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Developments in mycotoxin analysis: an update for 2018-19

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REVIEW ARTICLE

Abstract

This review summarises developments on the analysis of various matrices for mycotoxins that have been published in the period from mid-2018 to mid-2019. Analytical methods to determine aflatoxins, *Alternaria* toxins, ergot alkaloids, fumonisins, ochratoxins, patulin, trichothecenes, and zearalenone are covered in individual sections. Advances in sampling strategies are also discussed in a dedicated section. In addition, developments in multi-mycotoxin methods – including comprehensive mass spectrometric-based methods as well as simple immunoassays – are also reviewed. This critical review aims to briefly present the most important recent developments and trends in mycotoxin determination as well as to address limitations of the presented methodologies.

Keywords: sampling, multi-mycotoxin analysis, aflatoxin, *Alternaria* toxins, ergot alkaloids, fumonisin, ochratoxin A, patulin, trichothecene, zearalenone, quality control

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 review covering the mid-2017 to mid-2018 period (Tittlemier *et al.*, 2019a). As with the previous reviews in this series, our primary purpose is to raise awareness of the developments and advances in analytical methods for mycotoxins published between mid-2018 to mid-2019. This review will complement other recent general overviews of screening tools for mycotoxins by Santana Oliveira *et al.* (2019) as well as Nolan *et al.* (2019).

As with past years, a selection of the most novel and relevant advances in analytical methodology are discussed in this review, as opposed to incremental improvements of more 'mature' methodology. This review is not meant to be an exhaustive list of publications on mycotoxin analytical methods. Critical comments on the included methods, their validation parameters, or unique applications are included to guide readers in determining the impact of the developments. This review should therefore appeal to both mycotoxin veterans and newcomers to the field.

In light of the many novel approaches published over the past few years, we would like to strongly encourage analysts to progress beyond the 'proof of concept' stage of evaluating novel methodologies or technologies, and expand their validation. Validation must move beyond the assessment of performance over tightly controlled short-term scenarios that use simplified test materials, such as solutions of mycotoxins in solvent. A thorough evaluation of method performance that incorporates relevant matrices, relevant

analyte concentrations, as well as realistic variations in testing conditions that occur over time and with a variety of operators, must also be performed.

Specific topics included in this review are sampling (section 2), multi-mycotoxin methods (section 3), aflatoxins (section 4), *Alternaria* toxins (section 5), ergot alkaloids (section 6), fumonisins (section 7), ochratoxins (section 8), patulin (section 9), trichothecenes (section 10) and zearalenone (section 11).

2. Sampling

Research on sampling and sample processing published over the past year cover a wide range of issues. Topics ranged from the cost effectiveness of various sampling plans (Focker *et al.*, 2018), logistical considerations for developing sampling plans for research studies (Pitt, 2019), performance of different sampling processes (Mallmann *et al.*, 2018), novel sampling methods (Ahmed *et al.*, 2018), and the effects of sample processing on the accuracy and precision of mycotoxin analysis (Damiani *et al.*, 2019; Kumphanda *et al.*, 2019).

Focker *et al.* (2018) constructed a model using historical results from the Dutch monitoring program to determine the most cost effective sampling and analysis plan for assessing compliance with European Union (EU) regulations for deoxynivalenol (DON) in wheat and aflatoxin B_1 (AFB_1) in maize for human consumption or use in foodstuffs. Their model maximised the number of correct accept/reject decisions within a number of set budgets by varying the analytical method used (liquid chromatography with tandem mass spectrometry, enzyme-linked immunosorbent assay (ELISA), or lateral flow device (LFD)), the number of incremental samples taken from the grain lot and combined into an aggregate sample, and the number of test portions analysed from the comminuted aggregate. The most cost-effective plans for a bulk consignment of grain used instrumental or ELISA methods. For AFB_1 in maize, the optimal plan for all budgets over €500 collected as many incremental samples as possible, which was driven by the greater heterogeneity of aflatoxins in maize as compared to DON in wheat. The percentage of correct decisions ranged from 87.9 to 92.4% for these AFB_1 plans. For DON in wheat, there was minimal difference amongst the various sampling plans; the percentage of correct decisions ranged from 96.2 to 98.5%. Focker *et al.* (2018) included a very good discussion on how their cost assumptions impacted the model outputs, therefore readers are strongly advised to assess how their own costs align with the assumptions used in this paper before undertaking similar assessments of optimum plans.

The recent opinion article by Pitt (2019) on biocontrol for reducing aflatoxins in maize contains a very good discussion of the challenges with assessing aflatoxin concentrations in maize with regards to sampling and sample preparation. In particular, Pitt used the FAO Mycotoxin Sampling Tool available online (FAO, 2013) to determine confidence limits of aflatoxin results reported in published studies for the analysis of maize, as well as to estimate the relative contributions of sampling, sample processing, and analysis to the total variance of aflatoxin results. His discussion clearly demonstrates how a lack of replication at the sampling and sample processing stages in a study will greatly reduce, or even eliminate, the ability to statistically discern differences between treatments. Pitt's article also highlights the need to consider the impact of a thorough sampling plan through a thoughtful discussion on how the multiple kg of maize required to minimise variance through the use of replicates and large samples may not be feasible in situations where producers' yield may only be 1-2 tonnes.

Mallmann *et al.* (2018) assessed mycotoxin presence in maize and wheat using two sampling processes that used a pneumatic grain probe to sample grain stored in silos containing approximately 1000 tonnes of grain according to different probing patterns. Sample processing was well considered and thorough, as the relatively large samples (triplicate 4 and 12 kg samples for each location in the respective sampling processes) were coarsely ground prior to sub-division using a riffle-type divider and further comminution. Mallmann *et al.* did not observe a significant difference between the two sampling processes in the variability of results for aflatoxins, fumonisins, or zearalenone (ZEN) in maize or ZEN in wheat. They did, however, note that the DON coefficient of variation was significantly lower (10.1 vs 22.2%) for the probing pattern that sectioned the wheat silo into three horizontal layers along the height of the silo as opposed to covering the entire depth at a sampling point in one sample. While there was some discussion on how spatial variability in mycotoxin occurrence within a silo could be due to particle segregation during silo filling, the authors did not explore how removal of grain from the various locations in the silos could have disrupted the structure or size of localised mycotoxin contamination and impacted the variance amongst replicates. These considerations highlight the complexity in experimental design when assessing effects of sampling bulk commodities.

Proof of concept for sampling headspace around fungal colonies using novel passive and active methods was demonstrated by Ahmed *et al.* (2018). Volatiles associated with mycotoxin biosynthesis by *Aspergillus fumigatus* were collected using thermal desorption tubes containing a porous organic polymer (Tenax TA) and graphitised carbon black (Carbograph 5 TD) as sorbents. While not directly focused on the analysis of mycotoxins, this work is nevertheless relevant as the authors noted that some of the volatiles were specifically associated with early (pyrazine

and methyl-pyrazine) or later (terpenes) fungal growth phases. These observations emphasise the need to consider the time period of sampling when studying a dynamic system.

Damiani *et al.* (2019) examined the effect of particle size of comminuted maize on the extraction efficiency of fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2). Comminuted maize was fractionated based on particle size, and then analysed for the two fumonisins. Extraction efficiency from each fraction increased as particle size decreased for all four naturally contaminated samples. Concentrations in the smallest particle size fractions of <250 µm ranged from 1.3 to 4 times higher than concentrations measured in portions of unfractionated ground maize. The authors attributed this to greater surface area available for solvent extraction of the fumonisins and therefore little to no impact of analyte diffusion from inside of the solid material to its outer surface. This work demonstrates that comminution to smaller particle sizes could not only reduce variance of test results, but also affect accuracy of test results.

Kumphanda *et al.* (2019) also focused on the effects of sample processing on the analysis of mycotoxins in maize, within the context of minimising costs associated with the extraction and analysis of larger test portion masses commonly recommended to reduce data variance. This thorough study encompassed a number of experiments. One experiment characterised the sample preparation and analytical variance associated with the use of test portions (50 and 12.5 g) prepared by grinding laboratory samples on a Romer mill, as well as the optimum size of slurry test portion (25 g) determined in a preliminary experiment. The relative mean combined variances were 15:5:1 for the 12.5 g dry grind, 50 g dry grind, and 25 g slurry test portions, respectively. In fact, the variance in aflatoxin concentrations from the slurry test portions was low enough that it was comparable to the variance due to the particular fluorometric immunoassay used to quantitate the aflatoxins. The authors discussed the differences in variance observed amongst the different test portions in the context of sample mass and particle size, but they unfortunately did not characterise the particle size distributions produced by use of the Romer mill and the slurry mixing techniques. Since the Romer mill appears to produce a coarser particle size distribution than other grinders (Tittlemier *et al.*, 2017), the advantage of the slurry mix noted by Kumphanda *et al.* (2019) may not hold if more intensive dry grinding is performed to produce smaller particles. The authors also noted higher extraction efficiencies for the slurry mixing as was observed for the smaller particle size fractions of maize by Damiani *et al.* (2019), suggesting that the advantage of the slurry mix was due to smaller particle sizes than produced by the Romer mill. Kumphanda *et al.* (2019) also provided an excellent discussion on the 'worrisome trend' of reduction in test portion masses in publications covering the analysis of various commodities for mycotoxins.

3. Multi-mycotoxin methods

Given the global availability of comprehensive liquid chromatography-mass spectrometry (LC-MS) instrumentation, the use of generic sample preparation procedures based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) is nowadays well-implemented in routine analysis of multiple mycotoxins. This approach represents the most common strategy applied when investigating possibilities for the analysis of new mycotoxin/commodity combinations. Therefore, the main innovations this year were reported in testing novel materials to improve the efficiency of QuEChERS-based methodologies.

An ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) method based on the QuEChERS procedure, including the dispersive solid phase extraction (dSPE) step with a C18 sorbent, has been developed for the determination of mycotoxins (α-zearalenol (α-ZEL), ZEN, and AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2)) in edible oils (Hidalgo-Ruiz *et al.*, 2019). In contrast with the common trend of including as many mycotoxins/commodities as possible, the work by Hildago-Ruiz *et al.* (2019) addresses a scientifically sound target and is dedicated to only one commodity, while including a wide range of representative types, namely different types of olive oils (extra virgin olive oil, olive oil, lampante olive oil and refined olive oil), two types of pomace oil, two types of sunflower oil, soy oil, and maize oil. This enabled the researchers to deeply investigate variations in matrix effects to evaluate if one common matrix assisted calibration curve could be used. It was observed that matrix effects were comparable to extra virgin olive oil for most of the matrices apart from crude olive pomace oil, in which there was a strong ion suppression effect for aflatoxins, which was the only one requiring a specific matrix-assisted calibration curve. The method was validated obtaining quantification limits of 0.5 µg/kg for aflatoxins and 1 µg/kg for α-ZEL and ZEN, recoveries (evaluated in the range 0.5-25 µg/kg) from 80 to 120%, and intra and inter-day precision values lower than 20%. The expanded uncertainty, U, was also evaluated and it was below 32% at 25 µg/kg. The developed method was applied to the determination of the six mycotoxins in 194 oil samples, implementing an internal quality control procedure to guarantee the reliability of the results. This included injection of blank sample to check for interferences, a matrix-matched calibration curve using extra virgin olive oil, and fortified samples to check for extraction efficiency.

