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Review

Trace mycotoxin analysis in complex biological and food matrices by liquid chromatography–atmospheric pressure ionisation mass spectrometry

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Abstract

Mycotoxins are toxic secondary metabolites produced by filamentous fungi that are growing on agricultural commodities. Their frequent presence in food and their severe toxic, carcinogenic and estrogenic properties have been recognised as potential threat to human health. A reliable risk assessment of mycotoxin contamination for humans and animals relies basically on their unambiguous identification and accurate quantification in food and feedstuff. While most screening methods for mycotoxins are based on immunoassays, unambiguous analyte confirmation can be easily achieved with mass spectrometric methods, like gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS). Due to the introduction of atmospheric pressure ionisation (API) techniques in the late 80s, LC/MS has become a routine technique also in food analysis, overcoming the traditional drawbacks of GC/MS regarding volatility and thermal stability. During the last few years, this technical and instrumental progress had also an increasing impact on the expanding field of mycotoxin analysis. The aim of the present review is to give an overview on the application of LC–(API)MS in the analysis of frequently occurring and highly toxic mycotoxins, such as trichothecenes, ochratoxins, zearalenone, fumonisins, aflatoxins, enniatins, moniliformin and several other mycotoxins. This includes also the investigation of some of their metabolites and degradation products. Suitable sample pre-treatment procedures, their applicability for high sample through-put and their influence on matrix effects will be discussed. The review covers literature published until July 2006.

Keywords: Mycotoxin; Metabolite; Degradation product; Bioadduct; Bioconjugate; Trichothecene; Ochratoxin; Zearalenone; Zeranol; Fumonisin; Aflatoxin; Patulin; Moniliformin; Enniatin; Beauvericin; Mycophenolic acid; Mass spectrometry; High-performance liquid chromatography; LC/MS; Electrospray ionisation; Atmospheric pressure ionisation; Atmospheric pressure chemical ionisation; Atmospheric pressure photoionisation; ESI; APCI; APPI

Abbreviations: 3-/15-AcDON, 3-/15-acetyldesoxynivalenol; ACN, acetonitrile; AcOH, acetic acid; AFB_{1-2}, aflatoxin B_{1-2}; AFG_{1-2}, aflatoxin G_{1-2}; AFM_1, aflatoxin M_1; AFQ_1, aflatoxin Q_1; AFP_1, aflatoxin P_1; APCI, atmospheric pressure chemical ionisation; API, atmospheric pressure ionisation; APPI, atmospheric pressure photo ionisation; BEA, beauvericin; CID, collision induced dissociation; CRM, consecutive reaction monitoring; DAS, diacetoxyscirpenol; DOM-1, deepoxydesoxynivalenol; DON, desoxynivalenol; EA, EA_1, enniatin A, enniatin A_1; EB, EB_1, enniatin B, enniatin B_1; ELISA, enzyme-linked immuno sorbent assay; ESI, electrospray ionisation; FAB, fast atom bombardment; FA_{1-3}, fumonisin A_{1-3}; FAK_1, fumonisin AK_1; FB_{1-5}, fumonisin B_{1-5}; FC_{1-4}, fumonisin C_{1-4}; FD, fumonisin D; FP_{1-3}, fumonisin P_{1-3} = 3-hydroxypyridinium FC_{1-3}; FDA, food and drug administration; FL, fluorescence detection; FUS, fusaproliferin; F-X, fusarenone X; GC–ECD, gas chromatography–electron capture detection; GC/MS, gas chromatography/mass spectrometry; HFB_1, fully hydrolysed fumonisin B_1; HT-2, HT-2 toxin; IAC, immunoaffinity chromatography; LC/MS, liquid chromatography/mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MAS, monoacetoxyscirpenol; MeOH, methanol; MON, moniliformin; MPA, mycophenolic acid; MS/MS, tandem mass spectrometry; NanoESI, nano electrospray ionisation; NEO, neosolaniol; NIV, nivalenol; OTA, ochratoxin A; OTB, ochratoxin B; OTC, ochratoxin C; OTα, ochratoxin α; OTβ, ochratoxin β; PB, particle beam; $PHFB_1$, partially hydrolysed FB_1; QTrap, combination of triple quadrupole and ion trap mass analyser; RP, reversed-phase; SFC, supercritical fluid chromatography; SFE, supercritical fluid extraction; SIM, selected ion monitoring; SPE, solid-phase extraction; SRM, selected reaction monitoring; T-2, T-2 toxin; TFA, trifluoro acetic acid; TLC, thin-layer chromatography; TSP, thermospray; VER, verrucarol; WHO, World Health Organisation; ZAN, zearalanone; α-ZAL, α-zearalanol = zeranol; β-ZAL, β-zearalanol = taleranol; α-ZOL, α-zearalenol; β-ZOL, β-zearalenol; ZON, zearalenone

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Contents

1. Introduction

Mycotoxins are toxic secondary metabolites produced by about 200 identified filamentous fungi, as e.g. *Fusarium*, *Aspergillus* and *Penicillium* species, growing under a wide range of climatic conditions on agricultural commodities (grains, spices, fruits, coffee, nuts, etc.) in the field and during storage [1,2]. Their occurrence in food, beverages and feed has

been recognised as potential threat to human and animal health, either caused by direct contamination of plant materials or products thereof [1–3], or by "carry over" of mycotoxins and their metabolites into animal tissues, milk and eggs after intake of contaminated feed [4,5].

Although evidence of mycotoxicoses can be traced back to ancient times and the middle ages (ergotism), it was, however, not before 1960, that mycotoxins were identified as potential health hazard, when 100.000 turkeys died from an acute necrosis of the liver after consuming groundnuts infected by *Aspergillus flavus* and contaminated by aflatoxins (X-disease) [6]. The toxic properties of mycotoxins in humans and animals include severe nephrotoxic, neurotoxic, carcinogenic, immunosuppressive and estrogenic effects. Less critical compounds and chronic intake of small amounts of mycotoxins may reduce feed intake and weight gain in animals and cause diarrhoea in humans [7]. Several endemic diseases in Asia, Africa and Europe are now correlated to acute mycotoxin intoxication, such as Kwarshiorkor and Reye's syndrome (damage to liver and kidney caused by aflatoxins) and Balkan Endemic Nephropathy (tumours of the upper urinary tract caused by ochratoxin A) [8].

Some hundred different mycotoxin species have been discovered so far exhibiting a great structural diversity. Most of them offer considerable thermal and chemical stability. They cannot or can only partly be removed by food processing or by other suitable decontamination procedures [7]. Today, aflatoxins, trichothecenes, ochratoxin A (OTA), zearalenone (ZON), fumonisins, moniliformin and patulin receive by far the greatest attention due to their frequent occurrence and their severe effects on animal and human health [1,2,7]. Factors contributing to their presence or production in food and feed include storage, environmental and ecological conditions, as temperature, relative humidity, the substrate and the use of fungicides, though the interrelations between all these factors are not yet well understood and toxin production cannot reasonably be predicted.

Surveillance studies showed that mycotoxin contamination is a world-wide problem [9,10]. It is estimated that 25% of the world's crop production and 20% of crop production within the European Union may be contaminated with mycotoxins [3]. Economic losses deriving from that are tremendous including reduction of livestock production and agricultural production, health care, veterinary and regulatory costs. The situation is considered less severe in Europe and Northern America, due to technical, educational and climatic reasons [7]. Measures have been set up by authorities in many countries to monitor and control mycotoxin levels, especially for agricultural import products from third world countries. Maximum tolerable levels and guideline levels have been established for aflatoxins, OTA, ZON and desoxynivalenol (DON) down to the ppb to ppt level in different food and feed products [7,11–15]. Respective levels are under debate for other mycotoxins.

Driven by regulatory authorities, like the European Commission and the Food and Drug Administration (FDA), extensive analytical efforts have been made to enable fast and reliable analysis of a large number of samples for surveillance and monitoring purposes on the ppb to ppt level in a wide variety of complex food environments. Especially for the highly toxic and carcinogenic aflatoxins, fast and highly sensitive (ppt level) screening assays based on immunochemical methods are commercially available [16–18]. Similar test kits which are easily applicable to automation are also available for OTA and ZON as well as for other relevant mycotoxins, enabling the screening in complex food and feed matrices [16–18]. In this context chromatography coupled to different detection principles is used to confirm unambiguously positive findings and to enable exact quantification when a maximum tolerable level or a relevant guideline level has been exceeded. Depending on the nature of the mycotoxin different analytical approaches are preferred, as GC–electron capture detection (GC–ECD) for trichothecenes [19,20] and HPLC-fluorescence (FL) detection for ZON [21], OTA [22], fumonisins [23] and aflatoxins. A variety of different sample clean up protocols have been established in order to remove sufficiently the food/feed matrix and/or to concentrate the target analytes [19–24].

Unambiguous analyte confirmation by mass spectrometry still relies in mycotoxin analysis to a considerable extent on gas chromatographic–mass spectrometric (GC/MS) approaches, as published recently for trichothecenes [19,25,26], OTA [22,27], ZON [28], patulin [29] and fumonisins [23]. In contrast, liquid chromatographic/mass spectrometric (LC/MS) methods have rarely been used before 1996 predominantly applying thermospray (TSP), particle beam (PB) and fast atom bombardment (FAB) interfaces for trace analysis at ppb level of trichothecenes [30–32], fumonisins [33–35], aflatoxins [36] and ZON [31]. This situation changed rapidly from the mid 90s onwards when the benefits of atmospheric pressure ionisation (API) interfaces were realised compared to LC–TSP/MS and GC/MS. Atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) LC/MS became widely used in environmental and food analysis, owing to their robustness, easy handling, high sensitivity, accuracy and analyte selectivity and their compatibility with almost the whole range of compound polarities [37–39]. Consequently, GC/MS methods and LC methods coupled to less selective detectors have been increasingly replaced by LC–API/MS, in order to reduce considerably, or even to omit, sample preparation or/and derivatisation. More polar mycotoxins deriving from degradation, transformation and metabolism became accessible to structural elucidation, confirmation and quantification. This opened a completely new and more efficient approach to monitor the distribution of mycotoxins on trace levels in complex biological and food surroundings [37–39]. In general, GC/MS and LC/MS are now commonly accepted by authorities as highly reliable analyte confirmation tools in residue analysis in the course of regulatory and legal proceedings [40]. For this purpose a set of identification criteria has just recently been proposed by the European Commission basically relying on a system of identification points with e.g. one precursor ion and two product ions and their ratios being the minimum requirement for unambiguous analyte identification by LC/MS/MS [40–42].

The present review gives an overview on the impact of modern LC/MS methodology in the field of mycotoxin research and analysis. This includes all relevant groups of mycotoxins, as

trichothecenes, OTA, ZON, fumonisins, aflatoxins and others and covers both, quantitative (trace analysis) and qualitative (structural elucidation) aspects in complex biological matrices. In view of higher sample through-puts, emphasis is given to sample pre-treatment procedures that are frequently also the bottle-neck of a selective LC/MS method, and their applicability to automation. Furthermore, the influence of sample clean up on the accuracy and sensitivity of LC/MS results (matrix effects) is discussed.

2. Trichothecenes

Trichothecenes are a group of mycotoxins produced particularly by *Fusarium* moulds. Around 190 different structures have been discovered so far having all in common a tetracyclic, sesquiterpenoid 12,13-epoxytrichothec-9-ene ring system. They are divided into four groups which are characterised by specific structural features: type A: without a carbonyl group at position C-8, type B with a carbonyl group at C-8 (see Fig. 1), type C with a second epoxy group and type D with a macrocyclic structure [43].

The main source of trichothecene contamination in food and feedstuff are cereals (maize, oats, barley and wheat) which are infected by *Fusarium* fungi. While type A and B trichothecene are widely distributed in these commodities, the type C and D trichothecenes, though more toxic, occur only rarely in food and feed. In this respect, T-2 toxin and HT-2 toxin as type A representatives and desoxynivalenol (DON) and nivalenol (NIV) as type B representatives have gained most attention due to their high toxicity and their prevalent occurrence.

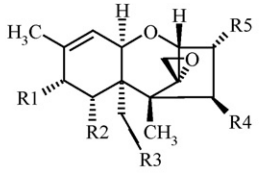
Trichothecenes initiate a wide range of effects in farm animals and man, as reduced consumption of feed, skin irritation, diarrhoea, multiple haemorrhage and immunosuppressive effects [2]. Several diseases have been directly correlated to trichothecene intoxication, such as the outbreak of alimentary toxic aleukia in Russia in 1913 and 1944. Due to its toxicity and frequent occurrence, several countries have established legal regulations or recommendations for DON, HT-2 toxin and T-2 toxin [44–46]. The FDA set up an advisory level of 1000 μg/kg in cereal products dedicated for human consumption [45], whereas various European regulations recommend maximum levels of DON between 100 and 1000 μg/kg for human consumption and 400 and 5000 μg/kg in feeding stuff [46].

2.1. Conventional trichothecene analysis

Accurate and reliable detection of trichothecenes is, therefore, required down to 100–500 μg/kg range in naturally contaminated samples, while a lower detection limit of 10–50 μg/kg is desirable in surveys, in order to establish data for risk assessment [47]. Suitable analytical methodology which has been summarised in two previous reviews [47,48] relies predominantly on GC/MS or GC–ECD following different sample clean up and derivatisation procedures. Major advantages of these methods are their high separation efficiency in mixtures and the cost effective and relatively easy way to achieve reliable results for type A and B trichothecene mixtures [48]. LC based methods without MS detection have less frequently been applied, mainly due to the low sensitivity of trichothecenes towards UV detection and the relatively low separation efficiency of HPLC compared to GC when multi trichothecene analysis is required. Furthermore, ELISA assays though frequently used for screening purposes, have recently been found to produce inaccurate quantitative data and to overestimate significantly trichothecene levels in biological samples [49].

2.2. Early LC/MS technology in trichothecene analysis

Early LC/MS methodology in trichothecene analysis relied predominantly on thermospray ionisation [30–32,50–53] while direct fluid injection [54,55], dynamic FAB [30,50,56], the moving belt technology [57], plasmaspray [30] and PB ionisation [58] were only rarely applied. Most of these papers deal with investigations of matrix-free reference solutions and focus on the mass spectrometric properties of trichothecene molecules, as ionisation efficiency [31,54], in-source fragmentation [31,32,55] and collision-induced dissociation (CID) reactions [55]. Only a few investigations were performed in biological matrices, as cereals and other plant materials [31,50,55], urine and faeces [32]. Depending on the individual trichothecene



	R1	R2	R3	R4	R5
Type A					
HT-2	O-isovaleryl	H	O-acetyl	OH	OH
T-2	O-isovaleryl	H	O-acetyl	O-acetyl	OH
Type B					
Nivalenol (NIV)	=O	OH	OH	OH	OH
Desoxynivalenol (DON)	=O	OH	OH	H	OH
3-AcetylDON (3-AcDON)	=O	OH	OH	H	O-acetyl
15-AcetylDON (15AcDON)	=O	OH	O-acetyl	H	OH

Fig. 1. Structures of type A and B trichothecenes.

molecules and the way of sample clean up and preconcentration, most of the methods provided detection limits in the ppb range. In this respect, LC/MS proved to be especially useful for structural elucidation and confirmation, as e.g. demonstrated for trichothecene metabolites in rat, cow and hen [32]. In contrast, quantitative aspects played only a minor role in all theses studies and validated quantification procedures were completely missing. Furthermore, interface technology was not robust enough to handle a large number of highly contaminated biological samples.

