitigated by the use of the internal standard (dihydroergotamine). The LOQs were somewhat high compared to other published methods at $2\ \mu\text{g}/\text{kg}$ for ergonovine (ergometrine) and $10\ \mu\text{g}/\text{kg}$ for the other ergot alkaloids, although the definition for LOQ was quite strict at signal-to-noise of at least 9:1 and $\text{RSD} < 20\%$. The method was used to survey wheat harvested in 2011, when ergot-infection was particularly widespread in western Canada. A strong linear relationship between the concentration of ergot alkaloids and the presence of ergot sclerotia was observed. Mulder *et al.* (2015) presented an LC-MS/MS method for ergot and tropane alkaloids in grain-based products for infants. In contrast to the previous method this used an acidified extraction solution, followed by ultrafiltration over a 30 kD ultrafilter. Alkaline mobile phase, using 6.5 mM ammonium hydroxide was used for chromatography allowing tropane and ergot alkaloids to be analysed in one LC run. In house validation of the method was performed, LODs were assessed using 14 different blank cereal samples. LODs (using S/N ratio =3) were reported to be between 0.1 and 0.5 $\mu\text{g}/\text{kg}$ for the ergot alkaloids. Extraction efficiency for ergot alkaloids ranged between 68 and 91% with RSD between 7 and 14%. However the number of replicates measured to determine these values was not reported. This method was used to simultaneously quantify ergot and tropane alkaloids in 113 cereal-based foods for infants and young children. Mean ergot alkaloid levels in the three sampling years were 11, 6.2 and 8.6 $\mu\text{g}/\text{kg}$, respectively (maximum: 115 $\mu\text{g}/\text{kg}$).

Jarmusch *et al.* (2016) investigated for the first time the application of APPI as an ionisation method for LC-MS/MS analysis of the ergot alkaloids ergonovine (ergometrine) and lysergic acid amide (ergine), and compared its performance to ESI. Samples of the grass *Achnatherum robustum* infected with the ergot producing *Epichloe* fungus were extracted using cold methanol and subjected to reversed-phase HPLC-ESI-MS and HPLC-APPI-MS analysis in selected reaction monitoring (SRM) mode. To allow comparison between the APPI- and the ESI- method the validation parameters were measured according to International Committee Harmonization guidelines. The performance of both methods was comparable, although APPI gave slightly better intermediate precision and accuracy (lower residuals). The LODs were 0.7 and 1.3 $\mu\text{g}/\text{kg}$, and 1.5 and 1.4 $\mu\text{g}/\text{kg}$ for lysergic acid amine and ergonovine using APPI-SRM and ESI-SRM respectively. Both methods showed very little matrix interference, with recoveries ranging from 82 to 100%. Intermediate precision ($n=9$, performed on 3 analyses on three days) ranged from 2 to

12% for HPLC-APPI-MS and 1.6 to 19% for LC-ESI-MS. There was no statistically significant difference between the concentrations of ergonovine and lysergic acid amide determined using ESI-MS and APPI-MS for the same samples. It seems apparent based on this data that APPI could also be used as an ionisation method for the other ergot alkaloids commonly analysed for in cereals.

The use of more advanced instrumentation, UHPLC-HRMS, for the target analysis and screening of a range of compounds was reported by Leon *et al.* (2016). A quantitative method for 77 banned veterinary drugs, mycotoxins and plant toxins, that also allowed post-target screening for 425 substances including pesticides and environmental contaminants in feed was developed. The method used QuEChERS-based extraction and the ergot alkaloids included in the method were ergosine, ergocornine, ergocryptine and ergocryptine and their 'inine' epimers. Method validation, including extensive matrix effect evaluation was carried out. Validation was performed using a protocol based on validation procedures from several guidelines, such as SANCO/12571/2013 (EC, 2013) and Commission Decision 2002/657/EC (EC, 2002). For each target substance a performance limit was established. Linearity, trueness/recoveries, precision and LOQ were also assessed. The LOQ was 20 $\mu\text{g}/\text{kg}$ and the dynamic range was 20-80 $\mu\text{g}/\text{kg}$ for all the ergot alkaloids in the method. Recoveries were 83-106% for all ergot alkaloids across two levels (LOQ and $2\times$ LOQ) with precision in terms of RSD from 8 to 23%. Full-scan accurate mass data were acquired with a resolving power of 50,000 FWHM and a mass accuracy lower than 5 ppm. The method was applied to feed samples, ergot alkaloids were detected, however they were either below LOQ or did not meet confirmation criteria. This work showed the high potential of the high resolving power of the LC-HRMS, the authors commented that it is a promising tool for control of residues and contaminants in feed. However the higher LOQ values found would mean it would not be suitable for food controls without further improvements. LC-Q-TOF-MS was also used in a study that compared three extraction techniques, ultrasound-assisted extraction in bath (UAE-B), or with sonotrode and microwave-assisted extraction (Nowak *et al.*, 2016). The method allowed determination of ergine (lysergic acid amide) and ergometrine as well as identification of other alkaloids. Only UAE-B proved suitable for ergot extraction, the method was optimised in terms of temperature, time and solvent composition. Recovery and matrix effect, repeatability, and intermediate precision were established during validation. Recovery ranged from 91 to 109% across three spiking levels for both compounds, with repeatability ranging from 3.7-8.4%. Limits of detection and quantification were 1.0 and 3.0 ng/ml, respectively, and were sufficient for determination of ergot alkaloids in *Ipomoea* seeds. The fact that the papers above reported either LODs and/or LOQs to discuss

method sensitivity, and that different criteria were used to assess them makes it difficult to make direct comparisons between the methods at least in terms of sensitivity.

A fast and easy screening method for the determination of the total ergot alkaloid content was developed using planar solid phase extraction (pSPE), otherwise known as HPTLC (Oellig and Melde, 2016). The pSPE was used to concentrate all ergot alkaloids in a target zone followed by detection as the sum by FLD. The method used ammonium acetate buffer for extraction, followed by a fast liquid-liquid partitioning pre-cleaning before pSPE was performed on HPTLC amino plates. The plates were developed with a single methanol development step to a migration distance of 50 mm after a pre-conditioning time of 15 min. This allowed the separation of the ergot alkaloids from the remaining matrix, and ensured all ergot alkaloids were focused in a single target analytes zone. For quantitation, the native fluorescence was used after dipping the plate in n-hexane/paraffin oil (low viscosity) solution for fluorescence enhancement. LOD and LOQ were 70 and 240 µg/kg in rye, respectively, expressed as ergocristine. Recoveries were 94 to 111% using spiking levels from 500 to 1,500 µg/kg for different rye flour samples. This method was efficient and reliable for screening for the total ergot alkaloid content in rye and offers a rapid alternative to the HPLC determination of individual alkaloids and their sum. A novel aspect of this method was the use of MS to analyse the target analyte zone using a TLC-MS interface. That way the presence of ergometrine, ergosine, ergocornine, ergocryptine, ergotamine and ergocristine was confirmed in a *Secale cornutum* sample. Identification was possible down to ~150 µg/kg in rye flour when a single alkaloid was present (ergocristine), or ~500 µg/kg for a mixture of ergot alkaloids expressed as ergocristine. Although the sensitivity of the MS detection was better than FLD the LOD and LOQ may still be somewhat high for the method to have a practical use for control or monitoring of ergot alkaloids in foods within Europe if the maximum permitted concentrations currently under discussion are introduced into EU regulations. However, the authors claim that the method would be applicable for monitoring and control at or below 1 mg/kg, the current quality criterion limit used as a guidance level in Germany for cereals, flour and cereal based foods.