Nuts, like oils, represent a complex matrix whose main components are lipids. An alternative approach replacing the traditional primary secondary amine (PSA)/C18 sorbent

with a new sorbent (Enhanced Matrix Removal (EMR) Lipid)) for the dSPE step in the QuEChERS process was proposed by Alcantara-Duran *et al.* (2019). The authors reported on the comparison of the two sample treatments with respect to matrix effects and recoveries, showing that the use of EMR-Lipid sorbent could significantly improve method performances in nuts (almonds, peanuts and pistachios). Even if the selection of target mycotoxins, which together with aflatoxins included some mycotoxins that are not likely to contaminate nuts, such as ergot alkaloids (EAs), might be questionable, the work by Alcantara-Duran *et al.* (2019) can be considered as a valuable example of development of a commodity-dedicated sample preparation that coped with specific issues arising from matrix composition. Overall, the method was fully validated revealing recovery rates from 75 to 98% and satisfactory precision with repeatability relative standard deviation (RSDr) values lower than 19%.

Among new sorbents to be applied in QuEChERS-like approaches, carbonaceous nanomaterials, and in particular multi-walled carbon nanotubes (MWCNTs), have been reported as a promising tool for mycotoxin analysis. Some applications developed in the last decade, and reviewed by Reinholds *et al.* (2019), have highlighted the advantages of magnetically modified MWCNTs which enable sample isolation through magnetic separation, reducing the interaction of nanoparticles, and enhancing mycotoxin recoveries. MWCNTs have special structures and characteristics, including a large and specific surface area, and can be modified, for example, by introduction of Fe_3O_4 nanoparticles that impart magnetic properties to the sorbent material. Separation of extracts and sorbents can be accomplished immediately with no need for centrifugal devices, decreasing analysis time.

This methodology has been deeply investigated either with respect to the extraction solvent composition and the type and amount of the Fe_3O_4-MWCNTs by Ma *et al.* (2019). The work produced a UHPLC-MS/MS method for the simultaneous determination of 20 mycotoxins in grains. Under optimised conditions, the method showed satisfactory linearity ($r^2 \geq 0.9965$), recoveries (73-113%), precision (1.3-12.7%), and suitable sensitivity (limits of quantification (LOQs) ranging from 0.002 to 5.4 µg/kg). However, this sorbent is not commercially available, therefore synthesis and characterisation (size and morphology) of the Fe_3O_4-MWCNTs composites must be accounted for in the analytical procedure. This would require the availability of techniques, such as transmission electron microscopy, X-ray diffraction, and Fourier transform infrared (FT-IR) spectroscopy analyses. Therefore, notwithstanding the satisfactory analytical performances of the validated method, there are questions about method reproducibility and transferability to other laboratories.

A further example of the use of homemade MWCNTs, in combination with a C18 sorbent, can be found in Jiang *et al.* (2018). This paper is discussed in more detail in section 10. A careful optimisation of the dSPE procedure with respect to toxin recoveries and matrix effects is described, however neither the source of MWCNTs nor the synthesis and characterisation protocol are reported in the manuscript, which challenges method reproducibility in other laboratories.

A broad scope method has been developed for the simultaneous analysis of citrinin (CIT) and ochratoxin A (OTA) in feed (chicken and pig) and food (cereal-based products, fruit, vegetable juices, nuts, seeds, herbs, spices, vegetarian and soy products, alcoholic beverages, baby food products and food supplements) by UHPLC-MS/MS (Meerpoel *et al.*, 2018). In spite of the simplicity of the applied QuEChERS (without dSPE) sample preparation procedure, particularly remarkable attention was paid to the optimisation of the extraction solvent composition. Commodity-dedicated mixtures were used to take into account differences (e.g. in water or fat content) among the targeted commodity groups. An extensive method validation was carried out according to the criteria described in Commission Regulation No. 401/2006/EC (EC, 2006a) and Commission Decision No. 2002/657/EC (EC, 2002). The method fitness-for-purpose was demonstrated by analysis of 90 feed samples of different composition, revealing the simultaneous presence of CIT (<LOQ-3.90 µg/kg) and OTA (<LOQ-5.60 µg/kg) in more than 50% of these products. Interestingly the authors discussed the applicability of the developed method to matrices belonging to the same commodity group, as defined in Commission Regulation No. 519/2014/EU (EC, 2014). A 'common' matrix-matched calibration was used for each commodity group. This last issue is definitely in line with incoming developments in validation guidelines for (multi)-mycotoxin methods. The classification of food/feedstuffs in 'commodity groups', which implies method validation in one representative commodity for each group ((EC, 2014) is one example of the more practical approaches borrowed from validation guidelines set for pesticide residues (EC, 2017).

The state-of-the-art validation guidelines and tools for performance evaluation of LC-MS methods intended for quantitative determination and for semi-quantitative screening of multiple mycotoxins has been reviewed by Pascale *et al.* (2019). The review's authors try to address the question of the fitness-for-purpose of the current acceptance and performance criteria for mycotoxin methods in view of the observed trend toward the use of in-house developed methods rather than collaboratively validated ones, and the transition from classic single mycotoxin standards to LC-MS/MS based multi-mycotoxin methods which is taking place rapidly, and indeed too fast for standards harmonisation to keep up with such

progress. The re-consideration of validation guidelines and performance criteria for LC-MS screening methods is also strongly advised. Specific guidelines for validation and verification of mycotoxin screening methods set in the Commission Regulation No 2014/519/EU (EC, 2014) are the first step forward in this direction.

Amongst the available quality control tools to ensure that performance criteria are met, isotopically labelled mycotoxins continue to be an essential tool to ensure accurate quantification, as well as a proper management of matrix effects, particularly in official control laboratories in spite of their high costs. Zhang and Xu (2019a) proposed the implementation of the stable isotope dilution assay (SIDA) approach for LC-MS/MS multi-mycotoxin analysis (aflatoxins, trichothecenes, fumonisins, ZEN) in edible oils. This was a follow up to previous validation studies demonstrating the fitness-for-purpose of SIDA LC-MS/MS methods for routine regulatory analysis of multiple mycotoxins within the United States Food and Drug Administration compliance testing and surveillance programmes. In this study, oil samples were fortified with ^{13}C uniformly labelled internal standards for the 12 targeted mycotoxins, followed by extractions using 50% acetonitrile/water (v/v). The SIDA approach enabled quantitation using calibration standards in neat solvent. Furthermore, the manuscript describes the application of a relatively easy-to-use principal component analysis statistical tool to study the effects of a variety of factors, such as sample matrix, fortification concentration, and/or individual mycotoxins, on method performance throughout the recovery study. Using this approach, possible outliers could be observed, and a root-cause analysis of suspect observations could lead to a better understanding of method performance. Recoveries of the optimised method over the tested spiking range (10 to 1000 µg/kg) were 80 to 120% with RSDr values lower that 20%. The method LOQs ranged from 0.1 µg/kg (AFB_1) to 6.4 µg/kg (ZEN). Among 16 U.S. market samples, ZEN was detected in three maize oil samples at 37, 185, and 317 µg/kg, respectively.

In a recent study, an liquid chromatography-high resolution mass spectrometry (LC-HRMS) method intended for the screening of all major regulated mycotoxins, and previously validated by a collaborative study, has been examined in a verification exercise in comparison with other immunoassay-based screening methods, i.e. ELISA, LFDs and a fluorescence polarisation (FP) immunoassay (Lattanzio *et al.*, 2019). The study was organised similarly to a collaborative trial and involved 20 technicians that used the methods for the first time. Reference materials for DON and AFB_1 in wheat were analysed. Results were statistically evaluated to calculate method precision, cut-off values and the rate of false suspect results, then compared with results from previous validation studies. In addition, the statistical analysis of the results allowed researchers to identify the major factors affecting the method precision. The LC-HRMS screening method showed fit-for-purpose cut off levels for DON (cut off 1,150 µg/kg for a screening target concentration of 1,600 µg/kg) and AFB_1 (cut off 1.23 µg/kg for a screening target concentration of 2 µg/kg), which were comparable to those obtained for the immunoassays tested in the same exercise. With respect to method precision, expressed as relative standard deviation that accounted for repeatability and between-technician variability, values of 10 and 33% were obtained for DON and AFB_1, respectively. A root-cause analysis indicated the main reason for the difference in precision between DON and AFB_1 was the difference in magnitude of the LC-HRMS instrumental response. Because the DON target concentration was almost three orders of magnitude higher than for AFB_1, LC-HRMS peaks obtained for DON were well above the instrument LOQ whereas for AFB_1 they were close to the LOQ where more variance in low intensity peak areas can be expected. This is a typical example of compromises in LC-MS multi-mycotoxin screening methods that cover various mass fraction ranges reflecting the natural occurrence of different mycotoxins. For less specific extractions (i.e. those processes that just use solvent extraction without clean-up using additional techniques such as SPE) more matrix materials will be co-extracted and could decrease the signal size of mycotoxins that are present at trace levels.

Among the possibilities offered by modern HRMS detectors, the data-independent acquisition (DIA) mode is emerging as a very versatile approach to combine target quantification and untargeted screening. DIA is used to perform untargeted fragmentation by segmenting the entire mass range in a number of subsequent fixed *m/z* precursor ion ranges to cover the entire range of precursor ions of interest. From the product ion spectra, characteristic product ions for identity confirmation of the targeted mycotoxins can be obtained. The combination of full scan and MS/MS makes the method fully compliant with the requirements set in the document SANTE/12089/2016 (EC, 2016) providing guidance criteria for mycotoxin identification that should be taken into account during method validation. However, the combination of several scan events increases the cycle time and leads to sensitivity losses, therefore a fit-for-purpose balance between method sensitivity and confirmatory power needs to be evaluated.

Jia *et al.* (2019) applied the DIA approach to screen mycotoxins and their transformation products in nutraceuticals from green tea. A fully automated on-line QuEChERS extraction procedure was used for sample preparation. For LC-MS/HRMS analysis, a full scan event (*m/z* 90-900) was combined with twenty consecutive data independent MS/HRMS events. Quantitative and confirmatory information for the targeted mycotoxins were obtained by extracting the exact mass of the selected diagnostic ions. In addition, full scan chromatograms

combined with untargeted fragmentation spectra could be exploited for retrospective analysis. Method analytical performances for target mycotoxins were compliant with requirements set in Commission Decision No. 2002/657/EC (EC, 2002), however any results from retrospective analysis of untargeted mycotoxin metabolites in real samples were not reported.