2.3. Modern LC/MS analysis of trichothecenes

2.3.1. Investigated trichothecene analytes and typical matrices

Modern LC/API–MS instrumentation has recently been applied to a wide range of different type A and B trichothecenes (Table 1). These papers were focused on the wide-spread NIV and DON [59–79], but also dealt with other type A and B trichothecenes occurring with relatively high incidence in agricultural commodities, including T-2 toxin [59,61,68–70,72,74,75,78,80,81], HT-2 toxin [61,70,72,74,75,78,80,81], acetyl T-2 toxin [67], 3-acetyl DON (3-AcDON) [61,64,65,68–70,72–75,78], 15-acetyl DON (15-AcDON) [61,65,68–70,72–75,78,79], monoacetoxyscirpenol (MAS) and diacetoxyscirpenol (DAS) [59,61,72,74,75,78,80,81], fusarenone X (F-X) [64,65,68,69,72–75,78] and neosolaniol (NEO) [61,67,72]. Most trichothecene analyses by LC/MS were performed down to the low ppb level in grains and products thereof, like corn meal, rolled oats or feed where trichothecene contamination is most likely to occur (Table 1). Very recently, Berthiller et al. detected DON-3-glucopyranoside in wheat and maize [82]. They found that this plant metabolite contributes to about 10% to the natural overall DON contamination in these grains, even exceeding the 3- and 15-AcDON levels. Since this compound is easily hydrolysed during digestion it should also be considered with regard to food and feed safety. Besides, Berger et al. and Bily et al. investigated a couple of different type A and B trichothecenes in fungal cultures [61,79] while a limited number of other groups determined carry over of DON into pig and human urine [65,76], pig serum [79], insect larvae [79], eggs [77], milk [70] and beer [83]. In this context, Razzazi-Fazeli et al. [65] and Sorensen and Elbaek [70] monitored together with DON also its major *in vivo* metabolite, deepoxydesoxynivalenol (DOM-1), in order to estimate more accurately actual DON contamination in pig fed with mycotoxin contaminated oats or the carry over rate of DON into milk. Finally, Young et al. investigated the oxidative degradation of type A and B trichothecenes in aqueous ozone. Degradation kinetics as well as structures of the main degradation products were determined by LC/MS/MS. Purpose of this study was to find a new chemical pathway to reduce trichothecene levels in contaminated food and feedstuff [75].

In distinct contrast, rarely occurring type C and D trichothecenes did not attract any attention, except for one paper of Tuomi et al. dealing with the determination of macrocyclic trichothecene mycotoxins in indoor environments, as building materials, gypsum boards and wooden materials [84].

2.3.2. Sample preparation

Sample preparation prior to LC/MS trichothecenes analysis is relatively simple and easy due to the high MS selectivity (Table 1). Solid matrices, like grains, are frequently extracted with acetonitrile/water mixtures of different ratios sometimes also applying pressure in order to speed up and to improve the extraction process [71]. Typically, extracts are afterwards cleaned with one or two MycoSep columns (mixture of charcoal, ion-exchange resins and other materials [85]) [60–62,65,68,69,71,72,74,80–83]. Biselli et al. reported improvement of trichothecene A recoveries when MycoSep columns were additionally washed after use [68,69].

Alternatively, solid-phase extraction (SPE) has been described for sample extract clean up applying either carbograph-4 material [64,73], immunoaffinity material [76,77], polymeric absorbent materials with RP and anion exchange functionalities [70] or reversed-phase materials partly in combination with a preceding liquid/liquid extraction step [77,84]. Royer et al. compared different absorbent materials and found MycoSep columns best suited for DON analysis when extraction is performed under pressurised conditions [71]. In general, to avoid underestimation of actual DON levels in urine (and possibly in other biological liquids), any kind of clean up should be preceded by a β-glucuronidase digestion since a considerable part of urinary DON was found as its polar glucuronide conjugate which is not well retained on non polar SPE sorbent materials [76].

Huopalathi et al. introduced supercritical fluid extraction (SFE) as suitable extraction/sample clean up tool for critical matrices like feed. By defatting the SFE extract with a further liquid/liquid extraction step with hexane they achieved sufficient LC/MS sensitivity in the medium ppb range [59]. Plattner and Maragos even proposed to omit any kind of sample clean up [62,63]. They injected the crude acetonitrile/water extract from grain materials into the LC/MS/MS system. Quantitative data down to the 50 ppb level were readily achieved when the crude extracts were diluted with water or, alternatively, when the liquid chromatographic separation of the analytes from co-eluting matrix components was strikingly improved. The method seems to work well for the determination of NIV and DON in grain though neither LC/MS chromatograms nor any method validation data were presented. Interestingly, the authors reported that an additional sample clean up step with MycoSep columns did not improve the quality of the developed method.

2.3.3. Typical LC conditions for LC/MS analysis

HPLC separation prior to MS detection is of major importance since trichothecene analysis typically deals with two or more analytes in complex biological surroundings. It exclusively relies on reversed-phase materials (RP-18) with acetonitrile/methanol/water [59,60,64,65], methanol/water [61–63,66,68,69,72,74,75–77,80,82,84] and acetonitrile/water mixtures [67,71,73,78,81,83] as mobile phases in both, the isocratic and gradient mode (Table 1). In this context, methanol was reported to enhance MS sensitivity especially for type B trichothecenes when compared to acetonitrile [68,69,74]. Acetic acid and ammonium acetate are predominantly applied as additives either

Table 1
Overview on LC–MS methods in trichothecene analysis

Analytes	Matrix	Sample preparation	Liquid chromatography		Mass spectrometry		Ref.
			Column	Mobile phase/additives	Ionisation/ion selection	Scan mode	
DON, DAS, T-2	Feed, corn meal, rolled oats	SFE with CO_2 + 5% MeOH, defatting by liquid/liquid extraction with hexane	RP-18	Isocratic: aqueous 3 mM NH_4OAc/MeOH/ACN—50:45:5	ESI + triple quadrupole	Full scan, SIM, SRM	[59]
10 macrocyclic trichothecene	Building materials, paper, gypsum, wooden materials	Extraction with MeOH/H_2O—95:5, liquid/liquid extraction with hexane, SPE with RP-8 columns	RP-18	Gradient: H_2O with 10 mM NH_4OAc/MeOH with 20 μM NaOAc	ESI + ion trap	Full scan, product ion scan	[84]
DON, NIV	Wheat	Extraction with ACN/H_2O—84:16, clean up with MycoSep 227 and 216 columns	RP-18	Isocratic: H_2O/ACN/MeOH—82:9:9	APCI − single quadrupole	Full scan, SIM	[60]
DON, NIV, DAS, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, NEO	Wheat, rice, fungal cultures	Extraction with ACN/H_2O—84:16, clean-up with MycoSep 227 and 216 columns	RP-18	Gradient: H_2O/MeOH + post-column 50 mM NH_4OAc in water for structure elucidation	APCI ± ion trap	Full scan, product ion scan	[61]
DON	Wheat	Extraction with ACN/H_2O—84:16 with and without clean up with MycoSep 225 columns	RP-18	Gradient: H_2O/MeOH + 0.3% AcOH	ESI − ion trap	Full scan, SIM	[62]
T-2, HT-2, AcT-2, DAS, MAS, NEO	Oats, barley, wheat, maize, feed	Extraction with ACN/H_2O—84:16, clean up with MycoSep 227 and 216 columns	RP-18	Gradient: H_2O/ACN both with 1 mM NH_4OAc	APCI + single quadrupole	Full scan, SIM	[81]
DON	Pure standard material	Dissolution in MeOH	RP-18	Gradient: H_2O/ACN	APCI − (APCI +) ion trap	Full scan, SIM	[67]
DON, NIV	Wheat, corn	Extraction with ACN/H_2O—84:16, filtration and dilution with water	RP-18	Gradient: H_2O/MeOH + 0.3% AcOH	ESI − ion trap	Full scan, SIM	[63]
DON	Human urine	Enzymatic deglucuronidation IAC	RP-18	Gradient: H_2O/MeOH	ESI + single quadrupole	Full scan, SIM	[76]
DON, NIV, 3-AcDON, F-X	Maize	Extraction with ACN/H_2O—75:25, SPE with carbograph columns	RP-18	Gradient: H_2O/ACN/MeOH	ESI − (ESI +/APCI ±), triple quadrupole	Product ion scan, SRM	[64]
DON, NIV, 3-AcDON, 15-AcDON, F-X, DOM-1	Maize, pig urine	Extraction with ACN/H_2O—84:16 (maize), clean up with MycoSep 227 columns	RP-18	Gradient: H_2O/ACN/MeOH	APCI − single quadrupole	Full scan, SIM	[65]
DON	Standard material	Dissolution in MeOH/H_2O—1:3	RP-18	Gradient: H_2O/MeOH	ESI + triple quadrupole	Full scan, product ion scan, SIM, SRM	[66]
DON	Wheat, eggs	Wheat: extraction with ACN/H_2O—84:16; egg: extraction with ACN, IAC	RP-18	Gradient: H_2O/MeOH	ESI − triple quadrupole	SRM	[77]
DON	Maize	Accelerated solvent extraction with ACN/H_2O—75:25, clean up with MycoSep 226 columns	RP-18	Gradient: H_2O with 5 mM NH_4OAc and HCOOH, pH 4/ACN	APCI + ion trap	Product ion scan, SRM	[71]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, NEO, DAS	Cereals	Extraction with ACN/H_2O—80:20, clean up with MycoSep 225 column	RP-18	Isocratic: H_2O/MeOH—65:35 with 0.1 mM NaCl	ESI ± single quadrupole	Full scan, SIM	[72]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X	Cereal based food and feed	Extraction with ACN/H_2O—85:15, clean up with MycoSep 226 columns	RP-18	Gradient: H_2O/MeOH	ESI ± (APCI ±), triple quadrupole	SRM	[68,69]
DON, NIV, 3-AcDON, 15-AcDON, F-X	Maize	Extraction with ACN/H_2O—75:25, SPE with carbograph-4 columns	RP-18	Gradient: H_2O/ACN	ESI − triple quadrupole	SRM	[73]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, DAS	Maize	Extraction with ACN/H_2O—84:16, clean up with MycoSep 227 or 226 columns	RP-18	Gradient: H_2O/MeOH each with 5 mM NH_4OAc	APCI ± QTrap	Full scan, product ion scan, SRM	[74]
HT-2, T-2, DAS	Poultry feed	Extraction with ACN/H_2O—84:16, clean up with MycoSep 225	RP-18	Gradient: H_2O/MeOH each with 5 mM NH_4OAc	APCI + QTrap	SRM	[80]
DON, NIV, 3-AcDON, HT-2, T-2	Malt, beer	Malt: extraction with ACN/H_2O—84:16, clean up with MycoSep 227 columns, beer: degassing, SPE with ChemElut 1020 columns	RP-18	Gradient: H_2O/MeOH/ACN with NH_4OAc	ESI ± triple quadrupole	SRM	[83]
DON-3-glucopyranoside, DON, 3-AcDON, 15-AcDON	Wheat, maize	Extraction with ACN/H_2O—84:16, Clean up with MycoSep 230	RP-18	Isocratic: H_2O/MeOH—85:15	APCI − QTrap	Full scan, product ion scan, SRM	[82]
DON, DOM-1, 3-AcDON, 15-AcDON, HT-2, T-2, T-2 triol, DAS, MAS	Milk	Enzymatic deglucuronidation, extraction with ACN/hexane—61:39, SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Gradient: H_2O/MeOH both either with or without 0.02% AcOH partly with 0.1 mM NH_4OAc in MeOH	ESI ± triple quadrupole	Product ion scan, SRM	[70]
DON, NIV, 3-AcDON, 15-AcDON, F-X, T-2, HT-2, NEO, MAS, DAS, T-2 triol, T-2 tetraol	Cereals, bread	Extraction with ACN/H_2O—84:16, clean up with MycoSep 227	RP-18	Gradient: H_2O with 0.00184 mM NH_3 and 0.13 mM NH_4OAc/ACN	ESI ± (APCI ±), triple quadrupole	SRM	[78]
DON, 3-AcDON, HT-2, T-2, 15-AcDON, F-X, MAS, DAS, NEO, VER and oxidation products	Aqueous ozone solution	–	RP-18	Gradient: H_2O/MeOH	APCI + ion trap	Full scan, product ion scan	[75]

DON: desoxynivalenol, NIV: nivalenol, 3-AcDON: 3-acetyldesoxynivalenol, 15-AcDON: 15-acetyldesoxynivalenol, DOM-1: deepoxydesoxynivalenol, HT-2: HT-2 toxin, T-2: T-2 toxin, F-X: fusarenon X, MAS: monoacetoxyscirpenol, DAS: diacetoxyscirpenol, NEO: neosolaniol, VER: verrucarol.

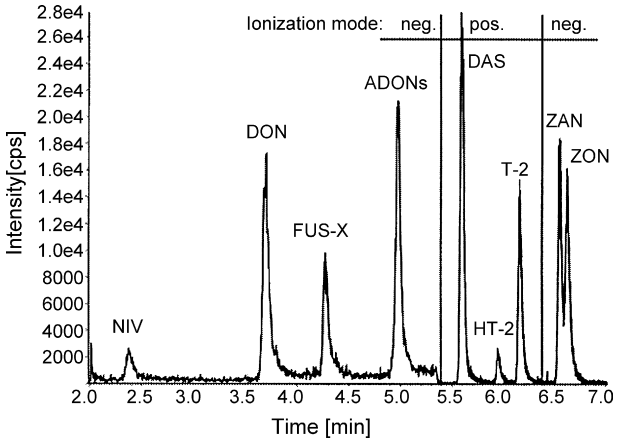


Fig. 2. Total ion LC/MRM chromatogram obtained after clean up with MycoSep 226 columns of a spiked maize sample (each mycotoxin 100 μg/kg). Vertical lines illustrate change of ionisation polarity. (Reproduced with permission from Journal of Chromatography A 1062 (2005) 209. Copyright 2005 Elsevier [74].)

to improve chromatographic separation or to force adduct formation for improved MS sensitivity or structural elucidation [59,61–63,70,71,74,78,81,84].

Depending on the analytes, matrix and column lengths, run times range between 6 and 30 min, e.g. Berger et al. was able to separate nine trichothecene mycotoxins plus two internal standards on a 125 mm RP column within 12 min [61]. Berthiller et al. monitored the presence of eight different trichothecene species together with ZON and zearalanone (ZAN) within 7 min (Fig. 2) [74], and also Tuomi et al. applied LC very efficiently and separated ten macrocyclic trichothecenes plus one internal standard within 20 min [84]. Furthermore, Razzazi-Fazeli et al. addressed the difficult issue to separate mass identical 3- and 15-AcDON isomers and evaluated several different RP-18 materials for this purpose. By applying a ternary gradient with water, methanol and acetonitrile, they were able to achieve baseline separation of both isomers [65]. Just recently, the workgroup of Humpf were able to achieve an excellent separation of this MS critical pair of isomers including the simultaneous analysis of 10 other type A and B trichothecenes although not all other analytes could be baseline separated by the H_2O/ACN/NH_4OAc/NH_3 gradient [78].

Another critical issue seems to be the sufficient separation of polar trichothecenes, like NIV and DON, from disturbing co-eluting matrix compounds. This has been reported in detail by Plattner and Maragos who increased their chromatographic retention to such an extent (∼15 min) that interfering matrix compounds were sufficiently separated from both analytes [63]. On the other hand a more extensive sample preparation protocol is equally feasible to avoid this problem and to elute NIV and DON within 3 min from the column without any matrix interferences [61].