8. Fumonisin

During the past year, progress was made in methods for fumonisin analysis and technologies for predicting fumonisin levels. Increasingly, FB₁, FB₂ and FB₃ have been incorporated into multi-analyte testing. The application of LC-MS methods, including LC-MS/MS and LC-HRMS, to the detection of fumonisins in foods was recently reviewed (Lucci *et al.*, 2015). From previous reports it is clear that many of the multi-mycotoxin MS methods do

not recover fumonisins as well as they do for many of the other mycotoxins. The type of extraction and extent of the clean-up have significant effects on ion suppression or enhancement. To reduce matrix effects, a novel solvent was investigated for extraction of FB₁, FB₂, DON and ZEA (García-Fonseca and Rubio, 2016). A supramolecular solvent was based upon oleic acid, aqueous hydrochloric acid, tetrahydrofuran, and acetic acid. Macromolecules were excluded from the extracts by a combination of both physical and chemical means. Milled samples were extracted with the solvent and centrifuged. The supernatant solution was diluted with the solvent and then subjected to LC-MS. For FB₁ and FB₂ the LODs for the method were 8 µg/kg and the LOQs were 25 µg/kg. Recoveries of FB₁ and FB₂ from Durum wheat, bread, and maize ranged from 87 to 105% with RSDs between 1 and 8%. The slopes of calibration curves from spiked wheat and maize extracted with the supramolecular solvent were not significantly different from those obtained in the solvent itself, suggesting the solvent successfully reduced matrix interferences. Extraction using QuEChERS-type procedures have previously been applied to fumonisins in various commodities. Recently, the technique, in combination with mixed-mode SPE was used to isolate FB₂, FB₄, FB₆, and OTA from green, roasted, or instant coffee (Nielsen *et al.*, 2015). Using an isotopic internal standard, the LOD of the method was 2 µg/kg. Interestingly, incorporating isopropanol into the gradient of the LC mobile phase increased the signal intensity of FB₂ by 55 to 75% for green coffee, 54 to 83% for instant coffee and 57 to 74% for roasted coffee. This suggests efforts to reduce ion suppression need not end with the sample extraction, but can continue to the chromatographic stages as well. For MS analysis, aqueous mixtures of ACN or methanol continue to predominate for fumonisin extraction. Recently a method was developed for FB₁ and FB₂ in maize that used two extractions: the first with 80% ACN/water and the second with 80% methanol/water (Vega, 2016). Fumonisin were isolated by immunoaffinity column and quantified by LC-MS. Using an external calibration of standards in solvent, recoveries from certified reference materials ranged from 70 to 110%, suggesting that the dual solvent extraction and clean-up were able to remove most matrix effects.

The discovery of new fumonisins and their metabolites has continued to be aided by MS. Several recent reports described methods for known, but uncommon, fumonisins or the discovery of new fumonisins or their metabolites. A HRMS method in combination with a QuEChERS-type clean-up was used to identify the *N*-acetylated analogs of FB₁, FB₂ and FB₃ (i.e. FA₁, FA₂, and FA₃) in maize samples (Tamura *et al.*, 2015a). The FA analogs were found in 10 maize samples that were contaminated with fumonisins. Mammals can metabolise fumonisins, and rats are known to *N*-acylate hydrolysed FB₁ (HFB₁ or AP₁) with fatty acids of various chain lengths to products collectively known as *N*-acyl-HFB₁. Recently it was demonstrated that rats also

N-acylate intact FB₁. The levels of a series of *N*-acylated derivatives in liver were similar to the levels of FB₁ in the same tissue (Harrer *et al.*, 2015). Analogs of the fumonisins continue to be discovered. Renaud *et al.* (2015) applied a semi-targeted approach to analyse fumonisins, AAL toxins, and related compounds in inoculated maize, tomatoes, and grapes. The method used product ion filtering in the negative mode to focus on detection of compounds containing the tricarballic (TCA) ester moiety common to both groups of toxins. During the chromatography, rapid polarity switching was used to collect MS/MS data on the suspect compounds in the positive mode. Perhaps not surprisingly, FB₁, FB₂, FB₃ and FB₄ were detected on maize, while FB₂, FB₄ and FB₆ were detected on grapes. However, interestingly, over 100 structurally related compounds (having the TCA ester) were also detected. The compounds included a hydroxyl-FB₁ and non-aminated fumonisins, found in maize and grapes respectively. Previously FB₁ has been shown to undergo a non-enzymatic reaction with glucose to yield an *N*-(1-deoxy-D-fructos-1-yl) derivative (i.e. NDF-FB₁). Products analogous to NDF-FB₁, but formed from FB₂ and FB₃ (NDF-FB₂ and NDF-FB₃) were identified in a sample of maize powder using HRMS (Matsuo *et al.*, 2015), further adding to the number of potential fumonisin analogs that may be present in maize.

Screening assays for fumonisins rely upon components that specifically recognise the toxins. In formats like ELISA, the recognition elements are antibodies. Immunoassays are often based upon competition between a labelled and unlabelled toxin for binding. Recent efforts have focused on improving both of these aspects: the recognition element and the labelled toxin. In previous reports we have described the progress in improving recognition elements, which include various types of antibodies and aptamers. For a number of years anti-idiotypic antibodies and mimotopes have also been developed. Both essentially mimic some attributes of the fumonisins by binding to a toxin-specific recognition element. Recently, Shu *et al.* (2016) developed a fusion protein consisting of an anti-idiotypic nanobody linked to alkaline phosphatase. Such a construct is analogous to the toxin-enzyme conjugates used in traditional competitive direct ELISAs. A chemiluminescence ELISA based upon the reagent demonstrated an LOD of 0.12 ng FB₁/ml, with a linear range of 0.29 to 2.68 ng/ml, indicating the high sensitivities possible and the potential for replacing toxin-enzyme conjugates with fusion proteins.

Fluorescence polarisation immunoassay (FPIA) is a technique that indirectly measures the rate of tumbling motion of a fluorescent molecule in solution. In competitive FPIA a tracer composed of a mycotoxin labelled with a fluorophore competes with unlabelled toxin for binding to an antibody. Binding slows the tumbling of the tracer, which is detected as an increase in polarisation. Among mycotoxin immunoassays the technique is relatively novel

because it has a homogeneous format (i.e. does not require separation of assay components). FPIA has generally been limited to single analyte quantification. This constraint has derived from the limits of the instruments used, which detect polarisation of fluorescence over a limited bandwidth. Recently Li *et al.* (2016) introduced assays to detect FB₁, DON, and T-2 toxin simultaneously. The technique uses tracers with three distinct emission maxima and an instrument capable of rapidly measuring at each of the three wavelengths. The time of analyses, including sample extraction was less than 30 min. Detectable ranges (IC₂₀ to IC₈₀) were 447 to 3,780 µg/kg (DON), 74 to 7,014 µg/kg (T-2 toxin) and 587 to 6,265 µg/kg (FB₁). While the ranges encompass many of the common regulatory levels for these toxins, the technique was not as sensitive as many of the existing immunoassays developed for the individual toxins. For 20 naturally contaminated maize samples, the correlation between the multiplexed FPIA for FB₁ and an LC-MS/MS method was good ($r^2=0.99$). With multiplexed immunoassays there is the possibility for non-specific binding of the tracer to other non-target antibodies used in the assay. The authors developed a mathematical treatment to measure such effects, a nonspecific factor (F). The F value for the FB₁ antibody with the T-2 tracer was 0.2, while for the other (non-matching) pairs the values were less than 0.1. The authors suggested that this had little impact on the assay. Results indicate the method can be successfully applied to the detection of FB₁ in maize and the technique is a significant step towards multiplexing of FPIA.