The availability of highly sensitive LC-MS methods for mycotoxin biomarker analysis in biological fluids and tissues has been increasing over the past years. Consequently, a number of studies describing application of these methods either to exposure assessment studies and to *in vivo*/*in vitro* mycotoxin metabolism studies have been reported. A comprehensive summary can be found in Vidal *et al.* (2019a). With respect to advances in analytical methodologies, it is worth mentioning a study by Lauwers *et al.* (2019) as a valuable example of efforts put into sample preparation to achieve suitable sensitivity and analytical performances meeting EU acceptability criteria for the determination of mycotoxins and their metabolites in complex biological matrices. In this study, pig plasma, urine, and faeces, and broiler chicken plasma and excreta were investigated. A simple and generic sample preparation based on protein precipitation alone (pig) or in combination with removal of phospholipids (chicken) was sufficient to achieve satisfactory method performances for plasma. However, a more intensive clean-up of the other matrices was needed. For these highly complex matrices the sample clean-up consisted of a pH-dependent liquid-liquid extraction (LLE) using ethyl acetate (pig urine), methanol/ethyl acetate/formic acid (75/24/1, v/v/v) (pig faeces) or acetonitrile (chicken excreta). For the extraction of pig faeces, additionally a combination of LLE using acetone and filtration of the supernatant on a phospholipid-removing cartridge was applied. LC-MS/MS was used to detect the targeted mycotoxins, belonging to the regulated groups (aflatoxins, OTA and *Fusarium* mycotoxins) and to two groups of emerging mycotoxins (*Alternaria* mycotoxins and enniatins). In addition, LC-HRMS was used to get qualitative information on phase I and II metabolites, for which analytical standards are not always commercially available. Finally, the applicability of the developed methodology was proven by analysing plasma, urine, faeces and/or excreta samples obtained during *in vivo* toxicokinetic studies with pigs and broiler chickens.

Most multi-class methods for the simultaneous determination of mycotoxins and other chemical contaminants reported in the last year are based on the largely explored dilute-and-shoot or QuEChERS approaches. In addition, an interesting alternative based on two-dimensional liquid chromatography (2D LC) has been proposed. Setting up a multi-class method requires a generic sample preparation and therefore implies selectivity loss. One possible strategy to improve method selectivity is represented by the use of a 2D LC to increase the resolving power of the analytical system. The main challenge in multi-analyte methods is represented by polar substances which are poorly retained by common reversed-phase LC columns and therefore suffer poor peak shape and high matrix effects. 2D LC is one possible chance for solving the problem as suggested in a couple of papers published in the last year. Kresse *et al.* (2019) proposed a 2D LC-MS/MS method for the simultaneous determination of 350 pesticides, mycotoxins, tropane alkaloids and growth regulators in grains. Analyte separation was achieved with a HILIC stationary phase to separate polar compounds and a C18 stationary phase to separate less polar compounds. The test portions were extracted with a mixture of acetonitrile/water (80:20) and extracts were directly injected into the 2D LC-MS/MS system without any further clean-up. Setting up the whole system was a quite complex procedure, but the resulting analytical methodology appeared quite powerful and reliable when looking at the data from the validation study performed according to the DG SANTE guidelines (EC, 2017). Three matrices with different compositions of gluten, fats, proteins and carbohydrates (wheat, maize and soybean) were selected as validation material. Validation criteria were fulfilled for nearly 90% of the pesticides and all the tested mycotoxins and tropane alkaloids. Recovery rates were between 70 and 120% and the RSDr values were below 25%. Method verification was accomplished by the analysis of certified reference materials and the participation in proficiency tests. Eight proficiency tests were passed successfully: three for the pesticide analysis, three for the mycotoxin analysis, and two for the analysis of the tropane alkaloids.

4. Aflatoxins

The number of published chromatographic methods for aflatoxins as the sole target analytes continued to decline and novelties in this field were sporadic. This is significantly driven by the fact that most progress focusses on the determination of larger groups of mycotoxins, in which aflatoxins are only one element among others.

However, chromatographic methods with mass selective detection are still used to investigate novel aspects of aflatoxin quantification. Vidal *et al.* (2018) studied different hydrolysis procedures for the determination of aflatoxins and modified forms thereof in maize. Different from a number of other modified mycotoxins, aflatoxins often break down to a number of other products under harsh conditions, such as alkaline environments, thus making analysis challenging. While trifluoroacetic acid has been used for pre-column derivatisation in older methods to reveal more fluorescent derivatives of AFB_1 and AFG_1, the acidic hydrolysis conditions discussed in this study did not reveal any changes in aflatoxin stability. The authors discuss in detail the pros and cons of pH driven hydrolysis

for a number of other mycotoxins that can co-occur with aflatoxins, such as conjugated DON and ZEN. The authors demonstrated that the use of α-amylase and cellulase, compared to other tested hydrolytic procedures, results in a significant increase of quantifiable aflatoxins, giving indication that aflatoxins are associated with carbohydrates.

An interesting approach for a new type of ELISA was followed by Mukhtar *et al.* (2019). Authors used a cyclic peptide mimicking AFB_1 to develop a peptide-based (pb) ELISA method. According to the validation data reported, the pb-ELISA enabled measurements at lower levels when compared with anti-AFB_1 antibodies previously synthesised. The developed pb-ELISA system showed rather low cross reactivity towards other commonly regulated mycotoxins like ZEN and OTA, but also for the other aflatoxins co-occurring with AFB_1, namely AFB_2, AFG_1 and AFG_2, all indicated with less than 1% cross-reactivity. The authors conducted a small within-laboratory validation with fortified rice and maize (10/20/40 µg/kg) and obtained recoveries ranging from 83-102% with relative standard deviations from 3 to 27% and a working range of 7-100 µg/kg. Taking into account the limited validation data, it appears that maize is the more challenging matrix for this ELISA.

A portable and regenerable prototype immunosensor system for the determination of AFM_1 in various types of milk was developed by Tsounidi *et al.* (2019). The sensor system makes use of polyclonal antibodies, which the authors obtained from an external source. The authors constructed their sensor system using state-of-the-art technical equipment from various suppliers, mainly those for optical appliances. The measurement is based on white light reflectance spectroscopy which allows measurements in an apparently optically 'open system' still being robust against distortion of daylight. The authors describe the optimisation of the sensor and validated their prototype with goat and cow milk over a range of 0.05 to 1 µg/l. Recoveries were reported as 93 to 100%. Other types of milk such as skim or full fat milk, as well as sheep and human milk, were shown to generate similar responses independent of milk type.

A Meizu smartphone model U10 was used in combination with Sicasys Spotxel® Reader software by Sergeyeva *et al.* (2019) to read fluorescence from molecularly imprinted polymer (MIP) membranes that were excited with an external UV source. Unfortunately, the described extraction protocol lacks information for readers to transform concentrations to a sample mass basis. However, the comparison of results with another method used as a reference shows that the experimental frame investigated resulted in a method with acceptable recovery at 7 µg/kg AFB_1. While the manuscript largely discusses the production and description of the MIP, it nicely demonstrates the possibilities to quantify aflatoxins for screening purposes with readily available technology.

Showing that unorthodox analytical approaches can lead to innovation, Chen *et al.* (2018) used a pH-meter to determine AFB_1. The basis for this approach is urease coupled to an AFB_1-oxime hapten. During measurement with this assay the urease activity leads to the formation of ammonia in the presence of urea, resulting in a change in pH. The pH change showed a linear relationship to the logarithm of the AFB_1 concentration from 0.6 to 23 ng/ml over a range of more than an order of magnitude in the test solution. The authors demonstrated the application of this prototype using a very small number of fortified maize and peanut samples. The resulting recovery values (82-94%) fell within acceptable ranges relevant for food law enforcement (EC, 2006a). Unfortunately, the authors did not fully characterise the performance of this method.

The presence of aflatoxigenic fungi and AFB_1 on peanuts or maize was predicted by vibrational spectroscopy using visible and near infra-red light in the range of 400-2,500 nm in two articles by Tao *et al.* (2019a,b). In their first article, the authors try to mimic a scenario in which aflatoxin contamination is derived from post-harvest treatment, resulting in surface contamination of the peanuts. As a result, individual peanut kernels were treated with 10 µl portions of aflatoxin solution to obtain different levels ranging from 10 to 1000 µg/kg on each kernel. The authors discussed their strategy to optimise the relevant spectroscopic aspects and statistical models used. They came to the conclusion that for two action levels relevant in the USA (20 µg/kg for foods and 100 µg/kg for swine feed) the chosen approach demonstrated the potential of identifying surface AFB_1-contamination of peanut kernels with the best overall accuracies of 89 and 93% for 20 and 100 µg/kg contamination level, respectively. Despite the effort that the authors undertook, a main challenge in their approach is that post-harvest contamination scenarios caused by dust particles from processing are likely to result in more homogeneous distributions of mycotoxins across the consignment, as opposed to pre-harvest contamination of individual kernels. This will make it harder to differentiate compliant from non-compliant parts of consignments. On the other hand, the research undertaken gives a good insight into the state-of-the-art technology employed for the identification of contaminated peanut kernels that should be subject to sorting prior to processing. In the second article, Tao *et al.* (2019b) focused on the relation of AFB_1 determination and the aspect of distinguishing aflatoxigenic vs nontoxigenic fungal infection of maize kernels. The relation of these aspects for the prediction models used was elucidated. Both articles depict – in combination – the complexity of identifying suitable screening tools to aid in the management of aflatoxins in agricultural commodities. In particular, the discrete design approaches for the two

commodities illustrate that a high level of expertise is needed for the understanding and possible implementation of this technology. It can therefore be concluded that skilled personal is also for this technology an asset during decision making, in particular for food and feed law enforcement.

Another spectroscopic method by Liu *et al.* (2019) came to the conclusion that terahertz spectroscopy in combination with chemometric methods offer the possibility to determine AFB_1 in soybean oil. Based on the details given in the article, it is not traceable to what degree the measured spectroscopic differences are derived from the solvent in which AFB_1 is dissolved. The authors gave no indication they compensated for this; the information presented suggests they rather added various amounts of AFB_1 spiking solution. While the authors only give reference to the supplier without specifying the product in detail, the available AFB_1 solutions on the supplier's website suggest that the solution used was AFB_1 in acetonitrile (2 µg/ml). In this situation, the AFB_1 is only a minute fraction of what has been added to alter the soybean oil composition, and calls into question the relevance and robustness of the spectroscopic method.

5. *Alternaria* toxins

Most of the analytical methods published in the last year for different mixtures of *Alternaria* toxins were matrix-specific methods based on LC-MS/MS (with or without SPE clean-up/concentration).