2.3.4. Mass spectrometry

Both, APCI and ESI were successfully applied in the positive as well as in the negative ion mode for the analysis of trichothecenes although this seems to be frequently dependent on the type of trichothecenes and even on the geometry and technical details of the MS instrumentation (Table 1). In this respect, a better and stable ionisation performance may be achieved and interface contamination reduced when especially the matrix and salt contaminated first part of the LC effluent is diverted into waste [70]. Razzazi et al. and other groups demonstrated highest ionisation efficiency for type A trichothecene in the positive ion mode [78,81] and for the type B trichothecenes in the negative ion mode [60,67,78]. This led Berthiller et al. [74] and Klötzel et al. [78] to MS polarity switching between negative and positive ionisation to achieve similar sensitivities for both types of trichothecenes within one LC/MS run (Fig. 2). Several authors added additives, as ammonium acetate [59,61,64,71,74,78,80,81,83], sodium acetate [84] and acetic acid [62,63,70] to the mobile phase, in order to support the ionisation process in the positive and negative ion mode via adduct ion formation. Other authors used post-column addition of ammonium acetate either to distinguish between type A and type B trichothecenes and their respective acetylation sites via their strikingly different ability to form acetate adduct ions in the negative ion mode and ammonium adduct ions in the positive ion mode [61] or simply to enhance the ionisation efficiencies of type A trichothecenes [70]. In distinct contrast to common MS handling, Dall'Asta et al. achieved with a small concentration (0.1 mM) of non-volatile sodium chloride a considerable increase of MS sensitivity by exclusive formation of charged sodium adducts in the positive ion mode and charged chloride adducts in the negative ion mode. Ions deriving from in-source fragmentation were almost completely absent [72]. With this method, both, type A and B trichothecenes can be reliably detected and quantified in the positive ion mode, though it has to be taken into account that the use of non-volatile sodium chloride may have a negative impact on the robustness and short-/medium-term performance of the interface.

APCI sensitivity was reported to be inferior to ESI [68,69,78]. This might be caused by a high APCI in-source fragmentation, as demonstrated by Royer et al., who observed complete degradation of DON in the positive APCI mode (loss of water [71]). Consequently, ESI is preferred in the majority of quantitative applications, since it also seems to be more robust than APCI [62–64].

On the other hand, APCI in-source fragmentation might be especially of interest for single-stage MS users who want to achieve unambiguous analyte identification by selected ion monitoring (SIM). Razzazi-Fazeli et al. and Berger et al. addressed this issue in detail and used some ion-source product ions as additional identification qualifiers [60,61,65], though neither these methods nor a couple of other MS methods [63–65,81] are in complete agreement with some official guidelines about compound identification in residue analysis [41,42] as only a maximum of two instead of four SIM ions were monitored. This is in distinct contrast to the set up of almost all tandem MS methods, since they enable unambiguous target compound confirmation by recording two or three selected reaction monitoring (SRM) precursor ion/product ion pairs per analyte [64,70,73,74,80].

Multi-stage MS applications with triple quadrupole [59,64,66,68–70,73,78,83] or ion trap instrumentation [61–63,71,84]

Table 2
Validation data of LC–MS methods in trichothecene analysis

Analytes	Matrix	Recovery (%)	LOD/LOQ (μg/kg) or (μg/L)	Linear range (μg/kg) or (μg/L) calibration procedure	Accuracy/precision (%)	Ref.
DAS, DON, T-2	Feed, corn meal, rolled oats	85–95	250/–	–	–/–	[59]
Macrocyclic trichothecene	Building materials, paper, gypsum, wooden materials	31–92	1–1000/–	1–500, internal calibration: reserpin	–/–	[84]
DON, NIV	Wheat	70–86	–/40–50	50–1000, external calibration in matrix	–/3.5–7.7	[60]
DON, NIV, DAS, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, NEO	Wheat, rice, fungal cultures	80–106	1–10/10–100	1–2500, internal calibration: VER/hydrocortisone	–/1.1–8.3	[61]
DON	Wheat	–	2000/–	–	–/–	[62]
T-2, HT-2, AcT-2, DAS, MAS, NEO	Oats, barley, wheat, maize, feed	77–01	–/50–85	85–500, internal calibration in matrix: [D_3]-T-2	–/5.7–25.2	[81]
DON, NIV	Wheat, corn	–	50/–	–	–/–	[63]
DON, NIV, 3-AcDON, F-X	Maize	81–98	–/1.5–10	5–1050, external calibration	–/4–10	[64]
DON, NIV, 3-AcDON, 15-AcDON, F-X, DOM-1	Maize, pig urine	64–102	–/25–150	25–1000, internal calibration in matrix: dexamethason	–/3.5–15.1	[65]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, NEO, DAS	Cereals	95 (DON)	20–50/100	100–2000, external calibration in matrix	4.9/∼5.0 (DON)	[72]
DON	Wheat, egg	84–94 (wheat), 80 (egg)	1.5/–(wheat), 0.01/0.025 (egg)	0.025–5 (egg), external calibration in matrix	–/15.2	[77]
DON	Maize	70	10/50	50–2000 m, internal calibration in matrix: VER	6.4–16.6/–	[71]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X	Cereal based food and feed	54–89	0.2/10 (T-2)	10–500, internal calibration in matrix: VER	–/–	[68,69]
DON, NIV, 3-AcDON, 15-AcDON, F-X	Maize	79–97	2–12/5, 20 (F-X)	5–5000, 20–5000 (F-X), internal calibration in matrix: nafcillin	–/3–10	[73]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, DAS	Maize	50–94	0.3–3.8/0.8–18.3	30–1000, external calibration in matrix	4.0/7.2 (DON)	[74]
DON, DOM-1, 3-AcDON, 15-Ac-DON, HT-2, T-2, T-2 triol, DAS, MAS	Milk	84–108	0.03–0.1 (CCα)/0.05–0.15 (CCβ)	–, external calibration in matrix	1–16/3.2–15	[70]
DON-3-glucopyranoside, DON, 3-AcDON, 15-AcDON	Wheat, maize	59 (DON-3-glucopyranoside)	20/- (DON-3-glucopyranoside), 6/-(DON)	–, external calibration	–/–	[82]
DON, NIV, 3-AcDON, 15-AcDON, F-X, T-2, HT-2, NEO, MAS, DAS, T-2 triol, T-2 tetraol	Cereals, bread	46–95	0.18–5.0/16	16–1600, internal calibration without matrix: DOM-1	–/1.2–16.5	[78]

DON: desoxynivalenol, NIV: nivalenol, 3-AcDON: 3-acetyldesoxynivalenol, 15-AcDON: 15-acetyldesoxynivalenol, DOM-1: deepoxydesoxynivalenol, HT-2: HT-2 toxin, T-2: T-2 toxin, F-X: fusarenon X, MAS: monoacetoxyscirpenol, DAS: diacetoxyscirpenol, NEO: neosolaniol, VER: verrucarol.

have been performed either to achieve accurate quantification down to the low ppb range by SRM scans [59,64,70,71,73,74,80,83] or for structural elucidation by product ion scans [61,66,71,75,82,84]. Several authors investigated in detail the collisonal induced fragmentation pattern of type A, type B and macrocyclic trichothecenes in ion trap and triple quadrupole instruments and proposed reasonable ion formation pathways [61,66,84]. In this context, Berger et al. developed a general identification scheme for type A and type B trichothecenes. It is based on the pattern of adduct and product ions which enables the differentiation between type A and B trichothecenes and reveals the number and location of substituents [61]. Tuomi et al. reported that macrocyclic trichothecene might be easily identified by an abundant $[M\text{-}230]^+$ ion in their product ion spectra. Interestingly, this ion seems to be absent in the fragmentation pattern of non-macrocyclic trichothecenes [84]. Besides, Berthiller et al. and Sorensen and Elbaek used characteristic differences in the fragmentation behaviour of 3- and 15-AcDON to enable their individual quantification by SRM even when both isomers co-elute from the LC column [70,74]. Another detailed LC/MS/MS investigation of DON standards isolated from natural sources, revealed trace amounts of hitherto unknown by-products. Based on their CID fragmentation pattern reasonable structure proposals could be made [66]. However, it remains unclear whether these compounds are artefacts of the sample preparation procedure or originate from natural sources.

2.3.5. Method validation and matrix effects

In general, LC/MS provides accurate quantification of trichothecenes down to the low ppb level in a couple of different biological matrices (Table 2). Recovery rates range predominantly between 70 and 108% and depend on the polarity of the analytes. Generally, polar trichothecenes, like NIV, DON and others exhibit the lowest recovery rates down to 31% in building materials, indicating significant losses of analytes during the sample clean up [65,70,73,74,78,83]. Increasing the polarity of the extraction medium was reported to enhance considerably the recovery rates of NIV although that led to strong ion suppression phenomena due to higher amounts of polar coextracted matrix compounds [78].

Typically, linear ranges are achieved with one or two orders of magnitude with good correlation coefficients though these values were frequently obtained in standard solutions [61,64,73,84] and not in the presence of the matrix [60,65,71,72,74,78,81]. Most of the authors did not use an internal standard to compensate for analyte losses, matrix effects or performance deviations of the MS detector. Three LC/MS methods applied internal standards (reserpin [84], dexamethason [65] and nafcillin [73]) that have no close chemical and physical similarity with trichothecenes. Consequently, they are only capable to compensate for frequently occurring LC–MS detector variations. Berger et al. [61], Biselli et al. [68,69] and Royer et al. [71] used verrucarol (VER), a semi synthetic trichothecene and Berger et al. applied a second standard (hydrocortisone) to check the recovery of the internal standard and the performance of the sample clean up process [61]. Just recently, Klötzel et al. evaluated VER and DOM-1 as internal standards and found DOM-1 more suitable since it offered superior MS properties along with a better chromatographic behaviour [78].

In the case of multi trichothecene analysis, it seems, however, doubtful whether one internal standard though structurally similar, may compensate sufficiently for varying effects of matrix components on the ionisation efficiency of 9–12 analytes eluting over a wide polartity range from the LC column [61,68,69]. Furthermore, matrix-matched calibration curves, standard addition protocols and improved sample preparation and/or chromatographic separation should be taken into account to cope with matrix related method validation problems. In some cases simply diluting sample extracts prior to LC/MS analysis might be able to solve these problems though overall method sensitivity decreases. One research group applied a stable isotope-labelled internal standard (deuterated T-2 toxin) which seems to be most suitable due to identical MS and LC properties with the analyte [81]. Interestingly, matrix effects were excluded by the authors, since data obtained with and without internal standard were in close agreement.

In this respect, it has to be mentioned that neither this group nor almost any other group presented validation data about the accuracy and robustness of their results or investigated in detail effects of matrix components on the ionisation efficiency of trichothecenes. Only Klötzel et al. monitored in detail matrix effects over a complete LC run of 25 min by constant post-column infusion of NIV and DAS after the injection of a blank food sample. Areas of ion suppression or ion enhancement were identified by a decrease or increase of the baseline in the respective LC/MS ion chromatograms [78].

Few authors compared LC/MS data with the results of other established analytical techniques [63,72,73,78] or validated their method accuracy with certified reference material [74]. Except Klötzel et al. who compared LC/MS results with LC-FL, they reported only moderate agreement of analytical data for several reasons, as e.g. cross reactivity of DON ELISA tests with other co-occurring type B trichothecenes [73]. Cavaliere et al. proposed LC/MS as suitable confirmation tool for ELISA tests to avoid false positives or cases of overestimation of DON concentrations [73]. For this purpose, Sypecka et al. defined some criteria for positive analyte identification including the chromatographic retention time (deviation compared to standard <2.5%) along with two SRM transitions and their abundance ratio (deviation compared to standard <20%) [77]. Just one group included partly current EU validation criteria into their method validation (e.g. CCα and CCβ, ruggedness, precision, recovery [41]) and investigated in detail matrix effects and their suppression by pH adjustment during SPE sample clean up [70]. From this point of view it seems doubtful if LC/MS methodology has been sufficiently elaborated in all cases, especially where none or only incomplete method validation data were presented [59,63,64,73].

3. Ochratoxins

Ochratoxin A, B and C (OTA, OTB and OTC, Fig. 3) are mycotoxins produced by several *Aspergillus* and *Penicillium* species in semitropical and temperate climates [1]. All

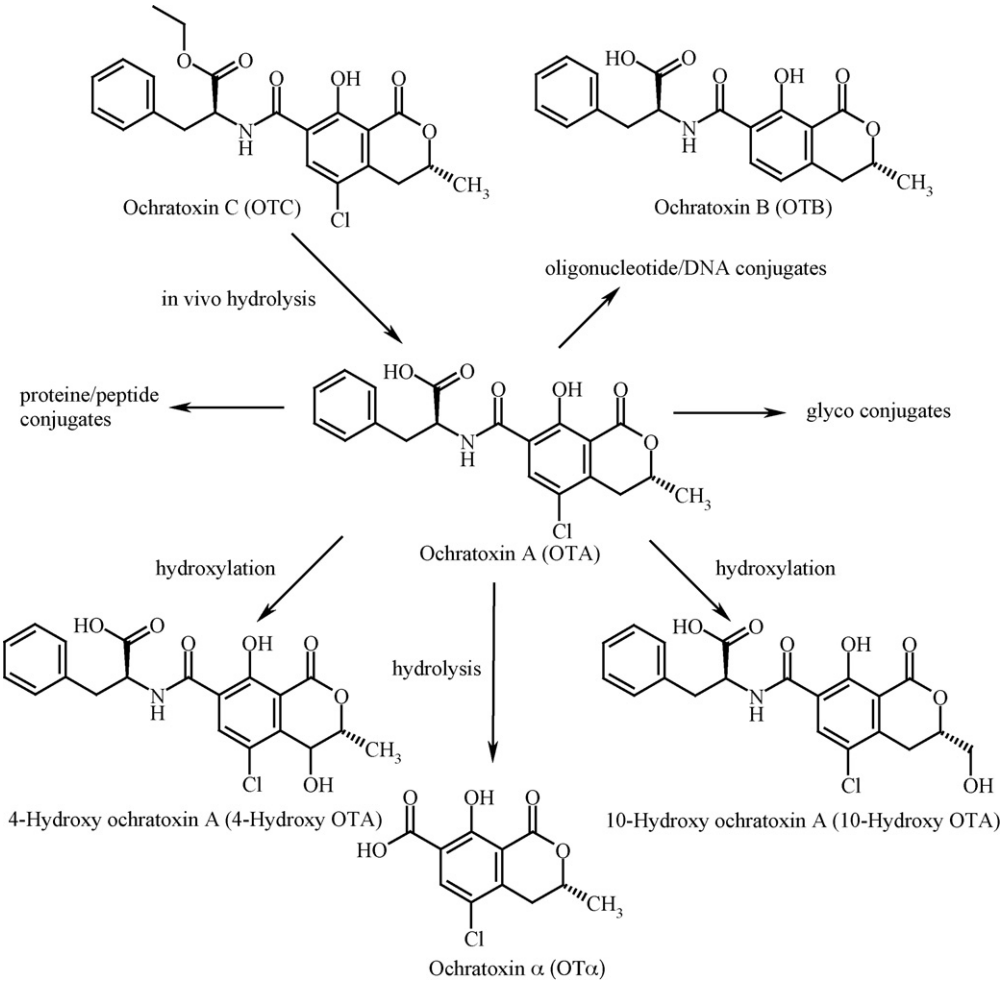


Fig. 3. Structures of ochratoxins and *in vivo* metabolism of ochratoxin A.

three compounds have in common a dihydroisocoumarin moiety linked over a 7-carboxy group to L-phenylalanine via a peptide bond. OTA attracted by far most attention since it is distinctly more toxic and prevalent than OTB (missing the chlorine atom) and is rapidly formed *in vivo* from ochratoxin C (ethyl ester of OTA).