An advantage of homogeneous assays is that there is no need to separate 'bound' and 'unbound' labels. One mechanism for avoiding the separation step is to measure the binding of the toxin to the recognition element, as was done recently with a label-free electrochemical immunosensor for FB₁ (Masikini *et al.*, 2016). The device was based upon the deposition of a polymer onto a glassy carbon electrode. In this case the polymer was a poly(2,5-dimethoxyaniline)-multi-walled carbon nanotube composite. The surface was further modified with fumonisin antibodies in order to capture the fumonisins for detection by electrochemical impedance spectroscopy. The charge transfer resistance increased with increasing FB₁ concentration, with a linear range of 7 to 49 pg/ml. Further exploration of the effects of various food matrices upon the performance of this sensor, as well as spiking and recovery experiments with maize, are warranted.

Immuno-chromatographic devices, also known as lateral flow immunoassays (LFIA), have been used to screen for fumonisins in commodities for a number of years, and many types are commercially available. Two approaches for improving such devices have been incorporating additional toxins and improving the signal from the gold nanoparticles commonly used in such assays. The use of labels that produce a chemiluminescent signal is one way to improve

sensitivity. A combined LFIA using chemiluminescence detection based on a CCD camera, was developed for AFB₁ and FB₁/FB₂ in maize flour (Zangheri *et al.*, 2015). The LOD for FB₁ was 6 µg/kg, with a dynamic range in maize flour of 6 to 1,500 µg/kg. Cross-reactivity of the fumonisin antibody towards FB₂ was reported to be 97%. Assays took approximately 30 min. Another approach to improving sensitivity involves using gold particles with different properties than the spherical colloidal gold that is typically used. Gold particles with 'urchin-like' surface features were compared to colloidal gold as labels in a fumonisin LFIA (Ren *et al.*, 2015). The label, which has a blue colour as opposed to the red colour typical of most colloidal gold LFDs, was used to develop an LFIA having a cut-off value (essentially complete inhibition of colour development at the test line) of 5 ng/ml, equivalent to 1000 µg/kg in grain. This was a 4-fold improvement over a similar device constructed with a colloidal gold label, suggesting modification of the properties of the gold label commonly used in such devices may yield further improvements in sensitivity.

As with previous years much of the literature has focused on detection of the fumonisins themselves. However, technologies for predicting levels of fumonisins based upon physical and chemical characteristics of the sample continue to be explored. Such technologies are of significant interest for screening purposes. An approach using NIR spectroscopy was used to predict fungal and fumonisin contamination in maize (Levasseur-Garcia *et al.*, 2015). Results were compared to a reference LC-FLD method. The study sought to establish whether a threshold exists for fungal contamination which can be used to classify samples according to the risk of fumonisin contamination. All of the samples with fumonisin levels above 4,000 µg/kg had a fungal count that exceeded 200,000 cfu/g, which was the threshold used in the NIR screen. This suggests NIR may be useful for assessing fungal contamination and, by extension, high levels of fumonisin contamination. Another screening technology is surface enhanced Raman spectroscopy (SERS). Recently a chemometric classification model based upon SERS was developed and evaluated for prediction of fumonisin contamination in maize (Lee and Herrman, 2016). Methanolic extracts of ground maize were mixed with silver dendrites and subjected to SERS analysis. Spectra were used to develop several classification and quantification models. The authors noted that the chemometric models exhibited moderately acceptable correct classification rates with little or no false-negative errors. Multiple linear regression models for quantification demonstrated good predictive power, with the slope of regression lines from 0.90 to 1.08 and high correlation coefficients (*r* from 0.95 to 0.97) and predictive accuracy (*r*² from 0.90 to 0.94). Although an extraction is still required, the potential for SERS as a rapid screening

tool for predicting the fumonisin content of maize was demonstrated.

9. Ochratoxins

As a general trend, the control of mycotoxins is moving more and more towards multi-mycotoxin analytical methods able to detect co-occurrence of the compounds of interest in the same food matrix. As OTA can co-occur with aflatoxins and CIT, the development and validation of analytical methods able to determine these three mycotoxins is increasing.

LC-MS/MS is the favourite technique used for control purposes. Regarding the latter, OTA is mostly measured with ESI+ using the protonated precursor ion of *m/z* 404 and product ions of *m/z* 358 and *m/z* 341. The above is consistent with the analytical methods used in proficiency testing schemes organised by FAPAS[®], where HPLC-FLD accounted for 79% and LC-MS/MS for 21% of all methods (FAPAS, 2015). A possible explanation of the preference of LC-FLD in this case is that LC-MS/MS is used mainly when multi-mycotoxin analysis is considered. Also in the year under consideration, the extraction phases were usually aqueous solutions of methanol or ACN in almost the same extent. A clean-up step was used in all cases, typically IAC. Seventy-five percent of the participants used accredited methods. As food matrices, the most recurring were roasted coffee, beer, red wine, dried vine fruits, cereal flour, cocoa and paprika. The performance of a LC quadrupole time-of-flight MS method for OTA determination in wine was evaluated by Rodriguez-Cabo *et al.* (2016). Under final conditions, wine samples (20 ml) were concentrated using a reversed-phase SPE cartridge, followed by OTA elution with ethyl acetate. The recoveries of the method were 91-121% and 90-113% (without and with internal standard correction, respectively), for wine samples fortified at three concentration levels. Both the precision (RSD_r < 12%) and the LOQ (0.05 µg/l) of the method were very good.

Moving to other detection techniques, the general trend to develop and improve innovative applications for different types of biosensors and aptamers is confirmed. Pagkaly *et al.* (2016) developed an optical biosensor for detection of OTA in beer samples. The biosensor consists of an array of ten Mach-Zehnder interferometers (MZIs) monolithically integrated along with their respective broad-band silicon light sources on the same chip. The MZIs sensing arms were functionalised with an OTA-ovalbumin conjugate. The chip was exposed to samples with an anti-OTA antibody, following a competitive immunoassay format. An external miniaturised spectrometer was employed to record the transmission spectra of each MZI continuously. The spectral shifts due to the immunoreaction were transformed to phase shifts through discrete Fourier transformation. The assay had a detection limit of 2.0 ng/ml and a dynamic