The QuEChERS approach was used by De Berardis *et al.* (2018) for the determination of tenuazonic acid (TeA), alternariol (AOH), alternariol monomethyl ether (AME) and tentoxin (TTX) in tomato- and fruit-based products. Samples were extracted with a mixture of acidic acetonitrile/water (80/20, v/v), $MgSO_4$, NaCl, and ceramic homogenisers. The upper organic phase was filtered and analysed by LC-MS/MS (electrospray ionization $(ESI)^+$). For the separation of the four mycotoxins, six reversed phase columns were tested and a phenyl-hexyl based column gave the best results by using a mixture of methanol/aqueous 1 M $(NH_4)_2CO_3$ (98.5/1.5, v/v) buffered at pH 8.8, as the mobile phase. *Alternaria* toxin limits of detection (LODs) ranged from 1-80 µg/kg, method recoveries from 63-109% and repeatability from 3-9%, but validation experiments were performed only for tomato paste spiked at very high levels of each mycotoxin (100 mg/kg). External calibration was used but no information on matrix effects was reported.

A similar approach was used by Guo *et al.* (2019a) for the extraction of TeA, AOH, AME, TTX, altenuene (ALT), and altenusin (ALS) from grapes. Sample extract (5 ml) was dried and reconstituted with 1 ml of acetonitrile/water containing 5 mmol/l ammonium acetate (20/80, v/v), filtered, and analysed by LC-MS/MS (ESI^+) for AOH, AME, ALT, TTX and TeA, and ESI^- for ALS. LODs, repeatability, and matrix effect (measured as signal suppression and enhancement) ranged from 0.03-0.21 µg/kg, 1.9-10.8 and 82.8-102%, respectively.

Wang *et al.* (2018a) used a QuEChERS approach for the extraction and LC-MS/MS (ESI^+) quantification of TeA, AOH, AME and TTX in jujube. Increasing amounts of $MgSO_4$ (0, 2 and 4 g) and NaCl (0, 0.5 and 1 g), as well as different ratios of sample/water (1/0, 1/2, and 1/4, m/v) were tested before adding acetonitrile/1% CH_3COOH. There was a parallel increase of mycotoxin extraction efficacy with mass of $MgSO_4$ used; no differences were observed with increases in the amount of added NaCl. In the optimised protocol, 10 ml water and 10 ml acetonitrile containing 1% CH_3COOH were successively added to 2.5 g of sample, which was then shaken. Immediately, 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were added and shaken to prevent agglomeration of the salts. The addition of PSA, Florisil, octadecyl silane (C_{18}), and graphitised carbon black was tested but not included in the final protocol because they produced a significant decrease of mycotoxin recoveries.

Zhang *et al.* (2019b) developed an LC-MS/MS (ESI^-) method for the determination of TeA, AOH, AME, ALT and TTX in drinking water consumed in China. After removal of residual chlorine and pH adjustment to 2.5, one litre of water was passed through an Oasis HLB column (Waters, Milford, MA, USA) at an approximate rate of 5-10 ml/min. Mycotoxins were eluted from the column with 5 ml MeOH and 5 ml MeOH/acetonitrile (1/1, v/v, containing 1% ammonia). The eluate was collected, dried and reconstituted with 1 ml of MeOH/water (1/9, v/v) solution for UHPLC-MS/MS analysis. TeA and TTX were found in 14% of 289 tested samples at very low concentrations, ranging from 0.16 to 2.7 ng/l and 0.21 to 2.2 ng/l, respectively.

Extraction with acetonitrile/water/formic acid (49/50/1, v/v), centrifugation, sample extract dilution 1/4 (v/v) with water, one night storage at 4 °C, filtration and LC-MS/MS (ESI^-) were used by Gambacorta *et al.* (2019) to determine TeA, AOH, AME, ALT, and TTX in 22 different types of spices, 18 herbs, 18 mixtures of spices, and 3 mixtures of herbs commercially available in Lebanon. The sensitivity of the method was good, with LODs <7 µg/kg and 89% of analysed samples contained at least one of the five monitored mycotoxins. The authors reported that concentrations observed in some spices and herbs could result in exposure to AOH or AME that exceed a threshold of toxicological concern (EFSA, 2011); however the masses of spices and herbs required to reach these thresholds were high, ranging from 0.5 g of white pepper to 4.4 g of thyme leaves.

A laborious LC-MS/MS (ESI^-) method was developed by Gotthardt *et al.* (2019) for the determination of TeA, AOH,

AME, TTX, altertoxin I (ATX I) and alterperylenol (ALTP) in infant food. Tomato purée and potato starch (as cereal model) were used for method development. Samples were extracted twice with a mixture of acetonitrile/water (84/16, v/v) and once with a mixture of acetonitrile/methanol/water (50/25/25, v/v/v). Within the five SPE columns tested for sample extract clean-up, a polymerically bonded trifunctional C18 silica sorbent (Discovery DSC-18) was selected and used for clean-up of crude extract. TeA, AOH, and AME were quantified using stable-isotopically labelled standards, whereas TTX, ATX I and ALTP were quantified using matrix matched calibration. For LC-MS/MS (ESI^-) determination it was necessary to use a Gemini-NX C18 column for TeA and a HyperClone BDS-C18 column for the other mycotoxins. Moreover, two different mobile phases were used in a binary gradient. Method LODs ranged from 0.05-1.25 µg/kg for starch and from 0.01-1.36 µg/kg for fresh tomato. Recoveries and repeatabilities ranged from 83-111 and 3-8%, respectively.

Extraction with acetonitrile, centrifugation, evaporation of extract, and reconstitution with MeOH/water (1/9, v/v), clean-up and extract concentration on OASIS HLB and LC-MS/MS (ESI^-) were used by Qiao *et al.* (2018) for the determination of TeA, AOH, AME, ALT, and TTX in cherries and derived products. A good sensitivity was reported with LOQ values ranging between 0.002-0.066 µg/kg.

A polyclonal antibody for AOH was produced in rabbits and used to develop a competitive indirect chemiluminescence enzyme immunoassay (ciCLEIA) to determine AOH in fruit juice, maize, and flour. No cross reactivity was observed for other *Alternaria* toxins. Analysis of naturally contaminated samples (1.2-13.8 µg/kg) by ciCLEIA showed a good correlation (R^2=0.9539) with results obtained by LC-MS/MS (ESI^-) (Yao *et al.*, 2019).

A colorimetric (Man *et al.*, 2018) and a fluorometric immunoassay (Man *et al.*, 2019) were developed to measure AME in fruits. Both methods used magnetic nanoparticles and SPE purification of crude extracts. The LODs were 0.25 ng/ml (equivalent to 5 µg/kg in fruits) and 0.25 pg/ml (equivalent to 0.01 µg/kg in fruits) for colorimetric and fluorometric assay, respectively. However, these methods were only tested on spiked cherry, orange, or apple, and no naturally contaminated samples were analysed.

6. Ergot alkaloids

Since the last review, a number of papers describing advances in EA analysis have been published. These papers describe five diverse techniques including: LC-MS; laser ablation electrospray ionisation (LAESI)-MS; enzyme immunoassay (EIA); high performance liquid chromatography-fluorescence detection (HPLC-FLD); near-infrared (NIR) and attenuated total reflectance Fourier transform mid-infrared (ATR-FTMIR) spectroscopy.

LC-MS/MS is the gold standard for EA analysis as it allows for detection of the largest number of compounds that might be present in infected crops. The power of using LC-MS analysis was demonstrated by Tittlemier *et al.* (2019b) for their studies monitoring the fate of EAs during durum processing and pasta production. LC-MS was key for this work because it allowed for the monitoring of changes in the ratios of *R*- to *S*-enantiomers, which would not be possible using the other techniques reviewed here. These data have important implications for food safety monitoring and exposure assessments. LC-MS was also used for monitoring of EAs in horsehair (Rudolph *et al.*, 2019). The described method was capable of simultaneously detecting a number of toxins that might be the cause of different syndromes in horses. The use of horsehair vs blood or urine allowed for long-term exposure monitoring of EAs in addition to a number of other toxins of interest. The authors were able to detect EAs in the low ng/kg range in spiked horsehair samples, but not in real samples likely due to their absence in the 13 horsehair samples tested.

Using the relatively new LAESI-MS technique, Bacon *et al.* (2018) were able to visualise the distribution of EA in different grass species infected with the symbiotic endophyte *Balansia epichloe*. LAESI does not require extensive sample preparation and allows for non-invasive measurement of the chemicals involved in microbe-host interactions during plant growth. Chanoclavine I and ergonovine were identified as the major EAs present in asymptomatic leaves. Due to the nature of this analysis, LAESI allowed localisation of the compounds in individual tissues throughout the plant development to be characterised. Interestingly, the data suggested that EAs were produced within the *Balansia* stromata and that their concentrations change during the growing season (Bacon *et al.*, 2018). The authors argued that LAESI provided a rapid and accurate method for screening and characterising EAs in *Balansia* infected grasses. One limitation of this study was that they only reported the detection of two EAs.

An EIA method was developed for the detection of total EAs in cereal flour and in bread (Gross *et al.*, 2018). The researchers produced antibodies against four EAs, and demonstrated that a competitive direct assay with antibodies to ergometrine was the most sensitive and validated it with HPLC-FLD. The method was able to reliably detect positive samples at 0.01-0.02 mg/kg and suitable for the determination of total EAs in a concentration range of 0.10-1.0 µg/kg. Cross reactivity was determined against 14 alkaloids; three of the EIAs were highly specific but the EIA for ergometrine, showed some reaction to all of the alkaloids. The authors argued that new EIA methods were needed as the two commercially available test kits did not

provide details on their antibody production or validation data in cereals. This work would have been strengthened by direct comparison of their EIAs with the two commercially available test kits.

HPLC-FLD was applied to the detection of EAs in rye (Holderied *et al.*, 2019). They suggested the use of HPLC-FLD over LC-MS based on the simplicity of the method and the cost of the instrumentation, they also noted the benefits of their method for use in developing countries. Their advancement to EA analysis was the introduction of lysergic acid diethylamide (LSD) as an internal standard for HPLC-FLD detection. Their validated method allowed for the quantitation of 12 representative EAs in rye flour and rye products after SPE clean-up. They suggested further optimisation of their method might be achieved by incorporation of the previously reviewed sample freeze-out clean-up step by Schummer *et al.* (2018).

The application of NIR and ATR-FTMIR spectroscopy for the determination of EAs in cool-season barley grown under cold climate conditions in Canada was tested (Shi *et al.*, 2019). NIR and ATR-FT/MIR are attractive techniques for mycotoxin screening due to their speed and non-destructive nature. LC-MS/MS was used to characterise and quantify EA levels in 67 barley samples, of which 49 were positive. NIR (680-2,500 nm) and MIR (4,000-700 cm^{-1}) spectra of each positive sample were collected and calibrated with EAs reference values. The data processing models indicated weak calibration and cross-validation parameters; the authors suggested that this may have been due to the relatively low EA levels in the barley. Their data indicate that these techniques offer theoretical advantages over LC-MS but have not demonstrated their usefulness yet in practical applications.