OTA occurs in a variety of food commodities of which cereals and cereal products, fruits, coffee, beer and wine are the most important sources of intake. Carry over from contaminated feedstuff has resulted in the detection of OTA in porcine and poultry tissues (liver, kidney, muscle, eggs) and pig blood while it is rapidly metabolised in ruminants to non-toxic ochratoxin α and to a low degree to 4- and 10-hydroxy OTA (Fig. 3) [86]. OTA is a potent nephrotoxin and hepatotoxin with teratogenic, mutagenic, carcinogenic and immunosuppressive effects even at trace levels. In humans, the consumption of OTA-contaminated food has been connected to the occurrence of Balkan endemic nephropathy [86], a disease characterised by severe kidney damage. In 1993, the International Agency for Research on Cancer classified OTA as possibly carcinogenic for humans (Group 2B) [87].

Dietary exposure results in detectable levels in human serum and reaches significantly high levels in patients showing symptoms of ochratoxicosis [86]. Consequently, there has been increasing concern on its presence in human blood and in other body fluids and tissues. In humans, OTA is metabolised very slowly with a half-life of more than 30 days [88]. It binds almost completely to plasma proteins and accumulates in kidney and liver. The World Health Organisation (WHO) has set a provisional tolerable daily intake level for OTA of 14 ng/kg body weight [89]. The Scientific Committee on Food of the European Commission suggested an even lower level of 5 ng/kg body weight per day [90]. Based on these recommendations, national and international guideline levels and maximum residue levels have been set in the range between 0.5 and 25 μg/kg in food and 5 and 300 μg/kg in feed [91,92].

3.1. Conventional ochratoxin analysis

Due to its high toxicity and strict regulations on maximum levels of OTA, accurate and sensitive detection is increasingly required down to the sub ppb range in a wide range of different food, feed and biological matrices. Relevant analytical techniques and protocols have been recently summarised and evaluated in two reviews [93,94].

Due to the strong native fluorescence activity of OTA, HPLC-FL is now established as the preferred routine analysis technique for OTA, OTB and OTC and their metabolites, since it offers a

better selectivity and sensitivity compared to other detectors. Sometimes, positive findings are confirmed by methylation of OTA and a second HPLC experiment [93]. Due to its robustness and its easy and cost-effective handling thin-layer chromatography (TLC) with FL detection is still routinely used in countries outside Europe and North America. Major disadvantage of this technique is the comparable low sensitivity towards OTA (ppb range) and frequently occurring interferences with the sample matrix [95]. GC-based methods found distinctly less attention since they suffer from a time-consuming and error-prone derivatisation protocol (methylation) needed to achieve sufficient volatility of the analytes. Nevertheless, GC/MS might be used to confirm unambiguously positive findings [93]. ELISA assays have been shown to be extremely suitable for a rapid screening of large sample numbers. They offer sensitivities towards OTA comparable to FL detection. Due to possible cross-reactivities with matrix components, confirmation by other techniques is, however, highly desirable to avoid false positive results or inaccurate and overestimated quantitative data [93,94].

In general, and independent of the applied detection technique there is need for a careful sample clean up and analyte enrichment by liquid/liquid partitioning or increasingly by SPE with RP, silica and immunoaffinity absorbent materials [93,94]. Low recovery rates are still a critical issue, since OTA binds very effectively to proteins and DNA affecting negatively the extraction/purification process in milk, tissue, plasma and urine samples [93].

3.2. Early LC/MS technology in ochratoxin analysis

Only few early LC/MS studies have been published for OTA applying TSP ionisation [96] and direct liquid injection [97]. They are focused on the mass spectrometric properties of OTA, such as ionisation efficiency and in-source fragmentation. Quantitative aspects played only a minor role and the analysis of spiked cereal samples demonstrated detection limits in the low ppm range which are three orders of magnitude less sensitive than HPLC-FL. Despite this lack of sensitivity and robustness, it was already recognised at this stage of early interface development that LC/MS might be extremely useful for structural elucidation and confirmation of OTA and its metabolites in animals and plants [93].

3.3. Modern LC/MS analysis of ochratoxins

3.3.1. Investigated ochratoxin-type analytes and typical matrices

In view of routine analysis and quantitative and qualitative LC/MS/(MS) method development, the analysis of ochratoxins is still exclusively focused on OTA since it exhibits the highest toxicity and most frequent occurrence of all known ochratoxins in biological and food environments. Due to its high toxicity, OTA analysis has to be performed down to the ppt range in agricultural commodities and feed, food and beverages thereof, such as cereals, coffee, wine, beer, milk and cheese (Table 3) to estimate the risk of OTA intake [94]. In addition, plasma and urine samples have been repeatedly analysed to monitor actual OTA contamination, distribution and excretion kinetics in humans and animals [98–104]. In this context, LC/MS is predominantly applied to confirm OTA positive results obtained by ELISA or HPLC-FL [93,94,98,100]. LC/MS is also an excellent tool to elucidate the structure of *in vivo* metabolites and OTA adducts in biological fluids and tissues, leading to a better understanding about the mode of action in OTA-induced mutagenesis and carcinogenesis in animals and humans [98–104]. In addition, Hancock and D'Agostino determined OTA in soil since it might also be considered as potential chemical/biological warfare agent [105].

Other ochratoxins, like OTB and OTC as well as products of the metabolic pathways of OTA, as ochratoxin α, 4-hydroxy OTA, 10-hydroxy OTA and glutathione, 3′monophosphate desoxyguanidine and carbohydrate conjugates of OTA have been only a matter of investigation in the course of a couple of metabolism and biological mode of action studies when LC/MS was used for structural confirmation or elucidation [103,104,106–110].

3.3.2. Sample preparation

Usually, solid samples are extracted with water [105], ethyl acetate [101], chloroform [102–104,111] and methanol–water [106,112], acetonitrile–water [68,69], hexane–acetonitrile [70] and dichloromethane–ethanol mixtures [113] containing additives like sodium hydrogen carbonate [99,112], phosphoric acid [101,102,113,114] or magnesium chloride, to enhance solubility and extraction efficiency of OTA [115,116]. Kokkonen et al. used a mixture of acetonitrile and hexane for cheese samples to enable simultaneous extraction and defatting [117]. Extracts as well as liquid samples are frequently submitted to SPE protocols with RP [118–120] but also with anion exchange material [102] and kieselguhr [102], or with a polymeric absorbent material that contains RP and anion exchange functionalities [70,114,120,121]. In addition, Gross-Steinmeyer et al. and Zepnik et al. applied preparative HPLC after RP SPE clean up to purify OTA metabolites from animal and human cells for reliable structural elucidation [108,109].

Alternatively, immunoaffinity columns have been applied [99,100,111,112,116,119–123] that are now in routine use in OTA screening protocols with e.g. LC-FL detection. These materials contain immobilised antibodies that exclusively retain OTA, thus producing cleaner extracts with a minimum level of interfering matrix components and excellent signal-to-noise ratios compared to less selective SPE sorbent materials [121,123]. However, it was demonstrated by Leitner et al. and Reinsch et al. that selective immunoaffinity columns in combination with highly selective LC/MS detection may also be regarded as analytical "overkill" in certain food matrices, since sensitivity and accuracy obtained with RP materials or RP/anion exchange material were found to be comparable with those of immunoaffinity materials [119,121]. Furthermore, immunoaffinity materials are expensive and distinctly less feasible to multitoxin analysis since they are highly specific for only one target mycotoxin (class). This is in contradiction to the perception of MS as a generally applicable detection tool though highly selective sample clean-up is sometimes

Table 3
Overview on LC–MS methods in ochratoxin analysis

Analytes	Matrix	Sample preparation	Liquid chromatography		Mass spectrometry		Ref.
			Column	Mobile phase/additives	Ionisation/ion selection	Scan mode	
OTA, OTB, OTα, OTβ, 4-hydroxy OTA, 4-hydroxy OTB, 10-hydroxy OTA	Rat urine, fungal cultures	Extraction with MeOH/H_2O—70:30, liquid/liquid extraction with $CHCl_3$, preparative TLC and preparative HPLC	RP-18	Isocratic: H_2O with 2% HCOOH/ACN 2:98	APCI − single quadrupole	Full scan	[106]
OTA	Serum	–	RP-18	No information given	APCI − single quadrupole	SIM	[98]
OTA	Beer, coffee	Mixing (beer)/extraction (coffee) with aqueous 0.4 M $MgCl_2$, liquid/liquid extraction with toluene, SPE with silica columns	RP-18	Gradient: 0.05% TFA in H_2O/MeOH	ESI ± triple quadrupole	Full scan, product ion scan, SRM	[115]
OTA	Plasma	SPE with RP-18 columns and IAC	RP-18	Isocratic: H_2O/MeOH/ACN—1:1:1 with 0.17% HCOOH	ESI + triple quadrupole	SRM	[100]
OTA	Beer	Liquid/liquid extraction with toluene, SPE with silica columns	RP-18	Gradient: 0.05% TFA in H_2O/MeOH	ESI + triple quadrupole	Full scan, product ion scan, SRM	[124]
OTA	Kidney, rye flour	Extraction with dichloromethane/ethanol/0.1 M phosphoric acid 75:15:10, liquid/liquid extraction with aqueous Na_2CO_3 and dichloromethane, OTA methyl ester formation with BF_3/MeOH	RP-18	Isocratic: aqueous 0.5% AcOH/MeOH/ACN—30:20:50	ESI + Triple quadrupole	Full scan, product ion scan, SRM	[113]
OTA	Plasma, coffee	Plasma: SPE with RP-18 columns IAC, coffee: extraction with MeOH/aqueous 3% $NaHCO_3$—1:1, IAC	RP-18	Isocratic: H_2O/MeOH/ACN—1:1:1 with 0.17% HCOOH	ESI + (APCI+), triple quadrupole	Full scan, product ion scan, SRM	[99]
OTA	Wine	SPE with RP-18 columns	RP-18 monolitith	Isocratic: aqueous 2.5% AcOH/MeOH—31.5:68.5	ESI + triple quadrupole	SRM	[118]
OTA, 4-hydroxy OTA	Rat liver microsomes	SPE with RP-18 columns, semi preparative HPLC	Direct infusion	Isocratic: H_2O/MeOH/AcOH—39.5:59.5:1.0	NanoESI − triple quadrupole	Full scan, product ion scan	[109]
OTA	Wine	SPE with RP-18 columns or IAC	RP-18	Isocratic: aqueous 2.5% AcOH/MeOH—68.5:31.5	ESI + triple quadrupole	SRM	[119]
OTA conjugate with glutathione	Aqueous solution	–	RP-18	Gradient: 0.1% HCOOH (v/v) in H_2O/ACN	ESI − ion trap	Full scan	[107]
OTA, 4-hydroxy OTA, OTA glyco/DNA conjugates	Rat and human hepatocytes	SPE with RP-8 columns, preparative HPLC	RP-8	Gradient: H_2O with 1% AcOH/MeOH/ACN	ESI ± triple quadrupole	Full scan, product ion scan	[108]
OTA, OTα, 4-hydroxy OTA, 10-hydroxy OTA, OTA glucuronide, OTA glyco/glutathione conjugate	Rat urine, blood, liver, kidney, faeces	Urine: dilution with H_2O/EtOH, faeces: extraction with aqueous HCl and liquid/liquid extraction with $CHCl_3$, plasma: dilution with EtOH and centrifugation, kidney/liver: extraction with 0.05 M Na_3PO_4, pH 6.5	RP-18	Gradient: 0.1% TFA in H_2O/MeOH	ESI − triple quadrupole	Full scan, SRM, precursor ion scan	[103]
OTA	Soil	Extraction with H_2O	RP-18	Gradient: 0.1% TFA in H_2O/ACN or isocratic: 0.1% TFA in H_2O/ACN—25:75	ESI + QTOF, TOF	Full scan	[105]
OTA	Wine	IAC	RP-18	Isocratic: H_2O/ACN—26:74 with 0.1% HCOOH	ESI + ion trap	Full scan, SIM	[122]
OTA	Coffee	Extraction with aqueous $NaHCO_3$, SPE with RP/anion exchange material	RP-18	Isocratic: 0.1% HCOOH in H_2O/ACN—60:40	ESI + single quadrupole	Full scan, SIM	[114]
OTA	Wheat flour, coffee, spices, wine, beer	(1) Extraction with aqueous 0.4 M $MgCl_2$ (only solid samples), liquid/liquid extraction with toluene, SPE with silica columns, or (2) extraction with aqueous Na_2CO_3, IAC	RP-18	Gradient: 0.05% TFA in H_2O/MeOH	ESI + (APCI+) ion trap	Full scan, product ion scan, SRM	[116]
OTA	Wine	Direct injection	RP-18, Ø 1 mm	Isocratic: 20 mM AcOH/NH_3 in H_2O/ACN—85:15	ESI − single quadrupole	Full scan, SIM	[125]
OTA	Cereal, cereal products	Mixing with RP-8 SPE material and extraction with MeOH/HCOOH—99:1	RP-18	Isocratic: 0.1 M HCOOH/MeOH—30:70	ESI − single quadrupole	SIM	[127]
OTA and its oligo-nucleotide conjugates	Aqueous standard solution	SPE with RP-18 columns, semi preparative HPLC	RP-8	Gradient: 5 mM NH_4OAc in H_2O/ACN	ESI − ion trap	Full scan, product ion scan	[110]
OTA	Pig kidney, liver, muscle	Extraction with 1 M phosphoric acid/ethyl acetate, liquid/liquid extraction with 0.5 M $NaHCO_3$, liquid/liquid extraction following pH-switch with 7 M phosphoric acid	RP-18	Gradient: 1% AcOH in H_2O/ACN	ESI + ion trap	Full scan, product ion scan, CRM	[101]
OTA	Kidney	Extraction with $CHCl_3$ with 2.6% phosphoric acid, SPE with anion exchange material or kieselguhr	RP-18	Gradient: 0.3% HCOOH in H_2O/ACN	ESI + triple quadrupole	Full scan, product ion scan, SRM	[102]
OTA	Cereal based food and feed	Extraction with ACN/H_2O—85:15, clean up with MycoSep 226	RP-18	Gradient: H_2O/MeOH	ESI − triple quadrupole	SRM	[68,69]
OTA	Rice, barley, what flour, beer, rice, wine	Extraction with MeOH/aqueous 3% $NaHCO_3$—50:50 (for solid samples), IAC	RP-18	Isocratic: H_2O/MeOH/ACN—1:1:1	ESI + triple quadrupole	SRM	[112]

OTA	Cheese	Extraction with 0.1% HCOOH in ACN/hexane—55:45	RP-18	Gradient: 0.1% AcOH in H_2O/ACN	ESI + triple quadrupole	SRM	[117]
OTA	Milk	Enzymatic deglucuronidation, extraction with ACN/hexane—61:39, SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Gradient: H_2O/MeOH	ESI − triple quadrupole	Product ion scan, SRM	[70]
OTB, 4-hydroxy OTB, 10-hydroxy OTB, OTβ	Rat urine, blood, liver, kidney, faeces	Urine: dilution with H_2O/EtOH, faeces: extraction with aqueous HCl and liquid/liquid extraction with $CHCl_3$, plasma: dilution with EtOH and centrifugation, kidney/liver: extraction with 0.05 M Na_3PO_4	RP-18	Gradient: 0.1% TFA in H_2O/MeOH	ESI − triple quadrupole	Full scan, SRM, product ion scan	[104]
OTA	Wine	SPE with anion exchange/RP material	RP-18	Isocratic: H_2O/MeOH/AcOH—29.5: 69:1.5	ESI + ion trap	Full scan, SRM, product ion scan	[121]
OTA	Grapes	Extraction with $CHCl_3$	RP-18	Gradient: H_2O/ACN each with 0.05% HCOOH	nanoESI +/(−) ion trap	Full scan, product ion scan	[111]
OTA	Beer	SPE with anion exchange/RP material	RP-18	Isocratic: H_2O/MeOH/AcOH—29.5:69:1.5	ESI + ion trap	Full scan, SRM, product ion scan	[120]

OTA: ochratoxin A, OTB: ochratoxin B, OTC: ochratoxin C, 4-hydroxy OTA: 4-hydroxy ochratoxin A, 10-hydroxy OTA: 10-hydroxy ochratoxin A, 4-hydroxy OTB: 4-hydroxy ochratoxin B, OTα: ochratoxin α, OTβ: ochratoxin β.

needed to overcome problems with matrix effects in LC/MS [119].