range 4.0-100 ng/ml in beer samples. Recoveries ranged from 91-116% and intra- and inter-assay coefficients of variation were 9 and 14%, respectively. A new optical aptasensor was introduced by Xu *et al.* (2016b), based on the side-by-side assembly of gold nanorods (GNRs) for the one-step determination of OTA. A thiol-modified DNA aptamer containing aptamer sequences against OTA hybridised with the DNA was decorated on the side sites of GNRs acting as probes. In presence of OTA, the GNRs disperse as a result of specific aptamer-OTA recognition and conformational changes in the aptamer. The resulting changes in the absorption spectra of the GNRs are used for sensing. A linear correlation was demonstrated between the absorbance of the GNRs at 708 nm and the concentration of OTA over the range 0.5-20 ng/ml, while the LOD was 0.22 ng/ml in buffer. The method was successfully applied for the detection of OTA in fortified red wine samples. Among the most interesting and innovative electrochemical aptasensors, Catanante *et al.* (2016) presented a type using methylene blue tagged anti-OTA aptamers. The best sensitivity was recorded by oxidation of amines using hexamethylenediamine on a screen-printed carbon electrode. Changes in the conformation/flexibility of the aptamer probe are recorded electrochemically. A dynamic range of 0.01-5 ng/ml was obtained for OTA with a LOD and a LOQ of 0.01 ng/ml and 0.03 ng/ml, respectively. The repeatability of the assay was about 4%. The developed aptasensor was used to screen cocoa samples for OTA contamination after MIP clean-up and showed recovery values of 84-85% for this matrix. After clean-up and dilution the assay was sensitive enough to quantify concentrations down to 2 µg/kg.

Taghdisi *et al.* (2016) developed a fluorescent aptasensor for the selective and sensitive detection of OTA, based on a complementary strand of aptamer and gold or silica nanoparticles coated with streptavidin. In the presence of OTA, the fluorophore and biotin-labelled complementary strand-silica nanoparticles coated with streptavidin conjugate is formed, thus resulting in a very strong fluorescence emission. The designed fluorescent aptasensor exhibits high selectivity toward OTA with a LOD of 0.1 nM (40 ng/l). Furthermore, the fabricated aptasensor was successfully applied for the detection of OTA in grape juice and serum with LODs of 44-60 ng/l. Lv *et al.* (2016) reported a novel fluorescence biosensing strategy for simple and sensitive OTA detection using an aptamer specific for OTA as recognition element and single-walled carbon nanohorns as fluorescence quenchers. Without OTA, the fluorophore labelled aptamer and the quencher are in close proximity, thus prohibiting emission. In the presence of the toxin, the OTA-specific aptamer forms an antiparallel G-quadruplex, which is resistant to being wrapped onto single-walled carbon nanohorns. The resulting fluorescent intensity as a function of OTA concentration was correspondingly measured. A detection

limit of 17.2 nM (7 µg/l) for the sensing platform with a linear detection range of 8-200 µg/l was obtained, which is far higher than the previous method. The biosensor was verified by testing red wine, diluted 100 fold with buffer solution spiked with a series of OTA concentrations. While recoveries (93-105%) and repeatabilities (2-5%) were good, this assay is just not sensitive enough (without further enrichment and clean-up) to measure naturally occurring levels of OTA in food. Sharma *et al.* (2015) demonstrated the development of a TiO₂ nanoparticle quenching based aptasensing platform for detection of OTA. When the toxin interacts with the aptamer, it induces aptamer G-quadruplex complex formation and weakens the interaction between the fluorescein-labelled aptamer and TiO₂, resulting in fluorescence recovery. At optimised experimental condition, the obtained LOD was 1.5 nM (0.6 µg/l) with a good linearity in a range of almost three orders of magnitude. The developed aptamer assay was evaluated for detection of OTA in beer samples at a rather high concentration of 100 µg/l (0.25 µM). Recoveries of 94-99% and RSD_r values of <4% were achieved. Liu *et al.* (2015) reported the development and performance of an evanescent wave optical aptasensor with a reversible ligand-grafted biosensing surface for rapid, sensitive and selective detection of OTA in food. In this system, OTA was covalently immobilised onto the surface of an optical fiber using glutaraldehyde and ethylenediamine as spacer. The aptasensor was developed for investigating the binding kinetics between the tethered ligand and free OTA-aptamer, the performance of the aptamer-based bioassay and the reversibility of biosensing surface. Using a competitive detection mode, a working range of 0.7-13 µg/l and a LOD of 0.4 µg/l were achieved. In spiked wheat samples 89-106% of OTA was recovered with RSD_r values <16%. Unfortunately only spiking concentrations in µg/l were given and the (missing) description of the sample preparations allows no calculation of the assay sensitivity in solid food samples.

Marechal and co-workers (2015) coupled an aptamer based miniaturised monolithic affinity preconcentration unit with capillary electrophoresis (CE) and laser induced FLD. A 1.5 cm miniaturised aptamer-based monolithic affinity support was in-line coupled to the end of the capillary used for CE. The preconcentration unit consisted of a fritless affinity monolith bonded with modified oligonucleotide aptamers. Photografting allowed to confine the binding reaction to the desired silica monolithic segment, upstream the empty section of the CE capillary using an UV mask. The optimal conditions to successfully perform the in-line coupling (preconcentration, elution and separation of OTA) with the composite capillary were adjusted depending on individual requirements of each step to ensure compatibility. Under optimised conditions, OTA was successfully preconcentrated and quantified down to 0.1 pg on column. Quantitative recovery of 93% was

achieved in a single elution of 30 pg percolated OTA, while RSDs were <10%. Beer (spiked at 4 µg/l) and wine (spiked at 1 µg/l) samples have been successfully analysed with this device, needing a total analysis time of 30 min per sample.

Malvano *et al.* (2016) developed an impedimetric label-free immunosensor for the detection of OTA. Oriented and non-oriented antibody immobilisation methods were compared. While a lower LOD (5 pg/ml) for the non-oriented immobilisation was obtained, the linear range was also far narrower (only one order of magnitude) in contrast to oriented anti-OTA immunosensors, which showed linearity from 0.01-5 ng/ml. Analysis of the atomic force microscopy images showed two different nanostructures, indicating that the use of oriented immobilisation created a more ordered and dense antibody surface. Finally, the oriented immunosensor was used to quantify OTA in spiked cocoa bean samples and the results were in good comparison with those from a competitive ELISA kit. Cocoa bean samples were ground and extracted with 0.1 M phosphoric acid/CHCl₃ for 15 min. The filtrate was treated with 0.13 M sodium bicarbonate solution and the upper aqueous phase was recovered and centrifuged. This procedure was applied to spiked (1.0, 1.5, 2.5 and 5 µg/kg OTA) cocoa bean samples. 96-109% OTA was recovered with RSD_r values ≤6% for all levels. A competitive fluorescent ELISA for the detection of OTA by using H₂O₂ induced fluorescence quenching of mercaptopropionic acid-modified CdTe quantum dots (QDs) was reported by Huang *et al.* (2016). Catalase was labelled with OTA as a competitive antigen, while the fluorescence signal output of the CdTe QDs is quenched by H₂O₂. A linear detection range of 0.05 ng/l (LOD) to 10 ng/l was obtained, making this assay far (~300 fold) more sensitive to OTA than conventional ELISA tests. Extraction of solid samples (50% aqueous methanol) and dilution with buffer to reduce the content of organic solvent for the assay yield a total dilution factor of 50. The (theoretical) LOD in cereals would be around 2.5 ng/kg, therefore allowing repeated dilutions to minimise matrix interference. From spiked wheat, maize and rice, 86-102% OTA were recovered with RSD_r values ≤18%.