The studies presented continue to support the use of LC-MS as the gold standard for EA analysis and highlighted the challenges of using methods involving EIA or NIR. The use of LAESI for *in situ* EA analysis presented a novel approach. This technique provides the deepest understanding of EA occurrence in grass but it still requires a mass spectrometer for detection, which will limit wider uptake by the mycotoxin community.

7. Fumonisins

A review of all aspects of fumonisins, including an overview of their determination, was recently published (Ponce-García *et al.*, 2018). The dependence of accurate analytical results upon the use of appropriate sampling, sub-sampling and sample comminution procedures was reinforced recently by a study that demonstrated the significant impact of sample comminution upon the extraction of FB_1 and FB_2 from maize (Damiani *et al.*, 2019). This work is discussed in section 2.

Although the article by Damiani *et al.* is an exception, over the past few years there has been a notable decline in the number of articles that describe methods focused solely on the fumonisins. This is particularly true for LC-MS-based methods for fumonisins in foods, and it is almost certainly the result of greater emphasis being placed on improved multi-analyte screens. The same trend is apparent with immunoassays, which has been concurrent with an increase in the number of reports of multi-toxin ELISAs, lateral flow immunoassays, and biosensors. The number of publications describing new or improved screening assays for fumonisins has continued to increase, with a significant proportion of such articles originating from Asia. This reflects the increasing impact of the region on mycotoxin research. Many of the recent reports describe modifications to the well-established antigen-immobilised (or 'competitive indirect', CI-) and antibody-immobilised (or 'competitive direct', CD-) ELISA formats. In particular, there were multiple reports of improving the enzyme-based amplification step by replacing the traditional horseradish peroxidase (HRP) with novel enzymatic or fluorometric labels. A few such reports are briefly summarised here.

A substitute for fumonisin-HRP (FB-HRP) was reported in the context of a four-plex assay for detecting FB_1, AFB_1, OTA, and ZEN in maize (Zhang *et al.*, 2018a). In the competitive portion of the assay a conjugate of FB_1-bovine serum albumin (BSA)-biotin competed against free FB_1. This step was followed by a washing step and the addition of an avidin-HRP conjugate. The substitution, along with using chemiluminescence detection, improved sensitivity. However, the assay required an additional washing step that can be avoided by using FB_1-HRP. HRP can be replaced with other enzymatic labels. Zhan *et al.* (2019) reported an amplification system based upon glucose oxidase. The enzyme converted glucose and O_2 to D-glucono-1,5-lactone and H_2O_2. The H_2O_2 then reacted with Au^{3+}, reducing it to Au on the surface of 5 nm gold 'seeds', causing them to grow and for the solution to change from colourless to purple. The visible cut-off limit was 1.25 ng/ml FB_1 and the IC_{50} was 1.86 ng/ml, about 13-fold lower than the corresponding HRP-based ELISA. Hydrogen peroxide also formed the basis for a different type of detection, in this case involving its conversion to H_2O and O_2 by catalase (CAT) (Lu *et al.*, 2018). Rather than FB_1-HRP, the assay used FB_1-CAT in the competition reaction. As a detection reagent, the assay used fluorescent quantum dots (QD) which were sensitive to quenching by H_2O_2. When FB_1 was present, less FB_1-CAT bound, which meant more H_2O_2 remained, quenching the fluorescence. When FB_1 was absent, the FB_1-CAT bound resulting in destruction of H_2O_2 and preservation of the fluorescence. The IC_{50} was 1.95 ng/ml FB_1, about 10-fold lower than that of an HRP-based colorimetric ELISA.

ELISAs that use labels yielding electroactive products have been described previously. A different approach was

recently reported that measured electroactivity without requiring an enzymatic label (Lu and Gunasekaran, 2019). An indium tin oxide electrode was constructed that contained a surface modified with gold nanoparticles (AuNP) and anti-fumonisin antibodies. The binding of FB_1 to antibodies immobilised on the electrode was measured by differential pulse voltammetry. The signal produced was proportional to the concentration of FB_1 applied. The LOD of FB_1 in buffer was 97 pg/ml, with a linear range of 0.3 to 140 ng/ml. Using matrix-matched calibration, the average recoveries of FB_1 spiked into maize at concentrations between 50 and 300 µg/kg ranged from 88.6 to 95.8%. Benefits of the method were the elimination of the enzymatic label and the direct proportionality of the response to the FB_1 concentration. A limiting factor was the incubation time that was required (50 min), a factor that may be solvable with further investigation.

Immunoassays on other types of electrodes have also been recently described. Cheng *et al.* (2019) measured changes in the electroactivity of an electrode modified with a nanocarbon surface. A surface consisting of an electroactive graphene oxide nanocolloid (GONC) was prepared and fumonisin-specific antibodies were immobilised non-covalently. Attachment of the antibodies lowered the electroactivity of the GONC. The binding of FB_1 to the antibody further decreased the electroactivity of the material. Because of this, the response of the sensor was directly related to the binding of FB_1. Unfortunately, the sensitivity (LOD of 294 ng/ml) was substantially poorer than seen with some of the other FB_1 detection platforms and the assay required a relatively long incubation time (1 h). Nevertheless, the proof-of-concept of this type of device was demonstrated.

Most fumonisin immunoassays use a primary (anti-fumonisin) antibody (Ab1). Some include a secondary antibody (Ab2) to bind to Ab1 for purposes of signal amplification. The literature has many examples of anti-fumonisin antibodies. But efforts continue to improve upon Ab1 by exploring alternatives to intact immunoglobulin G, such as single-chain variable fragment, and nanobodies. Furthermore, the use of anti-idiotype antibodies (a form of Ab2) and peptide fragments that mimic fumonisin (mimotopes) continue to be explored as ways to improve immunoassay performance. A novel immunoassay format using a fumonisin mimotope was developed and applied to spiked wheat samples (Peltomaa *et al.*, 2018). The assay was based upon a reagent created by linking the mimotope to yellow fluorescent protein (YFP). The mimotope-YFP competed with FB_1 for binding to a fumonisin antibody attached to AuNP. When the mimotope-YFP bound to the antibody the fluorescence of the YFP was quenched by the AuNP. In the presence of FB_1, the binding of the mimotope-YFP to the antibody was inhibited and the fluorescence was not quenched. Unlike most immunoassays, this format did not require separation of the labelled molecules (i.e. no washing steps). Furthermore, the fluorescence response was directly proportional to the concentration of free FB_1. The IC_{50} was 12.9 ng/ml FB_1, with a dynamic range of 7.3 to 22.6 ng/ml and a LOD of 1.1 ng/ml. In two samples of wheat, spiked at 2 and 4 mg/kg, recoveries were 86 and 103%, respectively. Relative standard deviations were less than 6.5%. The incubation required 20 min. This approach to shortening assays is very promising and should be investigated further with additional spiked and naturally contaminated samples.

Anti-idiotype antibodies are a form of Ab2 that bind in, or near, the site where Ab1 binds fumonisin. Because of this anti-idiotype antibodies can, in some cases, interfere with the binding of FB_1 to Ab1. Anti-idiotypes are subdivided into groups based upon functionality: those that do not (Ab2α) or those that do (Ab2β, Ab2γ) interfere with the toxin binding site. Importantly, Ab2 provide opportunities for new reagents to improve assay performance. Recently, a non-competitive immunoassay was developed for fumonisin detection using a combination of Ab1, Ab2β, and Ab2α (Shu *et al.*, 2019). The Ab2α used were lower molecular weight variants of antibodies (nanobodies) derived from a phage-display library. In this assay, when the Ab2β was present and toxin was absent, it prevented, through steric inhibition, the Ab2α from attaching to Ab1. In the presence of toxin, the toxin bound to Ab1 as did the Ab2α. Importantly, in this particular case, the Ab2β did not bind to Ab1 when FB_1 was present. The binding of Ab2α to Ab1 was detected with a secondary antibody-HRP conjugate. Clearly, this format required a complex set of interactions. However, a benefit of this format was that the response was directly proportional to the toxin content. The LOD was 0.19 ng/ml FB_1 and the concentration required to give 50% of the maximal signal was 0.68 ng/ml. Extracts of maize were cleaned-up over strong anion exchange SPE columns before testing. Average recoveries from maize spiked at levels from 10 to 1000 µg/kg ranged from 71.6 to 115%, with coefficients of variation from 2.79 to 12.7%. A comparison of the method with an LC-MS/MS method for naturally contaminated maize, wheat, and rice samples showed an excellent correlation between the two methods. While the assay format is decidedly complex, and the need for the SPE clean-up adds significant time to the process, the results suggest this is an alternative approach for achieving signals directly proportional to fumonisin content.

Most screening assays are based upon antibodies. However, antibodies have limitations and novel materials continue to be explored, with the goal of improving performance characteristics. Such materials include those based upon oligonucleotides (aptamers) and MIPs. A photoelectrochemical sensor was reported that used a MIP to bind FB_1 for detection (Mao *et al.*, 2019). The sensor was prepared by first covering the surface of an indiumtin

oxide (ITO) electrode with CdS QDs, graphene oxide (GO), and chitosan (CS). Upon this surface the MIP was prepared, using FB_1 as the template for imprinting. Before use, much of the FB_1 was removed by elution with ethanol. Light was used to induce a photocurrent in the device. Added FB_1 interacted with the MIP and lowered the photocurrent through the device. The LOD was reported to be 4.7 pg/ml FB_1, with a linear range of 0.01 to 1000 ng/ml. It is unclear how the spiking and recovery experiments were conducted, but recoveries from 3 samples were reported to be between 94 and 106% from maize and milk over the range of 0.1 to 10 ng/ml. The technique appears promising and would benefit from further research into matrix effects and testing of naturally contaminated samples. Although there are notable exceptions, it is common in the mycotoxin literature for MIPs to be synthesised with the toxin as the template. To prevent exposure during synthesis and leaching of toxin during use, investigation of less-toxic imprint molecules is encouraged.

Aptamers continue to be widely investigated antibody alternatives. Niazi *et al.* (2019) developed a novel sensor for measuring FB_1 and OTA. The format incorporated nanoparticles with a long fluorescence lifetime, allowing time-resolved fluorescence to be used for detection. The fluorescent nanoparticles were linked to magnetic nanoparticles through an aptamer 'bridge'. Binding of FB_1 by the aptamer disrupted the complex. Freed fluorescent nanoparticles (non-magnetic) were separated from the complex (magnetic) and the fluorescence of the complex was measured. The result was an extremely low LOD for FB_1 of 0.019 pg/ml (detection range 0.0001 to 0.5 ng/ml).