Though time-consuming, error-prone and not feasible for automation, liquid/liquid extraction with toluene or ethyl acetate is still in frequent use for solid as well as liquid samples due to the acidic/ionic nature of OTA. The organic layer containing OTA can be easily purified by SPE on a silica column [115,116,124]. Lindenmeier et al. demonstrated that the performance of this protocol is similar to that of immunoaffinity clean up, when LC/MS is used as detection system of wine and wheat samples. This observation seems to be highly dependent on the complexity of the food matrix, since the same authors reported strong matrix interferences in coffee samples that made IAC mandatory, even when LC/MS is applied [116]. Interestingly, Jorgensen and Vahl transformed OTA to its methyl ester in order to analyse OTA in pig kidney and rye flour by LC/MS/MS [113]. Though this approach is time-consuming, it offers the possibility to use the $[D_3]$-methyl ester of OTA as labelled internal standard that can be easily obtained by methylation of OTA with $[D_4]$-methanol.

Recently, Dall'Asta et al. demonstrated that sample clean up might be even omitted for LC/MS and HPLC-FL analysis of wine samples. Detection limits down to the sub ppb range were achieved when wine samples were directly injected into the HPLC/MS system [125]. No interferences of matrix components were observed in the LC/MS chromatograms though it remains doubtful that a similar procedure can be successfully applied to more complex food and biological matrices. The method offers an unexpected low detection limit that can partly be attributed to the use of 1 mm diameter LC columns. Though dirty samples may lead to fast clogging of such small diameter columns, this issue as well as method robustness was not addressed by the authors.

3.3.3. Typical LC conditions for LC/MS analysis

Chromatographic separation prior to MS detection is exclusively achieved on RP stationary phases (RP-18, RP-8) with methanol/water, acetonitrile/water and methanol/acetonitrile/water mixtures as mobile phases, both in the isocratic and gradient mode. RP chromatography of acidic ochratoxins is highly pH dependent and acids, like formic acid, acetic acid and trifluoro acetic acid (TFA) are used as additives to achieve sufficient chromatographic retention. Typically, LC/MS run times are less than 10 min on conventional RP columns since the analysis is focused on OTA in most applications [102,113,114,118–122,124]. Taking the high MS selectivity into account, chromatographic separation frequently also in combination with a proper sample preparation procedure, is therefore necessary to remove matrix components from the analyte to enhance MS sensitivity as well as accuracy and reproducibility of quantitative data [126]. Excellent LC/MS separation characteristics could also be achieved in the course of metabolism studies with mixtures of OTA and its oligonucleotide conjugates [110] or its hydroxylated and glycosylated metabolites [103,108]. Typically, run times of these separations are in the range of 20–35 min.

A dramatic reduction of run times to less than 2 min was demonstrated by Zöllner et al. who applied a monolithic column to the LC/MS/MS analysis of OTA in wine samples [118]. This

fourth generation HPLC material is operated at high flow rates while maintaining high separation efficiency which is especially attractive for high through-put applications. A direct comparison of monolithic with conventional RP columns proved comparable results for OTA in terms of LC/MS sensitivity, accuracy and robustness. A short overall MS dwell time of less than 0.2 s per transition was found to be mandatory to avoid quantification problems due to the very narrow chromatographic peak widths [118].

Most recently, RP capillary columns were successfully introduced into OTA LC/MS analysis. In combination with nanoESI or ESI it was possible to reduce considerably sample preparation efforts, sample volumes and reagent consumption during sample clean up along with an overall method sensitivity in grapes [111] and wine [125] on the ppt concentration level. Possible major disadvantage of this LC approach might be an easy clogging of the small diameter columns by matrix loaded samples.

3.3.4. Mass spectrometry

In general and independent of interface geometries and ionisation polarities, OTA offers an extensive fragmentation behaviour in APCI interfaces resulting in distinctly lower sensitivity compared to ESI [99,115] (Table 3). Consequently, ESI has been almost exclusively applied in qualitative and quantitative OTA analysis where it was found much more robust for routine analyses than APCI. OTA produces abundant adduct ions in the electrospray source when sodium or potassium ions are present in the mobile phase [99,109,115] whereas one group detected acetonitrile adducts of OTA in the negative ion mode [111]. Furthermore, single-stage MS users have reported a sufficient yield of in-source product ions in ESI sources to enable structural confirmation or elucidation of ochratoxins and structurally related compounds [103,114]. The abundance of these product ions, typically reflecting loss of water, formic acid and carbon dioxide or cleavage of the amide bond, can be further enhanced by adjusting MS parameters, like the cone voltage. In this respect, ochratoxin conjugates can be easily identified by the in-source loss of their carbohydrate, glutathione and glucuronic acid moieties [103].

MS/MS experiments of OTA as well as its derivatives have been performed with low-energy collisions in both, the positive and negative ion mode, in order to obtain reliable structural information about metabolic intermediates and metabolic products in different biological systems. The product ion spectrum of OTA which has been investigated in detail is independent in its appearance from the applied collision gas (argon, helium, nitrogen) [99,101,102,111,113,115,118] and offers an identical product ion pattern compared to in-source fragmentation. Reasonable fragmentation pathways have been proposed in the positive ion mode with the major product ions revealing losses of water and formic acid and cleavage of the amide bond [99,103,104,109,121]. Due to the rather non-specific fragmentation pattern in the negative ion mode – loss of carbon dioxide is commonly observed with a lot of carboxy acids and esters and cleavage of the amide bond is observed only at higher collision energies [109] – SRM experiments for OTA quantification were predominantly performed in the positive ion mode with the OTA specific product ion at m/z 239 reflecting cleavage of the amide bond [99,102,115,116,118,121]. Besides, Losito et al. performed consecutive reaction monitoring (CRM) experiments (MS^3: m/z 404 ⇒ m/z 358 ⇒ m/z 341) on an ion trap instrument and achieved striking improvement of the MS sensitivity compared to the SRM mode [101]. Except for two methods [116,121], two or three precursor ion/product ion pairs (in two cases also in combination with the respective ion intensity ratios [70,102]) were selected in SRM and CRM experiments which is in agreement with present regulations about unambiguous identification of target residues in food, feed and biological samples [40,41]. In contrast, none of the single-stage methods would meet these regulatory requirements since only two ions instead of four were monitored by SIM [125,127].

3.3.5. Method validation and matrix effects

Quantitative LC/MS analysis offers excellent sensitivity down to the ppt range in a wide variety of different food and biological environments (Table 4). Though this is by a factor of 5–10 less sensitive than LC-FL, LC/MS is either used to confirm OTA positive samples that have been analysed by HPLC-FL or ELISA [114,125,127] or as alternative detection technique to LC-FL [113,118,119,122]. Method recovery rates range predominantly between 70 and 121% and depend highly on the investigated sample matrix. Especially roasted coffee has been shown to offer such a complex matrix that sufficient LC/MS sensitivity and recovery values can only be achieved when the extracts are either submitted to IAC [116] or to SPE with a polymeric sorbent material that offers both, RP and anion exchange properties [114]. Similarly, insufficient ionisation efficiency and thus a dramatic decrease of sensitivity were reported for the analysis of OTA in dark beer brands [124].

Typically, linear ranges are achieved with one to three orders of magnitude and with good correlation coefficients though these values were not always obtained in the presence of matrix [111,115,121,124]. Zearalanone (ZAN) [118,119] and OTB [99–102] were applied as internal standards to enhance accuracy and reliability of LC/MS analysis. Both compounds have no close similarity to MS or LC properties of OTA and detailed study in wine demonstrated that ZAN is due to its structural difference only partially able to compensate for matrix effects, as it was not possible to obtain one common calibration curve for different wine brands [119]. It is interesting that the performance of this internal calibration protocol was strikingly improved (striking reduction of the dispersion of calibration curves) after the major part of sample matrix was removed by IAC [126]. In contrast, the use of OTB seems to be not that critical in plasma and tissue samples [99–102], though OTB elutes earlier than OTA from the LC column. Quantitative data obtained by external, internal and standard addition protocols were found to be in good agreement indicating that matrix suppression effects in plasma samples have in contrast to wine no serious influence on the results [99]. Also this calibration approach is, however, highly matrix dependent as others failed to use OTB as internal standard in kidney and rye flour samples [113].

More recently Lindenmeier et al. demonstrated, that stable isotope labelled standards are distinctly less problematic and

Table 4
Validation data of LC–MS methods in ochratoxin analysis

Analytes	Matrix	Recovery (%)	LOD/LOQ (μg/kg) or (μg/L)	Linear range (μg/kg) or (μg/L), calibration procedure	Accuracy/precision (%)	Ref.
OTA	Serum	87	–/–	–	–/–	[98]
OTA	Beer, coffee	95 (beer), 70 (coffee)	0.02/0.06 (beer), 0.03/0.09 (coffee)	0.06–2.5 (beer), 0.09–4.0 (coffee), external calibration	–/–	[115]
OTA	Plasma	87	0.12/–	External calibration and internal calibration: OTB	–/–	[100]
OTA	Beer	78–88	0.02/0.6	0.06–2.5, external calibration	–/–	[124]
OTA	Kidney, rye flour	104–121	0.02/0.5	0.5–10, internal calibration: [D_3]-methyl ester of OTA	–/6–16	[113]
OTA	Plasma, coffee	–	0.5/1.0 (plasma)	1.0–40, external and internal calibration: OTB, standard addition	–/–	[99]
OTA	Wine	100	0.025/0.5	0.5–100, internal calibration in matrix: ZAN, standard addition	3.6/–	[118]
OTA	Wine	94–101	0.05/0.15	0.15–10, internal calibration in matrix: ZAN, standard addition	–/–	[119]
OTA	Coffee	83	0.1/–	–	–/5.9	[114]
OTA	Wine	97	0.01/–	–	–/2.6	[122]
OTA	Wheat flour, coffee, spices, wine, beer	105	0.5/1.4	1.4–126, internal calibration: [D_5]-OTA	2.1/3.6	[116]
OTA	Wine	96	0.05/–	–	–/–	[125]
OTA	Cereal products	–	0.3/0.95	–	–/–	[127]
OTA	Pig kidney, liver, muscle	86	0.6/1.5	1.5–15, internal calibration in matrix: OTB	–/6 (intraday), 9 (interday)	[101]
OTA	Kidney	57–75	0.11 (CCα)/0.25 (CCβ)	No data given, internal calibration in matrix: OTB	–/3–10	[102]
OTA	Cheese	105	0.3/0.6	5–1000, external calibration in matrix	–/2.5	[117]
OTA	Milk	103	0.01 (CCα)/0.02 (CCβ)	–, external calibration in matrix	20/3.8–15	[70]
OTA	Wine	89–105	0.35/1.13	0.6–10, external calibration	4-16/8	[121]
OTA	Grapes	95	0.01/0.02	0.02–0.2, external calibration	–/3	[111]

OTA: ochratoxin A, OTB: ochratoxin B, OTC: ochratoxin C.

more reliable when dealing with frequently varying sample matrices [116]. They synthesised [D_5]-OTA in a simple two step reaction sequence and applied it as internal standard in the LC/MS analysis of OTA. The excellent accuracy and precision of the method proved to be independent of the investigated sample matrix. No extensive validation was necessary, since deuterated OTA offers almost identical physical and chemical properties to OTA thus compensating for any kind of matrix effects arising in different sample environments [116]. In contrast to other internal standards, labelled ones can be used in combination with IAC, since they are retained analogously to the non-labelled analyte. Therefore, ZAN and OTB can only be added after immunoaffinity sample clean up with the limitation to compensate only for matrix effects and detector response variations but not for analyte losses during sample preparation [119].

Further validation data were carefully evaluated by several authors, giving evidence that LC/MS methodology in OTA analysis offers good accuracy and precision [70,115,116,118]. Accuracies were determined by spiking samples with defined amounts of OTA [70,115,118,121] while Lindenmeier et al. used certified reference material (contaminated wheat flour) for this purpose [116]. Two groups [70,102] included in part current EU validation criteria into their method validation, such as CCα, CCβ, recovery, etc. [41]. In this context, Sorensen and Elbaek demonstrated a reduction of matrix effects and improvement of accuracy values by careful pH adjustment during SPE clean up of milk samples [70].

Finally, quantitative data were also directly compared with results of LC-FL [99,100,114,119–121,125]. The good agreement of both data sets gives further evidence that LC/MS might be an excellent alternative tool for HPLC-FL.