Kim and Lim (2015) developed a chemiluminescence immunoassay with targeted inhibition for the determination of OTA in food. For analyte isolation, (non-specific) amine-functionalised magnetic nanoparticles were synthesised and used to enrich OTA. Then, OTA was tagged with a primary antibody, followed by the immunoreaction with a horseradish peroxidase labelled secondary antibody. The concentration of OTA was determined by measuring chemiluminescence through the catalysed luminol/H₂O₂ system. In spiked rice samples 87-99% OTA were recovered with RSD_r values ≤12%. The obtained LOD of 1.4 ng/l, could not be calculated back to the OTA concentration in rice due to lack of information in the article. Finally, a competitive inhibition immunoassay for the detection of

multiple mycotoxins was developed on a portable imaging surface plasmon resonance (SPR) instrument (Joshi *et al.*, 2016). Actually two 3-plex assays were first developed for the detection of DON, ZEA, T-2, FB₁, AFB₁ and OTA using a benchtop SPR instrument and two biosensor chips and later combined to a single 6-plex assay. Mycotoxin-ovalbumin conjugates were immobilised on the chip via amine coupling. The SPR response was then recorded upon injection of a mixture of antibodies and the sample over the chip with immobilised mycotoxin conjugates. The chips were then regenerated for multiple uses up to 60 cycles. The LODs in barley were determined to be 0.6-26 µg/kg for the different mycotoxins, with 3 µg/kg for OTA for the 3-plex SPR assays, while the 6-plex nanostructured imaging SPR instrument yielded far higher LODs (e.g. 160 µg/kg for OTA), which were still acceptable for several other toxins.

10. Patulin

Some reports of methods for PAT using emerging technology were published from mid-2015 to mid-2016. Marsol-Val *et al.* (2016) described a novel method consisting of injection-port derivatisation coupled to gas chromatography-tandem MS. The method allows quantification of derivatives of 5-hydroxymethylfurfural (HMF) and PAT in apple and pear juices. The optimal conditions for the injection-port derivatisation were determined to be 270 °C, 0.5 min purge-off, and a 1:2 sample:derivatisation reagent. *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide served as the derivatisation reagent. LODs of 0.7 and 1.6 µg/kg and LOQs of 2 and 5 µg/kg for PAT and HMF, respectively, were obtained. Repeatability (RSD_r) was below 12% for both compounds. In addition, the method linearity ranged between 5 and 192 µg/kg for PAT and between 25 and 1000 µg/kg for HMF.

De Clercq *et al.* (2016) developed a LC-UV method for PAT based on the AOAC official method 2000.02 with the following modifications. After the ethyl acetate liquid-liquid partition step, the alkaline clean-up step was replaced with an Oasis HLB® clean-up (Waters, Milford, MA, USA). The method was in-house validated for apple puree agar medium (APAM), cloudy apple juice and apple puree. Average PAT recoveries for APAM at levels of 100, 500, and 1000 µg/kg varied between 95 and 113% over three days, with an interday precision of 5-10%. Recoveries of added PAT in apple juice at 50 µg/kg and apple puree at 10 µg/kg ranged from 80-101% (RSD_r=12%) and 77-100% (RSD_r=9%), respectively. This method offered a LOD of 3-4 µg/kg and a LOQ of 5-8 µg/kg for APAM, apple juice and apple puree. The expanded measurement uncertainty was 46% for both matrices.

Anene *et al.* (2016) reported that a MIP was developed as thin polymer films attached to the surface of a solid support. Such materials were made of Stober silica particles

chemically modified with methacryloyl groups, to which polymethacrylic acid or copolymers of methacrylic and maleic acids were grafted during their radical polymerisation. The polymerisation was carried out in the presence of the mycotoxin and the resulting materials were used as a matrix for separation of PAT. The uptake capacity of PAT by the MIPs was 1.55 mmol/g and the adsorption of PAT on the MIP reached a steady state in only 20 min. The adsorption followed pseudo-second order kinetics. The adsorption isotherm was of a Freundlich type. In addition to clean-up for analytical purposes, such MIPs could potentially be also employed as an adsorbent for the removal of PAT from complex media.

11. Trichothecenes

The vast majority of papers published from mid-2015 to mid-2016 dealing with the determination of trichothecenes reported the use of LC-MS/MS methods. Some studies comprising trichothecenes as a part of multi-mycotoxin methods are discussed in chapter 3 of this article. Only two studies describing analysis of trichothecenes together with other *Fusarium* mycotoxins (Habler and Rychlik, 2016; Tamura *et al.*, 2015b) have been considered here. The common topics of both articles were the applied SPE clean-up followed by separation of (isomeric) mycotoxins.

The article of Tamura *et al.* (2015b) introduced a method of high-resolution liquid chromatography coupled with Q-Orbitrap MS for simultaneous determination of 20 *Fusarium* toxins (NIV, FUS-X, DON, 3-ADON, 15-ADON, HT-2, T-2, neosolaniol (NEO), diacetoxyscirpenol (DAS), fumonisins, ZEA and metabolites) in cereals. In the paper, three fluorophenyl-based columns were examined in order to achieve the effective separation of stereoisomeric and regioisomeric mycotoxins. Electrostatic interactions between fluorine atoms in functional groups of the stationary phase, and hydroxyl groups of the mycotoxins, should assure the separation of 3- and 15-ADONs (and other isomers of fumonisins and ZEA metabolites). The best chromatographic separation with optimal peak shapes was achieved by using the pentafluorophenyl Mastro™ PFP column (Shimadzu GLC, Ltd., Tokyo, Japan). In this case, 3-ADON, where the hydroxyl groups are more proximate to each other, showed stronger electrostatic interaction with the PFP functional groups than 15-ADON. It is worth to notice that besides the chromatographic separation, distinguishing of these two important DON metabolites is also (partly) possible by detecting mycotoxin-specific product ions. However, when using the Q-Orbitrap MS instrument, where the product spectra are usually used for confirmation only, baseline separation of 3- and 15-ADONs was crucial. As regards the sample preparation, QuEChERS extraction, followed by purification on the multifunctional MultiSep® 229 cartridges (Romer Labs, Tulln, Austria), was performed. Acceptable recoveries for all of the