8. Ochratoxins

Challenges in OTA analysis are mostly caused by interferences from complex sample matrices, that overlap with analyte peaks, quench fluorescence, or suppress analyte ionisation for MS detection. In pure solvents, OTA can be easily detected by HPLC in the pg/ml range due to its intense fluorescence and excellent ionizability in positive and negative electrospray ionisation allowing detection limits even below those of FLD. Considering matrix effects causing signal suppression during ESI, OTA is less prone compared to some other mycotoxins such as the trichothecenes, but it is still susceptible. Keeping this in mind, it is obvious that procedures for selective extraction of OTA from interfering matrix are the key step for successful sample analysis. To that end, immunoaffinity columns (IAC) are mostly used in combination with HPLC-FLD. As these columns are rather expensive and sold as single use material, Liu *et al.* (2018) investigated the reusability of IAC for OTA extraction from malt and ginger and studied the best parameter for regeneration and retaining the binding capacity of these columns. Interestingly the authors identified two key factors for a successful regeneration of the IAC columns. They reported that the dilution solvent added to the sample before passing through the column is of high relevance and reported the following ranking: water > phosphate buffered Tween > phosphate buffered saline (PBS). Furthermore, the adjustment of the pH between 7 and 8 was recognised as crucial. With this system IAC columns showed recoveries above 70% for up to eight repetitions when malt extracts were purified and for up to three repetitions with dried ginger extract when IAC columns were regenerated after OTA elution by filling with PBS storing overnight at 8 °C. Besides these modifications, classical protocols for sample extraction (methanol/water, 70/30, v/v) and for elution of OTA from the IAC (methanol) were applied. The authors concluded that reuse of the tested IAC columns is possible and reproducible. However, with a detailed look into the data, a significant decrease of recovery can be already observed during the third clean-up of the malt extracts and the second ginger extract, thus lowering the accuracy and repeatability of analysis despite a recovery above 70%. In addition, carryover of remaining OTA that was not fully eluted and the questionable shelf-life of used cartridges furthermore challenges the reuse of IAC columns in control labs. In summary, if IAC are reused, this should be done only for screening purposes when a stable isotope labelled standard such as d5-OTA is applied to compensate for reduced binding capacity of the IAC.

Within the last five years, aptamers have become popular as a cheaper alternative to antibodies for the development of biosensors. More than 100 articles regarding nano-bodies, MIPs and traditional antibodies for development of new biosensors or test strips were published in the last five years. A review from June 2019 provides an overview of the systems developed so far (Alhamoud *et al.,* 2019). The authors summed up the available methods, their sensitivity, and matrices tested. Additionally, the properties of the above-mentioned recognition elements are discussed. However, it is still challenging to find the best currently available test system for a specific scenario, to keep up to date with the current developments, and especially with the commercialisation of published methods. Luckily, for OTA, as well as for other mycotoxins, the end user sensor tree (TEST) has recently been launched as a website (http://test.foodsmartphone.net) helping to navigate through the current developments (Nelis *et al.,* 2019). The idea of this website is to act as a guide through the commercially available test systems and provide readers with positive and critical aspects of the different analytical tools, as well as information on the commercial source and the required training.

As alternatives to laborious IAC columns, three promising affinity-based protocols for OTA enrichment and purification have been published since our last review. Cyclodextrins are known to be capable of incorporating hydrophobic secondary metabolites such as OTA in aqueous

polar environments and to release them with increasing concentrations of non-polar organic solvents. Following a previous publication, Appell and others optimised the synthesis of a polyurethane-β-cyclodextrin-copolymer for selective and efficient extraction of OTA from liquid samples and used this material for the preparation of SPE cartridges (Appell *et al.,* 2018). For analysis, 1 ml red wine or grape juice was loaded on the column, followed by washing with 1 ml water and elution of OTA with 10 ml methanol. Recoveries for OTA ranged between 77.0-89.4% for wine and 69.1-86.5% for grape juice, thus confirming the suitability of the method for purification. The published HPLC-FLD chromatograms show only few interferences and clearly demonstrate a high purification efficiency. Interestingly, the reported method does not require a washing step with mixtures of water and organic solvents. A critical aspect of the method could be the elution volume of 10 ml, which is larger compared to classical SPE methods and requires a rotary evaporator for efficient removal before HPLC-analysis. It would be interesting to find out to what extent this procedure allows a simultaneous analysis of ZEN and other mycotoxins or fungal metabolites besides OTA, as an interaction with cyclodextrins is reported for ZEN. In summary, this method is a promising extension of the OTA clean-up portfolio with potential application in single but also multi-mycotoxin methods.

Even higher selectivity for OTA was obtained by aptamer loaded monolithic HPLC columns as compared to cyclodextrins. In most assays, aptamers bound to gold nano particles (APT@AuNP) were utilised for OTA recognition and have also been attached to monolithic columns as stationary phases. However, agglomeration of the AuNPs and strong effects on the permeability have been reported as drawbacks of this approach. Thus, direct binding of the aptamers to the column material has become the method of choice, which has been successfully demonstrated for OTA by two different groups. Previously, Chen *et al.* (2019) polymerised polyhedral oligomeric silsesquioxane-methacrylate (POSS-MA) ethyleneglycol dimethacrylate (EDMA) and aptamer acrylamido-2-methyl propane sulfonic acid ester (AMPS-Apt). Using this column material, good binding of OTA to the aptamers was obtained, but also the hydrophobic column material itself showed strong interaction with the analytes and matrix components. By incorporation of hydrophilic linkers such as N,N'-methylene-bis-acrylamide (MBA), the hydrophobicity of the column material could be significantly reduced, resulting in less retention of interfering compounds from the extracts and improved purification of OTA (Chen *et al.,* 2019). POSS with poly(ethylenimine) (PEI) followed by coupling of 5'NH_2-aptamer via trichlorotriazine is an alternative immobilisation approach for OTA selective aptamers (Yu *et al.,* 2019). The hydrophilic character of this column material and the high surface density of up to 1,800 pmol aptamer per microliter was also seen as promising; studies with beer samples gave comparable results for the POSS-PEI and the POSS-EDMA columns. For both aptamer-systems, specific loading and elution solvents were applied. Analytes were identified by their elution times after switching to elution buffer and were detected by means of a FLD. So far, only beer and other simple liquid matrices have been analysed using this type of column. Although these results seem promising, it remains unclear how robust these columns will be in every-day routine analysis. Also, the approach to assign the elution time from the aptamer-column when the elution buffer is applied as a retention time is questionable, especially, when the column characteristics such as void volume are not described. On the other hand, the sharp elution profile from the aptamer column could be used for the application within an online SPE system where extracts from more challenging matrices are first enriched and purified with aptamers bound to monolithic columns and subsequently analysed by classical reverse phase-(U)HPLC-FLD or MS. It would be great if one of these columns would become available for a mycotoxin analysis laboratory for testing the capacity of these materials with OTA-relevant complex matrices such as cocoa or paprika extracts.

9. Patulin

From mid-2018 to mid-2019 several publications on patulin (PAT) analysis were published: two general reviews, four aptamer-based methods, and one MIP-based electrochemical method. Vidal *et al.* (2019b) described analytical challenges in the determination of PAT. Since PAT is highly polar and has a low molecular weight (154 g/mol), it is difficult to develop low-cost immunochemical methods for PAT. The most common method employs HPLC-UV although LC-MS/MS methods have increased in popularity; nevertheless, the small size of PAT makes interpreting MS data problematic. The authors note that exposure data for PAT is hard to obtain since no biomarker for PAT has yet been described.

Sadok *et al.* (2019) published a review article discussing methods reported within the past decade for PAT in fruits and fruit-based products with a focus on LC coupled with UV and/or MS detectors. It also covered sample preparation using LLE, SPE, QuEChERS, or MIPs to improve sample clean-up and pre-concentration. UHPLC followed by MS detection may be the method of choice because of realised time savings and solvent consumption reduction.

Khan *et al.* (2019) developed an aptasensor platform for the detection of PAT using a label-free approach. Preparing the aptasensor involved electrografting diazonium onto the surface of a working carbon electrode, covalent immobilisation of carboxyamine polyethylene glycol, and then covalent immobilisation of amine-aptamer. The anti-PAT aptamer served as a gate for electron flow, permitting

the flow of electrons from a redox probe to the surface of the electrode when no PAT was present in the sample extract. In the presence of PAT, the three-dimensional conformation of the aptamer-PAT changes to 'lock' the gate, consequently reducing the electron flow toward the electrode surface. Impedance of the flow of electrons is proportional to the concentration of PAT. The linear range was 1-25 ng/ml and the LOD and LOQ were 2.8 and 4.0 ng/l, respectively. Selectivity of the aptasensor was confirmed with a mix of mycotoxins comprising ZEN, OTA, ochratoxin B (OTB) and AFB_1 in apple juice substrate. Samples were vortexed with ethyl acetate and centrifuged. After the supernatant was dried, diluted in PBS and filtered, PAT standard was added. Up to 99% toxin recovery was achieved.

Ma *et al.* (2018) reported a fluorescent assay using carboxyfluorescein (FAM)-labelled PAT aptamer (FAM-AptPAT) which was adsorbed to the surface of a magnetised reduced graphene oxide (rGO-Fe_3O_4). The immobilisation process quenches the fluorescence of FAM, but in the presence of PAT, FAM-AptPAT is stripped from the rGO-Fe_3O_4 surface and the fluorescence of FAM is restored. DNase I was used for amplification of the fluorescence, increasing the sensitivity of the method for the detection of PAT. In the method, a FAM-AptPAT tris-buffer solution containing NaCl, KCl, and $CaCl_2$ is heated. An rGO-Fe_3O_4 solution is mixed with FAM-AptPAT solution to form a FAM-AptPAT-rGO-Fe_3O_4 complex. Different concentrations of PAT are added, the mixture is incubated, and the FAM-aptPAT-rGO-Fe_3O_4 is magnetically separated, after which the fluorescence intensity of the supernatant is measured. Recycling of DNase I-assisted target leads to a strong amplification of fluorescence and consequently to an assay with a LOD of 0.28 µg/l, about 13 times lower than that achieved without using DNase I. The method is rapid, sensitive and selective. However, the fluorescein labelled aptamer and enzyme are expensive.

He and Dong (2018) described an aptamer-based assay for PAT. A gold electrode (AuE) was modified with a composite made from ZnO nanorods (ZnO-NRs) and CS. The ZnO-NRs/CS composite was prepared by adding ammonia to a zinc acetate dihydrate solution, heating and then drying to obtain ZnO-NRs. ZnO-NRS and CS were dispersed in an acetic acid solution with ultrasonication to produce a ZnO NRS-CS homogenous suspension. ZnO NRS-CS was precipitated, and dried on the AuE using an infrared lamp to obtain ZnO NRs-CS/AuE. Electrodeposition of gold nanoparticles (DpAu) was performed to obtain DpAu/ZnO NRs-CS/AuE which was coupled with PAT-Apt to form Apt/DpAu/ZnO NRs-CS/AuE. To block the remaining binding sites of DpAu/ZnO NRs-CS/AuE 6-Mercapto-1-hexanol (MCH) solution was applied to the electrode. The surface of AuE was then rinsed thoroughly with Tris-HCl buffer to obtain MCH/DpAu/ZnO NRs-CS/AuE. In the presence of PAT, it will form a complex with the aptamer on the electrode surface that hinders electron transfer from the electrode to the redox probe hexacyanoferrate and reduces current, typically measured at 0.176 V (vs Ag/AgCl). The linear range was from 0.5 pg/ml to 50 ng/ml PAT and the detection limit was 0.27 pg/ml for the determination of PAT in spiked apple juice samples. Recoveries of the added PAT ranged from 96 to 105%.