4. Zearalenone and metabolites

Zearalenone (ZON, Fig. 4) is a nonsteroidal estrogenic mycotoxin with a phenolic resorcyclic acid lactone structure. Sometimes together with low amounts of α-zearalenol, it is frequently produced by *Fusarium* species, which colonise grains like maize, oat, barley, wheat and sorghum under prolonged cool and wet weather conditions in temperate and warm regions [2,128]. ZON offers only a low toxicity and there is limited evidence for its carcinogenicity based on animal studies [129,130]. On the other hand, this mycotoxin exhibits, due to its agonistic effect on the estrogen receptor [128–130], striking estrogenic and anabolic properties in several animal species resulting in severe effects on the reproductive system [129]. To avoid any of the symptoms of hyperestrogenism a guideline level of 200 μg/kg ZON in feed has been proposed [131]. ZON contamination of food is caused either by direct contamination of grains

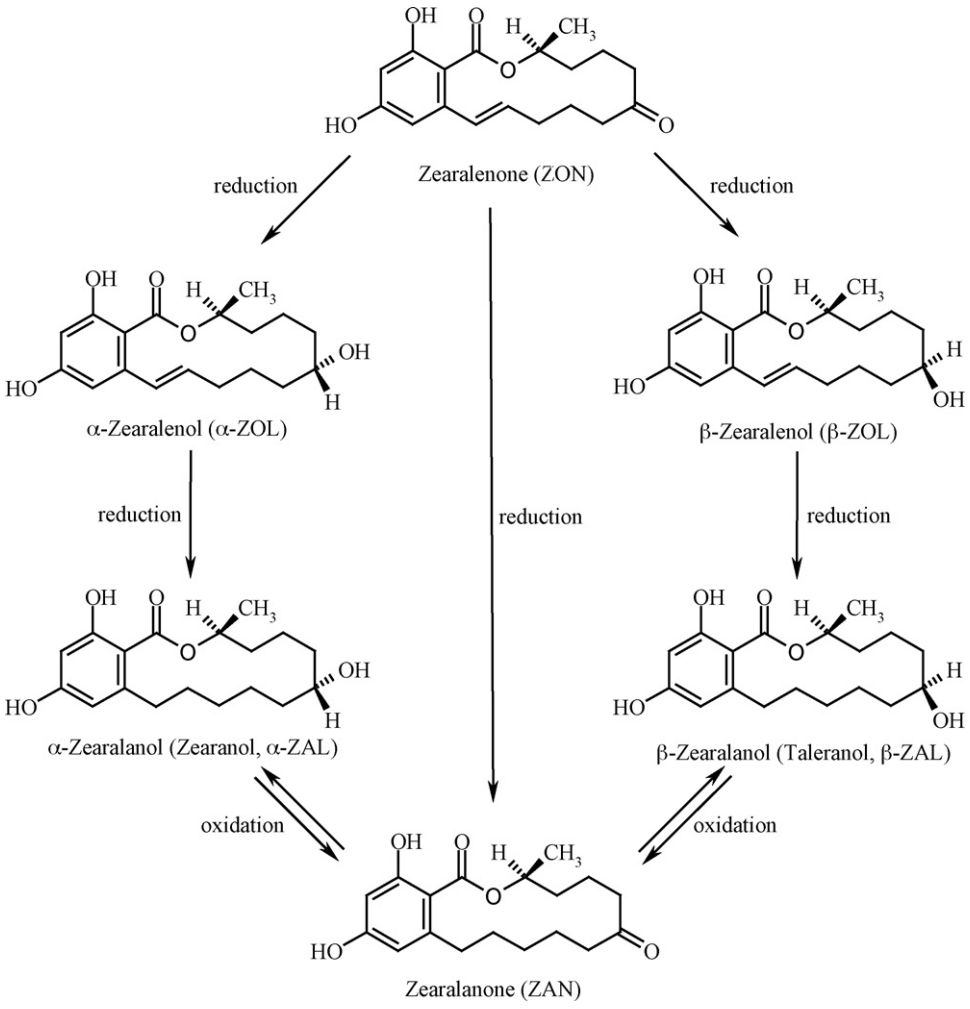


Fig. 4. *In vivo* metabolism of ZON (glucuronides and glyco conjugates are not depicted).

or to a distinctly minor degree by "carry over" of mycotoxins and their metabolites into animal tissues, milk and eggs after intake of contaminated feedstuff. Scientific evidence on the estrogenic effects of ZON in humans is still limited to a few investigations which are mainly based on a small number of individuals and incomplete data [129,132,133]. Nonetheless, the unequivocal results of numerous animal studies led to the establishment of tolerance levels throughout the world ranging between 30 and 1000 μg/kg in grains [134].

The ZON metabolism attracted considerable more attention than that of any other mycotoxin, since some of the metabolites (especially α-ZOL and α-ZAL) exceed considerably the estrogenic effects of ZON. α-ZAL has even been widely adopted as a growth stimulant since 1969 to improve fattening rates of cattle. Due to concerns about long-term health effects for humans, its use as well as its presence in food has been banned in the EU since 1985 [135]. In contrast to this view, the Joint FAO/WHO Expert Committee on Food Additives proposed in 1987 maximum residue levels in liver of 10 μg/kg and in muscle of 2 μg/kg [136] and the Food and Drug Administration (FDA) established safe concentration levels for total zeranol residues in uncooked edible tissues of cattle between 150 and 600 μg/kg [137].

The *in vivo* metabolism of ZON has been investigated in detail in several animal species and in humans. It has been shown that ZON is predominantly transformed into α- and β-zearalenol (α- and β-ZOL, Fig. 4) [128–130]. Concentration ratios of the metabolites, the precursor compound and their respective glucuronides vary strikingly with the species, e.g. a significant fraction of ZON was found in human and pig in the form of α-ZOL, while cows and the brewing strain of *Saccharomyces cerivisiae* predominantly metabolise ZON to β-ZOL [129,138]. Besides, it was demonstrated that a further reduction of α- and β-ZOL may occur in deer, goats, sheep, cattle and horses resulting in partly significant concentrations of zeranol (α-zearalanol, α-ZAL, Fig. 4) and taleranol (β-zearalanol, β-ZAL) in urine [139]. Further evidence for the natural formation of the growth promoter α-ZAL from ZON was achieved when considerable concentrations were found in bile, urine and muscle tissue of sheep, cattle and pigs that were most probably not treated with α-ZAL [140–142].

4.1. Conventional ZON analysis

In view of the strong estrogenic effects of ZON and its metabolites and the present legislation on maximum tolerance

levels and guideline levels, reliable determination between 10 and 100 μg/kg is needed in food and feedstuff. Even lower method sensitivities down to the sub ppb level are mandatory in biological matrices, as human and animal body fluids and tissues, to enable accurate risk assessment and studies on ZON metabolism. Current analytical techniques and protocols have been summarised in a review in 2001 [143].

Due to the strong native fluorescence activity of ZON and its metabolites, HPLC in combination with FL detection is presently the method of choice offering sufficient sensitivity, selectivity and separation efficiency after suitable sample clean up [143–145]. In contrast, the usefulness of GC-based methods is limited due to the need of time-consuming derivatisation of the phenolic hydroxy groups, and consequently, only GC/MS has been widely applied for the unambiguous confirmation of positive findings [146,147]. TLC [148] and ELISA [149] are frequently applied for a reliable screening for ZON and its metabolites. Its robustness and its easy and cost-effective handling favour especially TLC applications in countries outside Europe and North America though method performance characteristics are inferior to other methods in terms of sensitivity and selectivity. ELISA assays offer sensitivities comparable to FL detection. Due to potential cross-reactivities of the antibodies with matrix components, confirmation by other techniques is, however, highly desirable to avoid false positive results or inaccurate and overestimated quantitative data. For the same reason, the simultaneous determination of ZON and its metabolites is not possible by an ELISA assay [143].

Generally and independent of the detection method or sample matrix, sophisticated sample clean up and enrichment protocols are in most cases prerequisites to reach the requested sensitivity levels for ZON and its metabolites. In this respect, time-consuming and error-prone liquid/liquid extraction is increasingly superseded by SPE with reversed-phase [138,150,151], aminopropyl [152], and immunoaffinity absorbent materials [144,145,150] or by MycoSep columns containing a mixture of charcoal, ion-exchange resins and other materials [85,143].

4.2. Early LC/MS technology for ZON analysis

Early LC/MS methodology has been seldom applied to ZON analysis, e.g. dynamic FAB was used to investigate in detail the in-source fragmentation behaviour of ZON and structurally related compounds, like α- and β-ZOL [153]. Furthermore, Rajakylä et al. performed TSP experiments in biological surroundings. They achieved reliable qualitative and quantitative data of ZON in wheat samples down to the low ppm level and investigated in detail the optimisation of the thermospray ionisation process to achieve maximum MS sensitivity. However, a detailed method validation was not given [96].

4.3. Modern LC/MS analysis of ZON

4.3.1. Investigated zearalenone-type analytes and typical matrices

The finding that all ZON metabolites exhibit similar or even distinctly higher estrogenic effects than ZON has rapidly extended LC/MS methodology to the whole spectrum of *in vivo* ZON metabolites [73,77,126,138,142,151,154–162] in a wide diversity of different sample matrices including food/feed, biological and environmental samples (Table 6). Only applications dealing with agricultural commodities, as grains, focus exclusively on ZON determination with detection limits far below the guideline levels and maximum residue levels of different countries [68,69,71,74,79,150,163–166]. Depending on the sample matrix, method sensitivities can be achieved down to the ppt range in agricultural commodities [68,69,71,73,74,79,150,163–167] and in feed, food and beverages thereof, such as beer [138], milk [70] and eggs [77]. In addition, urine [142,151,154,156,157,161,162,171], tissue samples [79,142,151,154,156–159,168–171], plasma/serum [79,162,173] and faeces samples of animals [162] have been repeatedly analysed to monitor actual ZON contamination, ZON distribution/deposition and ZON excretion kinetics as well as ZON metabolism in different species, as e.g. in pig [142], heifer [156] and fish [158,159]. Since one of the ZON metabolites, α-ZAL, is used as growth promoter in cattle breeding, similar analytical procedures have also been provided for α-ZAL in animal urine, plasma and tissue samples [77,156,161,162,168–172]. Attention has also been paid to the corresponding metabolites β-ZAL and ZAN [77,156,157,161,162,171,172] that are typically formed *in vivo* in different species.

Interestingly, Lagana et al. determined ZON, α-ZAL and β-ZAL together with other endocrine disrupting chemicals in sewage and river water in order to estimate the risk of possible intake via these aquatic compartments. After suitable SPE sample clean up these analytes could be detected down to the sub ppt range [155,160].

Besides, Schneweis et al. also determined the glucopyranoside conjugate of ZON that was identified as a major ZON metabolite of wheat cells [167]. As pointed out by the authors, this ZON derivative should also be included into the risk assessment of mycotoxin contaminated corn materials since it is easily hydrolysed into ZON during digestion. Simultaneous determination of ZON together with other typical *Fusarium* mycotoxins, such as OTA, trichothecenes and fumonisins, has been also reported several times with LC/MS [68–71,73,74,79].

4.3.2. Sample preparation

Usually, solid cereals and grains are extracted with acetonitrile–water mixtures [68,69,71,73,150,163,167], animal tissues with methanol (muscle) [142,151,156] or with *meta*-phosphoric acid/acetonitrile mixtures (muscle, liver) [156,170] and fungal cultures with a mixture of methanol and aqueous sodium chloride [174]. For the analysis of fatty matrices sodium acetate in methanol–water has been used in combination with an extensive liquid/liquid extraction and preparative HPLC protocol. With this procedure, α-ZAL and β-ZAL could be detected together with 34 other anabolic compounds in kidney fat [169].

In addition to these conventional extraction procedures, several groups investigated microwave assisted extraction [164,165] and pressurised liquid extraction [71,166,175] as robust and time-saving alternatives, that seem to have the potential to enable automated sample handling. Though special

instrumentation is needed, both techniques provided reliable results in grains when used in combination with LC/MS detection without any further sample clean up. On the other hand, Royer et al. recommended SPE cleaning after pressurised liquid extraction since they observed increased levels of co-extracted matrix components being problematic even for selective MS/MS detection [71]. For other matrices, Huopalahti and Henion applied supercritical fluid extraction without any further sample clean up steps and achieved detection limits for α-ZAL of 100 μg/kg in bovine muscle tissue and liver [168] while Lagana et al. proposed matrix solid-phase dispersion as suitable extraction procedure for ZON, α- and β-ZOL in fish tissue [158,159].

With a few exceptions, as direct LC/MS analysis of raw extracts [77,163] or liquid/liquid extraction and preparative HPLC clean up [169,171], raw extracts as well as liquid samples (urine, beverages and water) are usually submitted to one or two consecutive SPE steps, if necessary, after complete enzymatic deglucuronidation of e.g. urine samples [70,151,161,171,172]. In this context, Mallis et al. demonstrated the suitability of automated on-line coupling of SPE sample clean up and LC/MS detection for the high through-put analysis of ZON and other phytoestrogens in plasma [173]. With a set up of two SPE trapping columns and one analytical column sample preparation and LC/MS analysis were performed within few minutes.

A wide variety of SPE absorbent materials, like RP/anion exchange polymers, immunoaffinity [150,162], Florisil [167], MycoSep [68,69,71,74,163] and carbograph-4 [73,158–160] (see also Table 5) has been applied but RP [138,142,150,151, 154–156,161,171,172] and polymeric absorbent materials with RP and anion exchange functionalities [70,142,156,170,173] have found widest applicability. In general, MycoSep columns offer low ZON recovery rates of 30–40% with type 226 material [68,69,71,74] or are even completely unreliable for ZON analysis as the analyte is completely lost due to irreversible trapping phenomena on MycoSep 227 material [74]. Again, immunoaffinity clean up was shown to be less suitable in combination with LC/MS detection, since its compound specificity is in contradiction to the multi-analyte detection capability of MS. In addition, detailed investigations with ZON demonstrated that the combination of highly selective sample clean up and highly selective MS/MS detection is in certain cases an analytical "overkill" [150]. This of course is dependent on the MS selectivity itself. Whenever single-stage MS instruments are used in the SIM mode, selective sample clean-up might be needed to remove any matrix interferences that are not visible with the more selective multi-stage mass spectrometer in the SRM mode [162].

4.3.3. Typical LC conditions for LC/MS analysis

Chromatographic separation prior to MS detection is of major importance since ZON and metabolites analysis deals frequently with two or more analytes with very similar or identical molecular masses and fragmentation behaviour rendering even SIM and SRM more difficult due to mutual influence on ion traces [172]. Furthermore, the wide range of different sample matrices in combination with less selective sample clean up leads, despite the high MS selectivity, to "visible" matrix interferences at the front of LC/MS chromatograms [71,138,142,151,156]. For accurate identification and quantification, these matrix components have to be sufficiently separated, especially from the early eluting β-ZAL and β-ZOL [142,151,156].

RP stationary phases (RP-18 and RP-8) with methanol–water, acetonitrile–water and methanol–acetonitrile–water mixtures as mobile phases, both in the isocratic and gradient mode, are commonly used (Table 5). Ammonium acetate [71,74,138, 150,151,156,171] and acids, like formic acid [71,167], acetic acid [164–166,170,175] and TFA [169] are frequently added, to enhance the chromatographic separation efficiency and/or the ionisation efficiency. Royer et al. observed non-reproducible retention times of ZON when the organic content in the injection solution was strikingly higher than in the mobile phase and recommended, therefore, a similar polarity of both solvents [71].

Depending on the analyte(s)/matrix combination analytical run times are in the range of 5–27 min on conventional RP columns [70,73,150,158,159,169]. LC/MS analysis of ZON alone can be easily achieved within 5–12 min [150,163, 164,166,173]. More recently, monolithic columns (see also OTA) were shown to enable a dramatic reduction of run times while maintaining sufficient separation efficiency for multi-analyte detection. Zöllner et al. cut down LC/MS analysis time for ZON and five of its metabolites from 15 min on a conventional RP column to 7 min on a monolithic column. Using identical mobile phase conditions baseline separation could be easily achieved for all six analytes on a commercially available monolithic column. Unfortunately, the authors performed their experiments in standard solutions and did not report any method validation data [126].

4.3.4. Mass spectrometry

Routine trace analysis of ZON can be performed with ESI and APCI in the positive and negative ion mode. Due to non-selective sample preparation, blocking of the APCI interface by pyrolysed matrix components has been reported during analyses of beer samples [138]. Splitting the first part of the matrix contaminated LC effluent into waste should be, however, a suitable measure to counteract this problem [71,74,166]. A better compatibility of ESI with higher LC flow rates above 200 μL/min could be achieved with a TurboIonSpray™ interface (PE Sciex, Concord, Canada) that enhances mobile phase evaporation during the ionisation process by a stream of hot nitrogen. Even splitting of the LC effluent prior to ESI might be completely omitted though this was surprisingly not done by Lagana and co-workers who used this type of interface with a liquid flow rate of 1 mL/min [73,158,159]. Several authors optimised carefully all ESI or APCI interface parameters [67,163,170] though these investigations are unfortunately not of general value, since the interface parameters are highly dependent on the interface geometries differing from manufacturer to manufacturer and on the individual instrument performance.