analytes, ranging between 71 and 106%, were achieved. Unfortunately, benefits of the follow-up SPE purification, as compared with simple QuEChERS extraction, as e.g. reduction of matrix effects or decreasing of LOQs, were not discussed. Habler and Rychlik, 2016 developed an LC-MS/MS method to determine trichothecenes (DON, DON-3-glucoside (DON-3G), 3-ADON, 15-ADON, FUS-X, NIV, HT-2 and T-2) together with other *Fusarium* toxins (ENNs, BEA and ZEA) in cereals. A stable isotope dilution quantification approach was validated for DON, 3- and 15-ADONs, HT-2, T-2, ENNs and BEA. All the isotopically labelled standards, with exception of [¹³C]₁₅-DON and [¹³C]₂₂-HT-2, were produced 'in house'. Chromatography focused mainly on baseline separation of DON and DON-3G, which was achieved by a Hydrosphere RP-C18 column (YMC Europe GmbH, Dinslaken, Germany). Authors highlighted the need of chromatographic separation of these two analytes for achieving accurate quantification of DON in ESI+. The 'in source' loss of glucose from DON-3G can occur in ESI+, so the co-elution of these two mycotoxins would lead to the overestimation of DON results. However, as described in many earlier studies (e.g. Vendl *et al.*, 2009; Veprikova *et al.*, 2015), the effective spectral separation of DON and DON-3G could be achieved in ESI-, marginalising the importance of the baseline separation. This possibility was not discussed by the authors group. As concerns 3- and 15-ADONs, they co-eluted under the given LC conditions. For their accurate quantification, different product ions (339.1>213.1 and 231.1 for 3-ADON and 339.1>261.1 and 137.1 for 15-ADON) have been used. SPE purification with multifunctional Bond Elut Mycotoxin cartridges (Agilent Technologies, Santa Clara, CA, USA) was used after the aqueous ACN extraction. The cartridges not containing activated carbon particles assured good recoveries for non-polar mycotoxins (as ZEA, ENNs and BEA). Method accuracy was verified by analysing certified reference materials for DON, HT-2 and T-2 with deviations below 7%. Similarly to the study of Tamura *et al.* (2015b), other benefits of the SPE clean-up, as matrix effects or LOQs reduction, when compared with analysis of crude extract, were not discussed.

Four other papers presented methods targeting exclusively trichothecenes. All of them focused on feed and/or animal products. Yang *et al.* (2015) developed the simultaneous determination of type A- and B-trichothecenes and their main metabolites in animal meat and liver. UHPLC coupled with triple-quadrupole MS/MS was used for analysis of T-2, HT-2, T-2 triol, NEO, DON, 3-ADON, 15-ADON, DOM-1 and NIV. Regarding distinguishing between 3- and 15-ADON, the authors used spectral separation with specific ion transitions, i.e. 339.5>213.0 and 230.9 for 3-ADON, and 339.5>137.2 and 321.1 for 15-ADON. The targeted trichothecenes were extracted with ACN:ethyl acetate (1:3, v/v). In comparison with extraction of cereals, where mostly ACN:water mixtures are being used, meat

and liver containing lipids and proteins required less polar extraction solvent for effective mycotoxins isolation. To minimise co-extracted matrix interferences, Oasis HLB® SPE cartridges were employed. According to the authors' results, polar mycotoxins as DON and NIV failed to be retained when the standard clean-up protocol was used, so a modified procedure was developed. This included the substitution of water:methanol (95:5, v/v) by pure water for washing, followed by defatting by n-hexane and using dichloromethane as the elution solvent. The mean recoveries of spiked samples ranged from 74-97%. The proposed method was successfully applied for analysis of real samples, with the primary results indicating that, compared to mycotoxins themselves, their metabolites are more likely to occur in animal tissue foods. Another study by Flores-Flores *et al.* (2015) developed a HPLC-MS/MS method for the simultaneous quantification of ten trichothecenes in ultra-high temperature processed cow milk. The method has been validated for NIV, DON, 3- and 15-ADONs, DOM-1, NEO, DAS, FUS-X, T-2 and HT-2. A number of extraction and clean-up procedures have been examined and were discussed in this study. Based on the best results achieved, the final sample extraction was realised with ACN, acidified with 0.2% formic acid, followed by extract purification in the presence of sodium acetate. The addition of this salt caused the separation of ACN layer containing mycotoxins and the aqueous phase with undesirable polar compounds. Without this clean-up step, evaporation of extract and further reconstitution in the mobile phase would be hampered by the presence of yellow viscous pellet containing unspecified milk components, causing other problems in method validation. Despite the performed clean-up step, authors indicated quite high matrix effects, especially for DON. The mean matrix induced suppression for this mycotoxin was 22% (100% means no matrix effect). For NIV, DOM-1, FUS-X, ADONs and T-2, the ionisation suppression was lower and ranged between 58 and 91%. For NEO, DAS and HT-2, a slight matrix induced ionisation enhancement occurred and was between 108 and 121%.

To study potential T-2 and HT-2 residues in shrimp tissues and organs, a LC-MS/MS method was developed as part of a toxicity study focused on metabolism of T-2 toxin in shrimps (Lu *et al.*, 2015). The method comprised simple ethyl acetate extraction of mycotoxins from homogenised fresh shrimp tissues, followed by a solvent exchange to methanol/water/formic acid before the LC separation. The method recovery for both analytes ranged between 84 and 111%, and the LOQs were 0.02-0.51 ng/g for T-2 and 0.17-4.48 ng/g for HT-2. Due to the low quantification limits achieved, the method was recommended be used for analysis of these toxins in real shrimp samples. Another group of authors focused on determination of type A trichothecenes (T-2, HT-2, NEO, T-2 triol and T-2 tetraol) in layer feed by using HPLC coupled with triple quadrupole

MS/MS in the positive ionisation mode (Bernhardt *et al.*, 2016). The method developed was intended to be used for monitoring of carry-over experiments with T-2 and laying hens, why it was important to include potential metabolites. Two fast and easy SPE clean-up approaches have been tested after the ACN/water extraction. The clean-up with MycoSep® 227 columns (Romer Labs) gave slightly better results than the purification by Bond Elut Mycotoxin cartridges (Varian, Inc., Walnut Creek, CA, USA), therefore the MycoSep® SPE was chosen for the final method. Satisfactory separation of mycotoxins was obtained by using the Pursuit™ XRs Ultra 2.8 column (Varian, Inc.) and 0.13 mM ammonium acetate/methanol gradient elution. The common ammonium adducts were detected and monitored in SRM mode. The LODs ranged between 0.9 and 7.5 ng/g.

One method published in 2015 utilised the nowadays rarely used gas chromatography (GC) approach (Escrivá *et al.*, 2016). Authors showed that despite the narrower analytical scope of GC methods, when coupling to tandem MS (GC-MS/MS), GC can be a very useful analytical technique with great sensitivity and selectivity. The analytes, NEO, DAS, DON, NIV, FUS-X, and 3- and 15-ADON, were determined in complex feeds based on cereals, vegetable oils, proteins and derived animal products, intended for laboratory rats. Simple extraction by the acidified ACN/water mixture followed by trimethylchlorosilane/*N*-trimethylsilylimidazole derivatisation ensured acceptable recoveries (62-97%), good linearity, repeatability and reproducibility. For compensation of matrix effects, matrix-matched calibrants were used. The achieved LOQs ranged between 1 and 10 ng/g for all studied trichothecenes, demonstrating that, unlike the previously published GC-based approaches (e.g. Labuda *et al.*, 2005; Shar *et al.*, 2014), this method is suitable for trace analysis.