Xu *et al.* (2019) described an aptamer-based impedimetric assay for PAT developed after evaluation of different functionalised electrodes. A glassy carbon electrode (GCE) was prepared via modification with black phosphorus nanosheets (BP NSs) and PAT aptamer. The electrode was further functionalised with AuNP to improve sensor performance. Detection of PAT was based on the variation of electron transfer resistance at the modified GCE surface. For the first electrode, the linear range was from 1.0 nM to 1.0 µM, with an LOD of 0.3 nM. The second electrode displayed improved performance, with a wider linear range (0.1 nM to 10.0 µM) and lower LOD (0.03 nM). Both assays were used to analyse fortified apple juice samples. Unfortunately, the authors did not report assay sensitivity and recovery in relevant units of concentration on a mass or volume basis for the juice samples, so the relevance of this technique for compliance testing is not clear. This method takes advantage of the high surface area of the BP NSs to increase the loading of AuNP and aptamers on the electrode surface, effectively amplifying the signal.

Huang *et al.* (2019) developed an MIP electrochemical sensor for determination of PAT. To construct the sensor, nitrogen doped graphene (NGE), platinum nanoparticle aqueous solution (PtNP, $H_2PtC_{l6}{\cdot}6H_2O$), hydrochloric acid, and ascorbic acid were ultrasonicated and centrifuged to obtain a black precipitate (PtNP-NGE) that was combined with N,N-dimethylformamide and thionine. After centrifugation the residues were dispersed in water to form a homogeneous thionine-PtNP-NGE suspension solution that was coated onto a GCE. The prepared thionine-PtNP-NGE/GCE was immersed in PBS solution containing thionine, patulin and $H_2PtCl_6{\cdot}6H_2O$. The electropolymerisation cycle was run 15 times. Afterwards, the polymer modified electrode was soaked in sulfuric acid to remove the template molecules (PAT) and MIP/thionine-PtNP-NGE/GCE was obtained. Thionine acted not only as a functional monomer for the MIP, but also as a signal indicator. Enhanced sensitivity was obtained by combining the electric conductivity of PtNPs, NGE, and thionine with multisignal amplification. The working range in PBS was 0.002-2 ng/ml with a detection limit of 0.001 ng/ml for PAT in PBS. The electrochemical sensor was used for the determination of patulin in real apple and grape juice samples. The samples were passed through a 0.22 µm nylon filter and the filtrate was diluted with PBS, and used for analysis. The recoveries were 100-113% for apple juice and 95-105% for grape juice samples. Sample

preparation for use with these electrodes was very simple, as it only included filtration and dilution with PBS.

10. Trichothecenes

From the eight most interesting papers dealing with trichothecenes analysis published from mid-2018 to mid-2019, only two presented chromatographic separation followed by MS or UV detection. Two papers were focused on immunochemical-based screening, two studies introduced biosensor-based detection, and two publications described simple and fast screening by utilising infrared mass spectroscopy and electronic nose technology.

The paper of Jiang *et al.* (2018), briefly discussed in section 3, focused on determination of multiple mycotoxins (of which nine were from the trichothecenes group) in maize and wheat by UHPLC–MS/MS, after purification of the aqueous-acetonitrile-citric acid extract by newly developed carbon nanotube-based SPE. Optimisation of several key parameters affecting the performance of the SPE procedure was the main aim of the study, where the types and filling amounts of the MWCNT and combinations with other different adsorbents (C_{18}, hydrophilic-lipophilic balance, cationic exchange, silica gel, and amino-propyl), were of the highest importance. The final method arrangement included 20 mg carboxylic MWCNT and 200 mg C_{18}. The recoveries and RSDs of the method ranged between 76-107 and 0.8-8.2%, respectively, with LOQs between 0.5 and 25 µg/l for all of trichothecenes. Generally it is worth highlighting that reporting of LODs/LOQs in 'weight to volume' units is not very useful for readers; it is much more valuable to report performance characteristics on a sample mass basis because the performance of the whole method, including the sample preparation, is reflected in this manner. In this particular study, the calculated matrix equivalent in the sample extract was 1 µg/kg, so LOQs were 0.5-25 µg/kg on a sample mass basis (Jiang *et al.,* 2018). Combination of the features of MWCNTs with other adsorbents has been shown to be a promising sorbent SPE material in recent years. When comparing to the majority of conventional SPE procedures, the developed method includes only one handling step, i.e. passing the extract through polypropylene tubes filled with sorbent between two frits. Although in this particular study, the method precision was excellent, the general problem of such 'in-house' clean-up methods can be the lower method ruggedness caused by less-repeatable column manufacturing (especially sorbent mixing and filling). This problem is eliminated by utilisation of commercially available products of similar 'one-step' arrangement, however, considerably higher costs have to be expected.

A HPLC-UV based method for simultaneous determination of DON, nivalenol (NIV), and their 3-β-D-glucosides ((DON-3G) and NIV-3-glucoside (NIV-3G)) in baby formula and Korean rice wine was developed by Lee *et al.* (2019). The method included the aqueous-acetonitrile extraction, immunoaffinity purification by the DON-NIV^{WB} columns (Vicam, Milford, MA, USA), HPLC separation on a C18 column with aqueous formic acid/methanolic gradient, and UV detection. The method accuracy expressed as analyte recovery ranged from 79 to 107%, RSD was less than 12% for all the analytes, and LODs and LOQs were below 4.4 and 13.3 µg/kg, respectively. The validated method was successfully applied to the analysis of 31 baby formulas and rice wines marketed in Korea, revealing the presence of DON-3G and NIV-3G in one sample of baby formula, at concentration levels of 13.5 and 9.8 µg/kg, respectively. The strongpoint of the method lies in the possibility to quantify both of the glycosylated type B trichothecenes at low concentrations in rather complex food matrices. While the analytical standards of DON-3G are commercially available, the glucosides of other trichothecenes, including NIV-3G, are still not available for purchase. For the purpose of this particular study, the NIV-3G standard was enzymatically synthesised and purified at the co-author's institution.

Moving from chromatographic methods to immuno-chemical-based screening, there are two studies that focused on detection of DON in different food and feed matrices. The first study of Zhou *et al.* (2019) describes the screening of DON in beer and maize extracts by indirect competitive immunoassay with europium-labelled antibodies and magnetic nanoparticles. After incubation of sample extract (or DON standard) with DON polyclonal primary antibody, Eu^{3+}-labelled secondary antibody and the DON-BSA coated magnetic nanobeads, the plate was positioned on a magnet, and the supernatant was discarded. After addition of the enhancement solution, the fluorescence of Eu was measured. This very simple and fast method, in combination with a relatively broad linear range (0.05 to 100 ng/ml), makes the method suitable for high throughput DON screening in cereal samples. Unfortunately, the cross-reactivity towards relevant DON derivatives, such as 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and DON-3G, was not examined, which may indirectly cause a misinterpretation of the data obtained. This is especially crucial in the case of beer, where the contribution of DON derivatives such as DON-3G from malt is expected, the overestimation of DON concentration is highly probable.

In the study of Kong *et al.* (2019), an indirect competitive ELISA and LFD were developed for quantitative analysis of DON in various complex food and feed samples. The different ecologically friendly solvent mixtures based on PBS, ethanol, and water were tested for DON extraction, with method recoveries ranging between 67 and 137%. Unlike the previous study, the authors produced their own anti-DON monoclonal antibody, and tested its cross-reactivity against acetylated DONs and other type B and A

trichothecenes (250 and 1.4% for 3-ADON and 15-ADON, and less than 0.1% for NIV, fusarenone X, HT-2 toxin (HT-2), T-2 toxin (T-2), and diacetoxyscirpenol (DAS)). The cross-reactivity to DON-3G was unfortunately not examined. The LODs were 0.24-0.66 µg/kg for ELISA, and 2.0-47 µg/kg for the LFD, depending on the particular extraction solvent used. The variability in the detection abilities was probably attributable to the different composition of matrix interferences extracted by solvents of different physico-chemical properties. Despite the fact that the ELISA method provided more sensitive and stable results, the main advantage of the developed LFD was the possibility to quantify the results even without the need of strip scan reader (the visual LODs were 10-150 µg/kg), and the simple 'on-site' handling, even by less skilled operators. Although pure water or PBS is usually sufficient for effective extraction of DON, the authors of the study optimised the above-mentioned extraction mixtures with a future vision to extend the method to other less polar mycotoxins, as multiplex detection is definitely the current trend.

Another two selected studies aimed to quantify T-2 in various complex matrices using biosensors, in particular electrochemical immunosensors (Wang *et al.*, 2018b), and aptamer functionalised nanoclusters with fluorescence resonance energy transfer (FRET; Khan *et al.*, 2018). The method of Wang *et al.* describes analysis of T-2 in animal feed and swine meat, where the electrochemical immunosensor based on AuNP, carboxylic group-functionalised single-walled carbon nanotubes and chitosan (AuNPs/cSWNTs/CS) was developed and employed for signal detection. The mechanism of detection was an indirect competitive binding of the anti-T-2 antibody to free T-2 present in standard or sample extract, and T-2-ovoalbumin immobilised on the electrochemical immunosensor surface. The amount of T-2 toxin was quantified through the electrochemical signal induced by binding of the enzyme-labelled secondary antibody and the appropriate substrate reaction. The detection limit of the method was 0.13 ng/ml (corresponding to 3.25 ng/g, when calculating on matrix equivalent in the sample extract). The developed method was verified by analysis of T-2 in fortified swine feed and meat, and showed satisfactory recoveries ranging between 91 and 102%. The specificity of the developed immunosensor was tested against three other mycotoxins, DON, NIV and neosolaniol, and the signal values were negligible. However, evaluation of the cross-reactivity of more structurally similar compounds, such as HT-2, would be more meaningful.

Khan *et al.* (2018) developed a method for sensitive detection of T-2 in wheat and maize extracts, based on one pot synthesis of aptamer functionalised silver nanoclusters (apt-AgNCs), and fluorescent detection of T-2, based on the principle of FRET. In the assay, the apt-AgNCs acts as an energy donor, developing a fluorescence, which is quenched by a molybdenum disulphide nanosheet, acting as fluorescent acceptor. Introduction of T-2 then leads to desorption of apt-AgNCs from the nanosheet, and recovery of fluorescence in a toxin concentration dependent-manner. The developed method showed very broad dynamic quantification of 0.005-500 ng/ml, and very low LOD of 0.93 pg/ml (corresponding to 9.3 pg/g of original sample matrix). Selectivity of the method was tested by examining the fluorescence intensities for DON, FB_1, AFB_1, ochratoxin C, OTB, and ZEN. The response from these mycotoxins did not exceed 20% of the T-2 response.