In general, the negative ion mode has been found to be strikingly more selective and sensitive than the positive ion mode

Table 5
Overview on LC–MS methods in ZON and metabolites analysis

Analytes	Matrix	Sample preparation	Liquid chromatography		Mass spectrometry		Ref.
			Column	Mobile phase/additives	Ionisation/Ion selection	Scan mode	
α-ZAL	Bovine muscle tissue and liver	SFE	RP-18	Gradient: H_2O with 20 mM NH_4OOCH/MeOH/ACN	APCI + triple quadrupole	SIM	[168]
ZON	Maize	Extraction with ACN/H_2O—75:25, IAC or clean up with MycoSep 224 columns or direct injection of extract	RP-18	Isocratic: H_2O/ACN—60:40	APCI +/(−) single quadrupole	SIM	[163]
α-ZAL, β-ZAL	Kidney fat	Extraction with 0.04 M NaOAc in H_2O/MeOH, extraction with MeOH, liquid/liquid extraction with hexane, liquid/liquid extraction with diethyl ether preparative HPLC	RP-18	Isocratic: H_2O with 0.1% TFA/MeOH—35:65	APCI + triple quadrupole	SRM	[169]
ZON	Maize, oats, wheat, barley	Extraction with ACN/H_2O—75:25 + KCl, SPE with IAC or RP-18 columns	RP-18	Isocratic: H_2O/MeOH—25:75 with 15 mM NH_4OAc	APCI − triple quadrupole	SRM	[150]
α-ZAL	Bovine muscle and liver	Extraction with 0.2% *m*-phosphoric acid/ACN, SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Isocratic: aqueous 0.005% AcOH/ACN—60:40	ESI − single quadrupole	SIM	[170]
ZON, α-ZOL, β-ZOL	Beer	SPE with RP-18 columns	RP-8	Isocratic: H_2O/MeOH—35:65 with 15 mM NH_4OAc	APCI − triple quadrupole	SRM	[138]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Pig urine, pig tissue	Urine: enzymatic deglucuronidation/desulfation, SPE with RP-18 columns, tissue: extraction with MeOH, SPE with RP-18 columns	RP-18	Isocratic: H_2O/MeOH/ACN—45:45:10 with 15 mM NH_4OAc	APCI − triple quadrupole	Product ion scan, SRM	[151,154]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	River water, waste water	SPE with RP-18 columns	RP-18	Isocratic: H_2O/MeOH/ACN—47:16:37 with 10 mM NH_4OAc	APCI − triple quadrupole	Full scan, product ion scan, SRM	[155]
ZON	Pure reference material	Dissolution in MeOH	RP-18	Gradient: H_2O/ACN	APCI ± ion trap	SIM	[67]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL, ZAN	Bovine urine	Enzymatic deglucuronidation/desulfation, SPE with RP-18 columns	RP-18	Gradient: H_2O/ACN	ESI − triple quadrupole	SRM	[172]
α-ZAL, β-ZAL	Chicken liver	Enzymatic deglucuronidation/desulfation, extraction with diethyl ether, liquid/liquid extraction with $CHCl_3$ and aqueous NaOH, SPE with RP-18 columns	RP-18	Gradient: 20 mM NH_4OAc/ACN	ESI − triple quadrupole	SRM	[171]
ZON	Wheat	Microwave assisted extraction with MeOH/ACN 1:1	RP-8	Isocratic: H_2O/MeOH each with 0.2% AcOH—45:55	APCI − ion trap	SIM	[164]
ZON	Corn	Microwave assisted extraction with MeOH/ACN 1:1	RP-8	Isocratic: H_2O/MeOH each with 0.2% AcOH—45:55	ESI − ion trap	SIM	[165]
ZON, ZON-4-β-D-glucopyranoside	Wheat	Extraction with ACN/H_2O—21:4, SPE with florisil columns	RP-18	Gradient: H_2O with 1% HCOOH/ACN	ESI + single quadrupole	Full scan	[167]
ZON	Wheat	Pressurised liquid extraction with ACN/H_2O—1:1	RP-8	Isocratic: H_2O/MeOH each with 0.2% AcOH—45:55	ESI − ion trap	SIM	[166]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL, ZAN	Liver, urine, muscle of pig and heifer	Urine: enzymatic deglucuronidation/desulfation, SPE with RP-18 columns, liver: extraction with 0.2% *m*-phosphoric acid/MeOH—4:6, enzymatic deglucuronidation/desulfation, SPE with *N*-vinylpyrrolidone/divinylbenzeneco-polymer columns, muscle: extraction with MeOH, SPE with RP-18 columns	RP-18	Isocratic: H_2O/MeOH/ACN—45:45:10 with 15 mM NH_4OAc	APCI − triple quadrupole	SRM	[142,156,157]
ZON	Pure standard material	Dissolution in HPLC mobile phase	RP-18	Isocratic: H_2O/MeOH/ACN—45:45:10 with 15 mM NH_4OAc	APCI − triple quadrupole	Full scan, product ion scan, SIM, SRM	[176]
ZON, α-ZOL, β-ZOL	Fish tissue and liver	Matrix solid-phase dispersion with RP-18 material, SPE with carbograph-4 columns	RP-18	Isocratic: H_2O/MeOH/ACN—47:16:37 with 10 mM NH_4OAc	ESI − (APCI−) triple quadrupole	SRM	[158,159]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Pure standard material	Dissolution in HPLC mobile phase	RP-18 monolithic	Isocratic: H_2O/MeOH/ACN—45:45:10 with 15 mM NH_4OAc	APCI − triple quadrupole	SRM	[126]
ZON	Corn	Pressurised liquid extraction with isopropanol/1% aqueous NH_3—1:1	RP-8	Isocratic: H_2O/MeOH each with 0.2% AcOH—45:55	ESI − ion trap	SIM	[175]
ZON	Rat plasma	Dilution with EDTA, on-line SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Gradient: H_2O with 0.02% triethylamine/ACN	ESI−/(+) triple quadrupole	SRM	[173]
ZON	Maize	Pressurised liquid extraction with ACN/H_2O—75:25, clean up with MycoSep 226 columns	RP-18	Gradient: H_2O + 1% ACN with 5 mM NH_4OAc and HCOOH, pH 4/ACN	APCI − ion trap	Product ion scan, SRM	[71]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Urine	Enzymatic deglucuronidation/desulfation, SPE with RP-18 columns, filtration through amino columns	RP-18	Isocratic: H_2O/MeOH—16:84	ESI − triple quadrupole	SRM	[161]

Table 5 (*Continued*)

Analytes	Matrix	Sample preparation	Liquid chromatography		Mass spectrometry		Ref.
			Column	Mobile phase/additives	Ionisation/Ion selection	Scan mode	
ZON, α-ZAL, β-ZAL	River water, waste water	SPE with carbograph-4 columns	RP-18	Isocratic: H_2O/ACN—50:50	APCI – triple quadrupole	SRM	[160]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Egg	Extraction with ACN	RP-18	Gradient: H_2O/MeOH	ESI – triple quadrupole	SRM	[77]
ZON	Cereal based food and feed	Extraction with ACN/H_2O—85:15, clean up with MycoSep 226 column	RP-18	Gradient: H_2O/MeOH	ESI ± (APCI±) triple quadrupole	SRM	[68,69]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Maize	Extraction with ACN/H_2O—75:25, SPE with carbograph-4 column	RP-18	Isocratic: H_2O/MeOH/ACN—50:15:35	ESI – triple quadrupole	SRM	[73]
ZON	Maize	Extraction with ACN/H_2O—84:16, clean up with MycoSep 226 (227) column	RP-18	Gradient: H_2O/MeOH with 5 mM NH_4OAc	APCI – QTrap	SRM	[74]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Milk	Enzymatic deglucuronidation, extraction with ACN/hexane—61:39, SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Gradient: H_2O/MeOH	ESI – triple quadrupole	Product ion scan, SRM	[70]
ZON	Fungal cultures	Extraction with MeOH/1% aqueous NaCl—80:20	RP-18	Isocratic: H_2O/MeOH—20:80	APCI – ion trap	SRM	[174]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL, ZAN	Urine, plasma, faeces	Urine: enzymatic deglucuronidation/sulfation, SPE with RP-18 columns, IAC, plasma: enzymatic deglucuronidation/sulfation, IAC, faeces: extraction with MeOH/H_2O—50:50, enzymatic deglucuronidation/sulfation, SPE with RP-18 columns, IAC	2*x* RP-18	Isocratic: H_2O/MeOH/ACN—35:30:35	APCI – single quadrupole	SIM	[162]

ZON: zearalenone, ZAN: zearalanone, α-ZOL: α-zearalenol, β-ZOL: β-zearalenol, α-ZAL: α-zearalanol (zeranol), β-ZAL: β-zearalanol (taleranol).

due to exclusive formation of deprotonated molecules $[M-H]^-$ [71,138,150]. This can be attributed to the acidic phenolic groups and to the instability of the protonated molecule $[M+H]^+$ in the positive ion mode [150,163]. Consequently, recording of negative ions has been predominantly applied for quantitative trace analysis though it is reported that variation of analyte signals is distinctly higher than in the positive ion mode [163]. The positive ion mode is used only when ZON is analysed together with other types of analytes as e.g. ZON glucopyranoside [167], that exhibit better ionisation efficiencies and in-source fragmentation characteristics in the positive ion mode.

The in-source fragmentation of the deprotonated molecule of ZON and its metabolites is limited to carbon dioxide elimination in the negative ion mode. Thus, structural information can only be obtained from MS/MS experiments on multi-stage MS instruments, even when the parameters like cone voltages are increased [163,170]. Therefore, none of the single-stage methods would be able to meet present official guidelines and regulations about unambiguous identification of target residues in food, feed and biological samples [40,41] since less than four ions are available for SIM [67,162–164,166,168,170].

The negative collision activated product ion spectra of ZON and its metabolites are independent in their appearance of the applied collision gas (helium, argon, nitrogen) [71,151,171]. As exemplified in Fig. 5a for α-ZOL, they all contain abundant ions reflecting neutral losses of water and carbon dioxide which are commonly observed for ester and lactones (Fig. 5b). Ions indicating more compound specific bond cleavages in the lactone ring system (Fig. 5a: *m/z* 160, *m/z* 174 of α-ZOL) can be observed in significant intensities only for ZON and ZAN unless collision energies are considerably increased [155,161]. Recently, this characteristic fragmentation pattern has been used to identify traces of by-products in ZON standards [176]. In addition, Jodlbauer and co-workers were able to show that the use of these very low abundant but compound specific product ions are extremely useful to enhance considerably SRM detection sensitivity for α- and β-ZOL (Fig. 5b and c: 319 ⇒ 275 versus 319 ⇒ 174) [151,157].

4.3.5. Method validation and matrix effects

LC/MS provides excellent sensitivity in the low ppb range which is well below the present guideline and maximum residue levels of ZON and α-ZAL in food and feed. For liquid matrices, like urine, beverage and plasma, LODs at the ppt level are easily feasible after suitable sample enrichment. Lagana et al. demonstrated LC/MS/MS sensitivities even in the sub ppt range for ZON, α- and β-ZAL in water [155,160]. The observed recovery rates range between 30 and 138% and are highly dependent on the analyte–matrix combination and the efficiency of sample clean up and LC prior to MS analysis [138,163]. In general, the relatively polar analytes β-ZAL and β-ZOL frequently exhibit the lowest recoveries indicating analyte losses during sample clean up [142]. Furthermore, several authors reported a striking decrease of recovery rates and method sensitivities when sample clean up and/or LC separation are diminished or even omitted [71,163,166].

Table 6
Validation data of LC–MS methods in ZON and metabolites analysis

Analytes	Matrix	Recovery (%)	LOD/LOQ (μg/kg) or (μg/L)	Linear range (μg/kg) or (μg/L), calibration procedure	Accuracy/precision (%)	Ref.
α-ZAL	Bovine mucle tissue and liver	58 (muscle tissue)	100/–	–	–/12.1	[168]
ZON	Maize	78–130	0.12/0.17	0.17–35, external calibration	<8.0/1.1–7.4	[163]
ZON	Maize, oats, wheat, barley	96–104	0.5/1	1–1000, internal calibration in matrix: ZAN	4.5/–	[150]
α-ZAL, β-ZAL	Kidney fat	–	Low ppb level/–	–	–/–	[169]
ZON, α-ZOL, β-ZOL	Beer	99–106	0.03–0.06 / 0.07–0.15	0.15–500, internal calibration in matrix: ZAN, standard addition	2.1–3.3/–	[138]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Pig urine, pig tissue	92–102 (urine), 86–91 (tissue)	0.1–0.5 / 0.5–1 (urine), 0.5/1 (tissue)	LOQ—100 (urine), 1–100 (tissue), internal calibration in matrix: ZAN	1.6–8.2/– (urine), 2.7–5.6/– (tissue)	[151,154]
α-ZAL	Bovine muscle and liver	85 (muscle), 79 (liver)	0.5 / 2.5	0.5–200, external calibration	–/2.0	[170]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	River water, waste water	81–92	–/0.0004–0.002	LOQ—0.1, internal calibration in matrix: 4-octylbenzenesulfuric acid	2.5–5.8 / 3.5–6.3	[155]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL, ZAN	Bovine urine	92–107	0.02–0.3 / 1	1–10, internal calibration in matrix: $[D_4]$-α-ZAL and $[D_4]$-β-ZAL	–/7.3–27	[172]
α-ZAL, β-ZAL	Chicken liver	74	–/1	1–10, external calibration in matrix	–/8–13	[171]
ZON	Wheat	93	–/30	Internal calibration in matrix: ZAN	6 / 12	[164]
ZON, ZON-glucopyranoside	Wheat	69 (ZON-glucopyranoside), 89 (ZON)	–/10	10–1000 external calibration	–/–	[167]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL, ZAN	Urine, muscle, liver	94–105 (urine), 86–91 (muscle), 55–85 (liver)	0.1–0.5 / 0.5–1.0 (urine), 0.5/1.0 (muscle), 0.1–1.0 / 0.5 –3.0 (liver)	0.5/1.0–100 (urine), 1.0–100 (muscle), 1.0/3.0–100 (liver), internal calibration in matrix: $[D_2]$-ZAN	1.6–8/–(urine), 3.7–5.6/–(muscle), 2–10/–(liver)	[142,156]
ZON	Wheat	93–103	5 / 15	80–800, internal calibration in matrix: ZAN	4.5 / 8.4	[166]
ZON, α-ZOL, β-ZOL	Fish tissue	83–103	0.1–1.0 / –	–	<11 / –	[158]
ZON, α-ZOL, β-ZOL	Fish tissue and liver	85–92	0.1–0.2 / –	–, internal calibration: ZAN	–/7.0–9.3	[159]
ZON	Rat plasma	–	0.1 / 1.0	1–1000, internal calibration in matrix: 3-chloro-4-fluorophenyl-2-morpholine-4-yl-kacetamid	–/5.0	[173]
ZON, α-ZAL, β-ZAL	River water, waste water	91–98	0.0003–0.0084 / –	–, internal calibration: ZAN	–/<8	[160]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Egg	74–79	0.1 / 0.3	0.3–5, external calibration in matrix	–/15–20	[77]
ZON	Maize	40	3 / 10	50–1000, internal calibration in matrix: α-ZAL	4.1–8.3 / –	[71]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Urine	101–138	0.2–0.3 (CCα)/0.4 (CCβ)	–, internal calibration in matrix: $[D_4]$-α-ZAL, $[D_4]$-β-ZAL, $[D_4]$-α-ZOL, $[D_4]$-β-ZOL	1.9–7.7 / –	[161]
ZON	Cereal based food and feed	92	–/10	10–200, internal calibration: ZAN	–/–	[68,69]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Maize	89–106	3–6 / 5–10	5–5000, internal calibration: α-estradiol	–/5–10	[73]
ZON	Maize	30	0.9 / 3.2	10–1000, internal calibration in matrix: ZAN	5.0 / 9.6	[74]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Milk	82–106	0.02–0.06 (CCα)/0.03–0.08 (CCβ)	–, external calibration in matrix	2–20 / 4.4–15	[70]
ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL, ZAN	Urine, plasma, faeces	57–108 (urine), 89–101 (plasma), 92–108 (liver)	0.1–0.5 / 0.5–1.0 (urine), 0.1–0.5 / 0.5–0.6 (plasma), 0.1–0.2 / 0.5–1.0 (faeces)	LOQ–100, internal calibration: $[D_2]$-ZAN	5–21 / 3.9–8.4 (urine), 2.5–6.7 / 2.0–10.7 (plasma), 1.0–9.4 / 2.0–4.8 (faeces)	[162]

ZON: zearalenone, ZAN: zearalanone, α-ZOL: α-zearalenol, β-ZOL: β-zearalenol, α-ZAL: α-zearalanol (zeranol), β-ZAL: β-zearalanol (Taleranol).