As concerns immunochemistry-based methods, Yoshinari *et al.* (2015) developed an approach exploiting a novel fluorescent biosensor called Quenchbody used for the quantitative determination of DON. This biosensor is based on the antigen-dependent removal of a quenching effect on a fluorophore attached to antibody domains. The anti-DON Quenchbody was synthesised from the sequence information of a monoclonal antibody specific to DON. The cross-reactivity testing of the anti-DON Quenchbody showed that it was most specific to DON (half-maximal effective concentration (EC_{50}) was 0.03 mg/l) followed by HT-2 toxin (EC_{50} was 0.55 mg/l). The EC_{50} values for NIV, T-2, OTA and ZEA were considerably higher (more than 1 mg/l). Unfortunately, the DON-derivatives frequently occurring in wheat as ADONs and DON-3G were not examined. Within the method validation, a set of naturally contaminated wheat samples was analysed by both the Quenchbody method, as well as by a reference LC-MS/MS method. The authors concluded that the DON

concentrations quantitated by LC-MS/MS correlated nicely with those obtained by the Quenchbody method ($r^2=0.98$). The group of Arola *et al.* (2016) demonstrated a novel rapid one-step immunoassay for determination of HT-2, utilising a pair of recombinant antibody antigen-binding fragments (Fab). The primary Fab recognises HT-2 and the secondary Fab binds specifically to the immune complex formed by the primary antibody and HT-2. Although the antibody used within the study recognised both HT-2 and T-2 toxins, the immune complex assay was highly specific for HT-2 alone. Matrix-matched calibration improved the quantitation of HT-2 in terms of matrix effects reduction. The advantage of this assay over the traditional ELISA is that it can be performed in solution, i.e. no immobilisation and washing steps are needed. The simplicity makes it suitable for rapid on-site testing of various samples. A potential drawback of the assay is that it might even be too selective, as T-2 does not give a signal. The authors concluded that a corresponding anti-immune complex antibody could potentially be developed for T-2, allowing the measurement of both toxins separately and specifically.

12. Zearalenone

This chapter focusses on the determination of ZEA in food and feed. As the mycotoxin is also often measured in other matrices, the interested reader is referred to two recent reviews. Analytical procedures for the determination of ZEA in environmental (and biological) samples have been summarised (Kwasniewska *et al.*, 2015). Also, the current state-of-the art in biomonitoring of ZEA and the application to human exposure assessment has been reviewed (Mally *et al.*, 2016).

As in previous years, the majority of newly published analytical methods for the determination of ZEA in food and feed continue to be LC-MS(/MS) based multi-mycotoxin methods, covered in chapter 3 of this article. However, also dedicated chromatographic methods to determine ZEA (along with its metabolites or other related compounds) in a variety of foodstuffs were developed and published within the last 12 months. Three of such methods are targeting naturally occurring estrogens in milk or milk products. Capriotti *et al.* (2015) developed a UHPLC-ESI-MS/MS based method to analyse milk for ZEA, α -ZOL, β -ZOL, α -ZAL, β -ZAL and zearalanone (ZAN) as well as estrone, α -estradiol, β -estradiol and estriol. Milk was diluted with water and passed through graphitised carbon black SPE columns. The cartridges were sequentially washed with water, acidified methanol and methanol, before analytes were eluted with a mixture of CH_2Cl_2 and methanol. After evaporation and uptake, 5 μl of the samples were injected into a flow of 300 $\mu\text{l}/\text{min}$ of a water/ACN gradient on a reversed phase column. Postcolumn addition of 50 $\mu\text{l}/\text{min}$ aqueous ammonia in methanol enhanced deprotonation for the subsequent ESI source in negative ion mode. The

triple quadrupole mass spectrometer was operated in SRM mode and total run times of 20 min were achieved. Depending on the spiking level, 85-100% ZEA, 97-110% α -ZOL, 85-106% β -ZOL, 92-101% α -ZAL, 92-102% β -ZAL and 92-102% ZAN were recovered. Matrix effects for all reported analytes were minimal, while RSD_r values of 5-7% were achieved for ZEA. LOQs ranged from 0.01-0.1 $\mu\text{g}/\text{l}$ for all analytes. Another UHPLC-MS/MS based method to determine a total of 19 endocrine disruptors, including ZEA and α -ZOL, in milk was proposed by Wielogorska *et al.* (2015). After a QuEChERS like salting out and partitioning step with NaCl, MgSO_4 , acetic acid and ACN, Oasis HLB[®] SPE clean-up was applied to the milk samples. Purified samples were injected on a reversed phase column and analytes were eluted in gradient mode with mixtures of aqueous ammonium acetate/ACN and methanol/ACN. The estrogenic compounds were ionised either in ESI+ or ESI- (e.g. ZEA and α -ZOL) mode and measured with a triple quadrupole instrument in time-sectored SRM mode. 105-107% of both mycotoxins were recovered and the RSD_r values were 7-8%. Compared to the previously mentioned method (Capriotti *et al.*, 2015), slightly higher LOQs were determined and a method calibration range from 0.5-20 $\mu\text{g}/\text{kg}$ could be set for all analytes. Interestingly, the third covered method to determine estrogenic compounds in milk (and yogurt) uses a completely different analytical setup. D'Orazio *et al.* (2015) used DLLME before determination of 11 analytes, including ZEA, α -ZOL, β -ZOL, α -ZAL and β -ZAL, with micellar electrokinetic chromatography (MEKC) coupled to an iontrap mass spectrometer. Prior to DLLME, proteins were precipitated with ACN and the samples were subsequently defatted with n-hexane. Afterwards, samples were diluted with brine and filtered. A mixture of ACN (as dispersion solvent) and CHCl_3 (as extraction solvent) was rapidly introduced into the aqueous extract, followed by vortex shaking. After centrifugation, the CHCl_3 droplet was evaporated, reconstituted and injected hydrodynamically in the MEKC-MS system. An aqueous solution of ammonium perfluorooctanoate at pH 9.0 served as MS friendly background electrolyte. After positive ionisation, mass spectra were acquired in a range of m/z 250-350. For ZEA, recoveries of 86-109% were achieved in different types of milk with RSD_r values of 10-19%. Validation parameters for the other analytes were similar. The major drawback of this method is that the achieved LOQ values are very high. For instance the linear range for ZEA was defined as 45-900 $\mu\text{g}/\text{l}$ of whole cow milk. With LOQs at least 100 times higher as for the other two presented methods, it is unsurprising that in none of the analyzed samples estrogenic compounds were detected.

Two methods employed novel extraction strategies for ZEA determination. Bozkurt and Işık (2015) evaluated ionic liquids as extraction solvents in DLLME followed by HPLC-FLD analysis of beer and cereal samples. Therefore, beer samples were degassed, while cereals were extracted

with methanol, diluted with water and the pH adjusted to 5.0. For DLLME, methanol was used as dispersive solvent. Two ionic liquids, 1-butyl-3-methyl- and 1-methyl-3-octyl-imidazolium bis(trifluoromethanesulfonyl) imide were tested as extraction solvents, with the former showing higher recoveries. Subsequent HPLC-FLD used isocratic elution with water/ACN on a reversed phase column. Extracts of test portions could be concentrated almost 100 times and a linear range of 1-750 µg/l was achieved. Depending on matrix and spiking level 80-100% ZEA were recovered and repeatabilities verified through triplicates were around 1-3%. Garcia-Fonseca and Rubio (2016) used an oleic acid based restricted access supramolecular solvent for extraction to minimise subsequent matrix-induced ionisation effects in LC-MS. Macromolecules such as proteins and carbohydrates were excluded from extraction by the physicochemical mechanisms of the extraction solvent, which was composed of inverted hexagonal aggregates of oleic acid. These aggregates self-assemble and coacervate from acidified oleic acid in mixtures of tetrahydrofuran and water and are stable at least for one month when stored at 4 °C. Extraction was tested for cereal samples, which were extracted with the two-fold volume of solvent prior to LC-ESI-MS analysis using an iontrap. 89-104% of ZEA were recovered from durum, maize and bread with repeatabilities of 1-7% and very similar figures were also obtained for DON, FB₁ and FB₂. External calibration with neat solvent standards was possible for all analytes.