Generally, it can be said that biosensors seem to provide a simple and very sensitive analysis of trichothecenes, and provide an alternative to immunochemical-based methods as well as instrumental chromatography-based approaches. Nevertheless, the chromatographic methods still remain the gold standard and best tool in terms of selectivity, confirmation, and multiple mycotoxin detection.

The last two studies quoted in this section aimed at fast prediction of mycotoxin presence by non-destructive fingerprinting methods, with minimum demands on sample preparation and expensive laboratory facilities. Both of the studies were focused on analysis of DON in wheat bran samples, and assessed the reliability of classification of samples into 'below-' and 'above-maximum limit' groups, when considering the EU maximum limit of 750 µg/kg for wheat bran (De Girolamo *et al.*, 2019; Lippolis *et al.*, 2018). In the first study of Girolamo *et al.*, the applicability of Fourier transformed near-infrared (FTNIR) and mid-infrared (FTMIR) spectroscopy, in combination with advanced chemometric methods as partial least squares-discriminant analysis and principal component-linear discriminant analysis, was investigated. Depending on the classification model, overall discrimination rates were from 87 to 91% for FTNIR and from 86 to 87% for the FTMIR spectral range (De Girolamo *et al.*, 2019). In the second study of Lippolis *et al.* (2018) prediction of DON contamination was realised by use of a metal oxide semiconductor-based electronic nose, in combination with discriminant function analysis. The recognition ability of the developed cross-validated model was 89%. Moreover, several markers, mainly acetic acid hexyl ester and 2,3-butanediol, associated with an increased DON content, were identified by a HS-SPME/GC-MS technique (Lippolis *et al.*, 2018).

Both of these fast screening approaches examined, in combination with appropriate mathematical-statistical modelling, were shown to be a useful tool for rapid assessment of compliance of DON with EU regulations, which possess a potential to reduce the economic burden on laboratory bodies by reducing the number of costly instrumental analyses.

11. Zearalenone

The scientific literature published from mid-2018 to mid-2019 on ZEN detection in food, feed, and biofluids, can be described as strongly polarised. On one hand, advanced LC-MS methods are used for analysing ZEN together with a number of other mycotoxins and food toxicants under the multi-toxin approach. On the other hand, a number of immune-based methodologies have been proposed for the development of rapid detection assays.

The performance of MS based multi-toxin methods are improving year by year in terms of the number of analytes covered, sensitivity, and quality control parameters, while instruments are becoming more affordable, thus allowing a rapid spread of the methods from the academic community to control labs performing routine analysis. As a consequence, MS- and chromatography-based studies focused on the detection of ZEN alone are rapidly declining. However, the number of papers proposing rapid methods or biosensors for detecting ZEN in food and, mainly, feed, are steadily growing.

In consideration of the high sensitivity due to the fluorescence quenching mechanism, QD technology is often exploited for the development of LFDs. However, the main pitfall of most studies published so far, detection parameters of QD-based LFDs are often tested only on mycotoxin solutions, without a thorough evaluation of the method performance over time and under real conditions. Therefore, although promising, such an approach should be moved to the next stage of validation, before entering the market and being used for routine analysis. Among the advancements reported in the public literature this year, an interesting improvement in QD-based assays was proposed by Li *et al.* (2018) for the development of an LFD based on carbon dots and silver nanoparticle FRET system. The underlying molecular mechanism involved the energy transfer from carbon dots emitting at 459 nm towards silver nanoparticles absorbing at 430 nm. The conjugation of carbon dots with ovalbumin (donor signal probe) and of silver nanoparticles with a ZEN-specific antibody (acceptor signal probe) allowed for the recognition of ZEN in cereals with LODs in the range 1-2.5 µg/kg. Although promising, the system was tested on a limited number of samples, and cross-reactivity was tested only for structurally unrelated mycotoxins (i.e. AFB_1, T-2, OTA, DON, FB_1). A similar assay was described for the application of ZEN-imprinted particles based on the encapsulation of carbon QDs with high luminescence properties, in a hydrophilic silica matrix (Shao *et al.*, 2018). The advantages of such QD-particles over other QDs can be found in the excellent molecular-specific fluorescence quenching, which was responsible for the good sensitivity (LOD: 20 µg/l as S/N 3:1) of the proposed system. However, although the validation parameters reported by the authors were satisfactory in terms of recovery (78-105%) and specificity, it should be noted that the method performance was tested only on spiked maize samples. In addition, specificity was challenged towards other mycotoxins, without taking into consideration possible interference from compounds with similar structures.

A promising bioprinting strategy was followed by Pidenko *et al.* (2018). After a denaturation step, proteins were reorganised as scaffolds for the generation of binding cavities with a high specificity against ZEN. The bioprinting was obtained using ZEN as the template compound, and glutaraldehyde as the crosslinker. Afterwards, the imprinted protein was immobilised on a microwell plate or prepared in a tube, and used for detecting ZEN in naturally incurred maize and wheat samples at contamination levels of about 100 µg/kg. The same group further exploited the bioimprinting approach together with QD sensing, for the implementation of a multiplex immunosorbent assay for the simultaneous detection of ZEN and DON in wheat and maize (Belaglazova *et al.*, 2019). LODs of 100 µg/kg for ZEN and 700 µg/kg for DON were obtained in naturally incurred samples, and results were successfully confirmed by LC-MS/MS.

Although still preliminary, the design of bioimprinted proteins may offer an alternative to the antibody-based detection and clean-up. Because the binding pocket is stabilised by covalent crosslinks, imprinted proteins may overcome several technical issues of antibodies such as the instability towards changes in pH and temperature. Therefore, a bioimprinting strategy may be seen as a promising substitute to the use of monoclonal and polyclonal antibodies in immunoaffinity detection.

The design of aptamers is another recognition-based strategy often proposed for ZEN detection in recent years. Usually, aptamers are built up using a systematic evolution of ligands by exponential enrichment methodology. The design is optimised for having high selectivity and good specificity. Among the research studies carried out in the field over the last year, interesting work was performed by Luo *et al.* (2019), who developed an aptasensor based on self-enhanced chemiluminescence. The luminophore was obtained by combining amine-functionalised $Ru(bipyridyl)_3^{2+}$-doped silica nanoparticles and NGE QDs. The main advantage of the system, mainly due to the combination of donor and acceptor probes on the same nanoparticle, was due to the short electron transfer distance and the decrease in energy loss, resulting overall in a higher luminescence efficiency and thus increased sensitivity. Although tested only at a preliminary lab-scale, the assay showed an impressive sensitivity reaching an LOD of 1 pg/l for ZEN standard solution.

An innovative strategy for the bioassay-based detection of ZEN, involves the use of *Photobacterium phosphoreum* bacterial cells as biosensitive element (Senko *et al.,* 2019). *P. phosphoreum* is a gram-negative bioluminescent bacterium often found in marine environment. It can emit bluish-green light (490 nm) following a reaction between flavin mononucleotide, luciferin and oxygen, catalysed by luciferase. The reaction can be quenched by cytotoxic compounds, therefore bioluminescent cells may lose their luminescence when exposed to several toxicants, among them mycotoxins or pesticides. The main improvement proposed by this work is related to the *P. phosphoreum* cells immobilisation in poly(vinyl alcohol), thus enabling detection under flow-through conditions. The proposed bioluminescent flow-through assay allowed for the decrease of the overall time of analysis from 30 to 10 min. The assay was initially tested using a ZEN standard solution prepared in water, in the concentration range 0.026-16.7 mg/l. The use of the assay to prove the enzymatic detoxification of mycotoxins is particularly intriguing amongst possible future applications. Although an optimisation of the analytical parameters and an extensive validation is required before real-life application, the paper demonstrated the existing potential in the use of immobilised bioluminescent cells for the determination of ZEN as well as other mycotoxins. The effect of multiple mycotoxins, as is often observed in samples, needs to be determined in order to fully explore the applicability of this strategy.

As already reported in the previous years, FP in often used for the detection of fluorescent mycotoxins, such as ZEN and OTA. Moving from the very first applications in the field, FP assays have achieved great progress thanks to developments in materials science and labelling techniques, as recently described in the review paper from Zhang *et al.* (2019c). An innovative FP assay was recently reported for the detection of ZEN, its modified forms ZAN, α-ZEL, β-zearalenol, α-zearalanol and β-zearalanol, as well as AFB_1, AFB_2, AFG_1 and AFG_2 in maize flour (Zhang *et al.*, 2018b). The authors developed a dual-wavelength, homologous and high-throughput FP immunoassay, using different fluorescein-labels and broad-specific antibodies, allowing for the group detection of the two mycotoxin classes. In particular, the group-LOD values in maize flour samples were approximately 5 µg/kg for aflatoxins and 11 µg/kg for ZEN and its modified forms. The results obtained in the assay were successfully confirmed by LC-MS/MS. Also in consideration of the short time required for the analysis (overall, <30 min), the multi-toxin group-detection offered by the proposed FP assay is a promising tool for screening of parent and modified mycotoxins in food and feed, prior to multi-toxin MS-based confirmatory analysis.

Besides biosensors, several optical sensors have been proposed for the detection of ZEN as well, usually exploiting its natural fluorescence. Llorent-Martinez *et al.* (2019) proposed a flow-through optosensor, based on a multi-commutated manifold equipped with microbeads able to retain ZEN. The fluorescent signal of ZEN was measured on the solid phase, reaching satisfactory sensitivity levels in order to meet EU maximum limits for feed (EC, 2006b). The detection system was tested on naturally incurred feed, using a QuEChERS procedure for sample preparation. The proposed optosensor represented a quick and easy-to-handle detection tool for assessing ZEN in feed. The main advantage of the system was that it did not require antibodies or other biosensing agents, thus having increased stability under real-life fluctuations in pH, temperature, and humidity.

Among spectroscopic techniques, Raman spectroscopy has been often proposed for mycotoxin analysis. However, due to the low sensitivity of this fingerprinting technique compared to chromatography, a strong chemometric data handling is usually required. Guo *et al.* (2019b) explored the feasibility of Raman-based screening for detecting ZEN in maize. Several multivariate models were built and compared for data treatment, i.e. partial least squares (PLS), synergy interval PLS (siPLS), and colony optimisation PLS (ACO-PLS). The best model was based on siPLS-ACO due to its accuracy. The LOD was unsatisfactory according to the authors; however, no values were provided to support this claim. In addition to poor sensitivity, another important drawback is the extensive data treatment required, which can be performed only by trained staff. However, Raman spectroscopy coupled with multivariate statistics allows for an in-situ non-destructive analysis of large sample batches, thus showing a good potential for industrial use as a high-throughput screening tool.

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Conflict of interest

The authors declare no conflict of interest.

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