Several novel immuno-analytical methods for the determination of ZEA have been published. An indirect competitive ELISA based on magnetic nanoparticles and biotin/streptavidin-horse radish peroxidase was developed for the determination of ZEA in cereals (Zhang *et al.*, 2015). After extraction with 70% aqueous methanol, extracts were only diluted 1:1 with buffer before analysis and around 1:1 in the wells by the other reagents, roughly resulting in a seemingly still acceptable exposure of the antibodies to 17% methanol. A corresponding working range in cereals of 0.7-24 µg/kg was obtained, while the IC₅₀ value for ZEA was 0.37 ng/ml. Cross-reactivities for α-ZAL, ZAN and α-ZOL were 27, 15 and 11%, respectively, but below 1% for β-ZOL and β-ZAL. Recovery rates of 92-115%, with RSD_r values <9% were achieved and ZEA concentrations of 12 cereal samples fitted nicely to those obtained by LC-MS/MS measurements. Zhan *et al.* (2016) developed a direct competitive fluorescent ELISA using ZEA labelled catalase as a competing antigen with H₂O₂-sensitive QDs for signal transduction. Maize samples were extracted with 60% aqueous methanol, diluted 4-fold with buffer and measured with the developed test. Orange mercaptopropionic acid capped CdTe QDs delivered a fluorescent signal, which was quenched by increasing concentrations of H₂O₂. Recovery rates of ZEA in spiked maize samples were 84-109% with RSD_r values <4%. As expected, the assay was not cross-reactive to other co-occurring classes of mycotoxins, but

no ZEA metabolites were tested. The main benefit of this assay is the excellent detection capability, as seen with an IC₅₀ value of 0.075 ng/ml, compared e.g. to conventional ELISAs. The corresponding linear range in cereals was around 0.08-25 µg/kg. Beloglazova *et al.* (2015) tested silica-coated liposomes loaded with QDs as labels for fluorescent immunoassays and developed an assay for concurrent ZEA and AFB₁ determination in cereals. The hollow spherical structure of liposomes allows encapsulation of multiple water-insoluble QDs, amplifying their signal. Silica coating not only provided conjugation groups, but also stabilised the liposomes against fusion and internal leakage. Cereals were extracted with 80% aqueous methanol and five-fold diluted before analysis. QDs of different colours were used for the analytes, allowing differentiation of the signals and multiplexing of the assay. The used anti-ZEA monoclonal antibody showed cross-reactivities versus α-ZOL (69%) and ZAN (22%), while again the cross-reactivities towards β-ZOL and β-ZAL were <1%. IC₅₀ values for ZEA in wheat and maize were 0.8-0.9 ng/ml, corresponding to rather high 16-18 µg/kg. Still, recoveries (87-121%), RSD_r values (<17%) and the comparison of naturally contaminated cereals with LC-MS/MS derived concentrations were acceptable. Urusov *et al.* (2015) tried to unify protocols and to shorten total analysis times for the determination of ZEA, OTA and AFB₁ with indirect competitive ELISAs. Monoclonal antibodies were biotinylated with an elongated spacer together with a streptavidin-polyperoxidase conjugate to generate signal. The durations of each reaction step, i.e. for the competitive immunoreaction or for the enzymatic reaction, were reduced as far as possible. In the end, the duration for the individual assays was 20 mins, while the similar protocols for each assay allow the parallel determination of the three toxins within 25 mins. Samples were extracted with 70% aqueous methanol, which after dilution was used as extract for each individual toxin assay. For ZEA, 96-108% were recovered from spiked maize samples, with RSDs <15%. A working range of 5-500 µg/kg was defined for ZEA, while the LOD was 3 µg/kg. Cross-reactivities and IC₅₀ values were not reported in this study.

Two multiplex LFIA have been developed. Sun *et al.* (2016) developed a colloidal gold-based competitive immunochromatographic test strip to determine ZEA and OTA in cereals. Samples were acidified and extracted with chloroform. The organic phase was re-extracted with NaHCO₃ solution and 100 µl of the aqueous phase was then used for the assay without further clean-up. In case of absence of both toxins, three red lines (control and both test lines containing the mycotoxin-protein conjugates) are seen. The final result was determined after 5-10 mins. The LOD for ZEA was determined as the smallest concentration showing no visible signal and was 20 µg/kg in cereals. Using optical readers a quantitative result can be obtained (as long as a weakened band can be detected) and recoveries of 86-104% with RSD_r values <13% were achieved from spiked

maize samples. Using the optical reader the LOD was 1.2 µg/kg in maize. Chen *et al.* (2016) developed a LFIA for the simultaneous determination of ZEA, AFB₁ and OTA in maize, rice and peanuts. Samples were extracted with 70% aqueous methanol, diluted four-fold with buffer treated with Tween 20 till a final concentration of 0.5%. Similar to the assay described above (Sun *et al.*, 2016), colloidal gold was used to visualise antibody-antigen bindings and in case of a negative sample, 4 red lines are visible. A visual detection limit of 50 µg/kg was verified for ZEA, while 10 µg/kg were obtained for AFB₁ and 15 µg/kg for OTA. Using a strip reader for quantitative analysis, LODs of around 0.1 µg/kg (AFB₁) to 0.5 µg/kg (ZEA) were obtained and the maximum detection range was close to corresponding the visual detection limits. Mean recoveries of all mycotoxins ranged from 86-115% with RSD_r values <17%.

Finally, two electrochemical methods to determine ZEA have been published. Afzali *et al.* (2015) introduced their electrochemical sensor for the determination of ZEA in beverages using a carbon paste electrode modified with multi-walled carbon nanotubes. A differential pulse voltametric method was employed to study the behaviour of ZEA. The analytical procedure included a closed-circuit accumulation step onto the modified electrode. An anodic peak, related to the oxidation of accumulated and reduced ZEA on the electrode surface, was observed at 0.4 V. Beverages were degassed and adjusted to pH 5 before analysis. 96-104% of ZEA were recovered from spiked samples, with RSD_r values <3%. A LOD of 0.6 µg/l was achieved and the linear range of the method was 2-50 µg/l. The same first author published a method to determine ZEA with a glassy carbon electrode modified with palladium nanoparticles and a conductive polymeric ionic liquid (Afzali and Fathirad, 2016). Electrocatalytic oxidation was performed in a mixture of 20% ACN with 80% 1 M perchloric acid. Cyclic voltammetry and square wave voltammetry revealed an electrocatalytic peak current at +0.69 V vs Ag/AgCl. Spiked cereal based food samples were extracted with 80% aqueous methanol, filtered and diluted. Non-alcoholic beers were boiled, diluted and pH adjusted to 7.2 before measurement. A LOD of 0.01 µg/l and a linear range of 0.03-35 µg/l were verified in neat solvents, but the applied dilution factor was not given for food samples in the article. Still, solid foodstuffs spiked at levels of 2 and 5 µg/kg were successfully analysed and gave recoveries between 92-104% for ZEA.

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