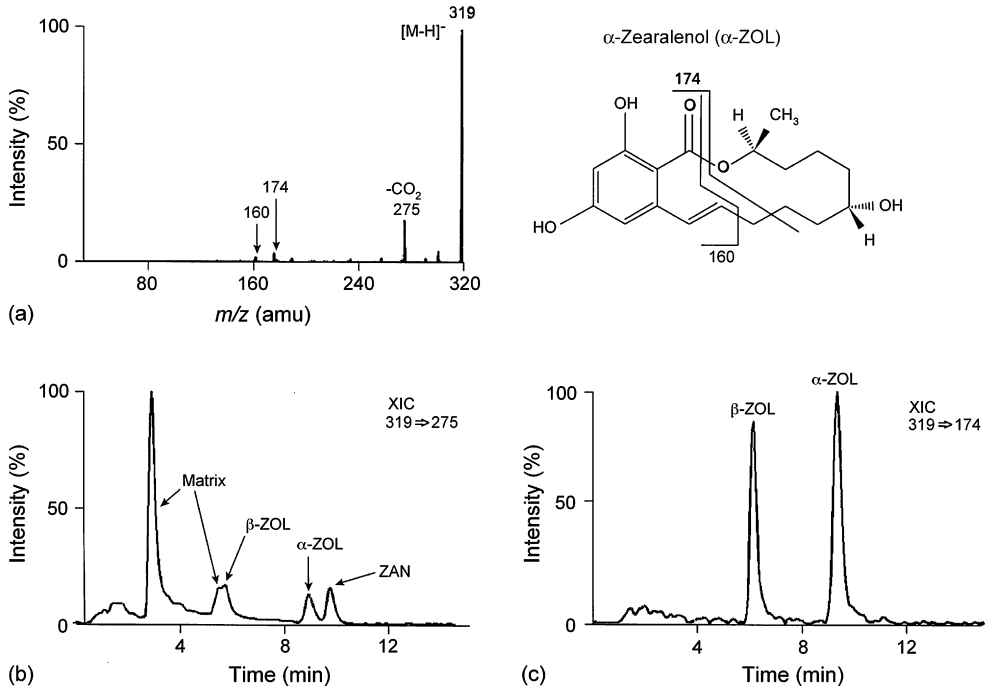


Fig. 5. (a) Product ion mass spectrum of α-ZOL (negative ion mode); (b) ZOL selective SRM chromatogram of a pig urine sample spiked with ZON and its metabolites (5 μg/L of each analyte). Recorded fragmentation pathway 319 ⇒ 275 u; $[M\text{-}H\text{-}CO_2]^-$; (c) α-ZOL selective extracted reaction monitoring chromatogram (XIC) of the same pig urine sample. Recorded fragmentation pathway: 319 ⇒ 174 u. (Reproduced with permission from Chromatographia 51 (2000) 681. Copyright 2000 Friedrich Vieweg [151] and LC–GC Europe 16 (2003) 354. Copyright 2003 Advanstar [157].)

Typically, linear ranges are achieved with one to three orders of magnitude. As demonstrated for the analysis of ZON and ZOLs in grain and beer, co-eluting MS "visible" and "invisible" matrix components might have, however, also tremendous impact on the analyte ionisation efficiency and consequently on the accuracy of quantitative data, when the analytes are not sufficiently separated from these components by either sample clean up and/or LC [138,150]. In this respect, the polar analytes β-ZAL and β-ZOL offer less accurate data since they elute in the front of the chromatograms where they are not sufficiently separated from disturbing matrix components [142]. Setting up matrix matched calibration curves or even performing a standard addition protocol [138] has been shown to be frequently mandatory and the use of internal standards improved considerably the accuracy when calibration curves varied between different grain, beer and biological matrices [138,150,162]. Due to the high probability of matrix effects in food analysis, internal standards such as ZAN [68,69,74,138,150,151,159,160,164,166], $[D_2]$-ZAN [142,156,162], α-ZAL [71], $[D_4]$-α-ZAL [161,172], $[D_4]$-β-ZAL [161,172], α-ZOL [161], β-ZOL [161] and to a less extent also 4-octylbenzene sulfuric acid [155], 3-chloro-4-fluorophenyl-2-morpholine-4-yl-kacetamid [173] and α-estradiol [73] are in frequent use. Zöllner et al. demonstrated in detail that similar or identical MS and LC properties are of major importance for the suitability of an internal standard [126]. Taking this into account, stable isotopically labelled internal standards, if available, should be absolutely preferred, while the use of 4-octylbenzenesulfuric acid, 3-chloro-4-fluorophenyl-2-morpholine-4-yl-kacetamid or α-estradiol can only compensate for performance variations of the LC/MS system since all three compounds exhibit completely different physical and chemical properties than ZON or any of its metabolites [155,173]. In this respect, only van Bennekom et al. [172] and Launay et al. [161] addressed the problem of multi-analyte detection and applied two and four stable isotopically labelled internal standards, respectively, to cover varying matrix influences over the whole chromatographic elution range of ZON metabolites. However, even with these extensive precautions matrix effects could not always be fully excluded, as reported by Launay et al. who observed ZON recovery rates in urine far beyond the 120% level [161].

LC/MS results were also shown to be in good agreement with data of other analytical techniques typically applied in ZON analysis, as LC-FL [139,147], GC/MS [161,169] and ELISA [73,150,171]. Zöllner et al. analysed ZON in maize in the course of an interlaboratory comparison test and achieved reliable results in comparison with several other analytical techniques [150], giving evidence that LC/MS is a powerful and convenient tool to confirm positive screening findings in ZON analysis.

With one exception [173] all MS/MS methods are in agreement with current guidelines about unambiguous compound identification in residue analysis since a minimum of two precursor ion/product ion pairs are selected for SRM experiments [40,41]. Besides, Launay et al. [161] as well as Sorensen and Elbaek [70] validated their LC/MS methods according to current EU validation criteria and determined e.g. the decision limit CCα and the detection capability CCβ which have been set up by the European Commission to replace LOD and LOQ in residue analysis [40,41]. In this context, it has to be mentioned that

the above cited regulations are currently only obligatory for the detection of α-ZAL, as this veterinary agent has been prohibited in food producing animals within the EU.

5. Fumonisins

Fumonisins are nongenotoxic carcinogens whose structures have been elucidated in 1988 [177]. They are diesters of propane-1,2,3-tricarboxylic acid and either 2-acetylamino-12,16-dimethyl-3,5,10,14,15-pentahydroxycosane (A-fumonisins) [178], 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxycosane (B-fumonisins) or 1-amino-11,15-dimethyl-2,4,9,13,14-pentahydroxynonadecane (C-fumonisins) with related homologues differing in the presence or absence of hydroxy groups at position C-5 and C-10 of the C-20 aminopentol backbone (Fig. 6). More recently, several other natural modifications, such as an *N*-linked 3-hydroxypyridine moiety (P-fumonisins) [179] and hydrolysis and oxidation of the ester group at position C-15 [180] have been identified.

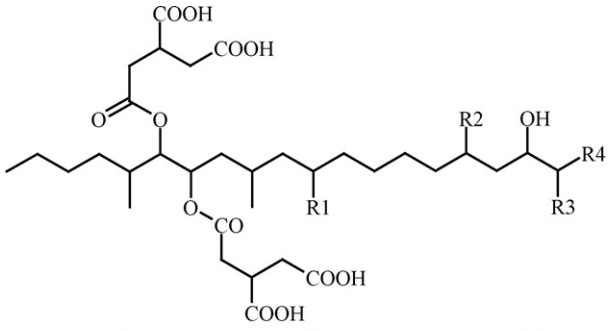
Fumonisins are primarily produced by the fungi *Fusarium monoliforme* and *Fusarium proliferatum* and occur at high incidence in corn and corn products all over the world [181]. They cause severe disorders in animals, as equine leukoencephalomalacia [182], pulmonary oedema in pigs [183] and oesophageal and hepatic cancer in horses and rats [184]. Though such severe acute toxic effects have not been described for humans and the available toxicological data give no clear evidence on their long-term toxicity [185–188], fumonisins are suspected to be the causative agents in human oesophageal cancer when ingested at high levels of 1–10 ppm [188]. For this reason the FDA recommended in 2001 maximum levels of total fumonisin contents in corn-based food (2–4 ppm) and feed (5–100 ppm) and the European Commission suggested a maximum tolerable total fumonisin intake of 2 μg/kg body weight [186–188].

Fumonisin B_1 (FB_1) attracts most attention since it comprises about 70% of total fumonisin content of *Fusarium* cultures and of contaminated corn samples and exhibits highest cancer promoting activity of all fumonisins. Its *in vivo* metabolism as well as its degradation during food and feed processing relies predominantly on partial or full hydrolysis of one or both ester groups [189]. Though FB_1 is relatively heat stable and persists most conditions in food manufacturing, it is transformed into its *N*-carboxymethyl analogue when it is heated in the presence of reducing sugars (baking, frying and cooking) [190,191]. Besides, partial hydrolysis has been observed in the presence of water under elevated temperatures [192].

5.1. Conventional fumonisin analysis

Present fumonisin trace analysis is focused on grains and products thereof, since the main source of fumonisin contamination has been identified in these matrices. To reach appropriate detection limits in the low ppb range sample purification and analyte enrichment are mandatory after analyte extraction from solid samples with water/methanol or water/acetonitrile mixtures. SPE is nowadays the method of choice with anion exchange, RP and more recently immunoaffinity materials as sorbent materials [24,193].

Due to their high molecular mass and polarity, fumonisins mixtures are typically separated by LC. Since they do not have suitable chromophores, their free amino group is gen-



	R_1	R_2	R_3	R_4
FA_1	OH	OH	CH_3CONH	CH_3
FA_2	H	OH	CH_3CONH	CH_3
FB_1	OH	OH	NH_2	CH_3
FB_2	H	OH	NH_2	CH_3
FB_3	OH	H	NH_2	CH_3
FB_4	H	H	NH_2	CH_3
FB_5	OH	H	NH_2	CH_3
FC_1	OH	OH	NH_2	H
FC_3	OH	H	NH_2	H
FC_4	H	H	NH_2	H
FD*	H	H	H	H
FP_1	OH	OH	3-hydroxypyridine	H
FP_2	H	OH	3-hydroxypyridine	H
FP_3	OH	H	3-hydroxypyridine	H

*Hydroxy group between R_1 and R_3 replaced by hydrogen atom.

Fig. 6. Structures of different groups of fumonisins.

erally derivatised for FL detection to reach detection limits between 10 and 50 μg/kg. A number of precolumn derivatisation reagents have been evaluated for this purpose but presently *o*-phthaldialdehyde is predominantly used though its chemical stability is critical and pushes for a fast and automated sample handling [193].

In contrast to LC, GC has only found limited use in fumonisin analyses [193]. Taking their high molecular mass and polarity into account, hydrolytic break down of fumonisin species into smaller fragments and suitable derivatisation are necessary but critical and time-consuming sample preparation steps that reduce considerably valuable structural information [24,193].

For screening purposes, low cost TLC and highly selective immunoaffinity-based techniques as ELISA have found wide acceptance [24,193]. Both techniques can be automated and enable high sample through-puts. Nevertheless, positive findings of both techniques have to be confirmed by other analytical techniques and especially ELISA is actually not capable to differentiate between individual fumonisin species.

5.2. Early LC/MS technology for fumonisin analysis

As mentioned above GC/MS is not able to deal with intact high mass fumonisins. Consequently, structural elucidation and confirmation in the course of environmental, biological and food/feed studies were performed by LC/MS [194]. The first experiments in the beginning of the 1990s relied predominantly on flow FAB [195–198] and TSP ionisation [189,195,199] and to a less degree on liquid secondary-ion MS [198,200] and PB MS that was used after methylation of the carboxy groups [201]. A considerable part of these papers focused on the mass spectrometric properties of fumonisins, as ionisation efficiency, adduct ion formation, in-source fragmentation and CID reactions [195,199,201]. FAB MS was found to be best suitable for fumonisin analysis when directly compared with thermospray and electrospray [195]. It was applied in several studies in food and feed and other biological surroundings either to confirm the presence of fumonisins or to elucidate their structures [195–198]. In contrast, thermospray MS exhibited only sufficient performance in the negative ion mode when ammonium acetate was present in the mobile phase [199].

5.3. Modern LC/MS analysis of fumonisins

5.3.1. Investigated fumonisin-type analytes and typical matrices

Due to its high and frequent incidence and its highest cancer promoting activity of all fumonisins, LC–API/MS applications have been clearly focused on the detection and quantitative determination of FB_1 [70,71,198,202–216]. Other series of fumonisins, as type-B [70,205,213–218], type-A [178,217], type C [202,219] and type-P fumonisins [179,217] have been almost exclusively analysed either together with FB_1 or other fumonisins. The majority of published fumonisin analyses by LC/MS was performed down to the low ppb level in grains and products thereof, like fried tortilla chips, masa and feed, where fumonisin contamination is most likely to occur. Other matrices, as garlic bulbs, asparagus spears [210], milk [70] or porcine tissues and body liquids [212] and hair [220], have rarely been monitored (Table 7).

Furthermore, liquid and solid fungal cultures were investigated in order to detect new fumonisin species, to elucidate biosynthetic pathways or to isolate sufficient fumonisin material for MS studies or further structural analysis [178,179,198,202,217,221,222]. In this respect, LC/MS and LC/MS/MS methodology played a major role in the 1990s to discover new series of fumonisins (FA, FC and FP) and to elucidate together with NMR spectroscopy their structures and biosynthetic formation pathways [178,179,202,221,223].

Besides, the fate of FB_1 during food processing and under environmental stress was extensively investigated. Partially and fully hydrolysed FB_1 ($PHFB_1$ and HFB_1) were identified as major hydrolytic degradation products [222,224] also frequently occurring together with FB_1 in corn and corn products [62,207,208,225–227]. *N*-(carboxymethyl)-FB_1 was shown to be the major reaction product of FB_1 with reducing sugars during baking, frying and cooking [225–229]. Poling et al. [227] and Lu et al. [228] characterised major intermediates of this reaction pathway and established the FB_1-glucose reaction kinetics.

A considerable part of fumonisin analyses was undertaken in aqueous model systems. Purpose of theses studies was to investigate the adduct formation ability of fumonisin with biomolecules [227,228,230,231], their hydrolytic [224] and oxidative [232] behaviour and fumonisin degradation during different food processing procedures [227–229]. Seefelder et al. identified FB_1 glyco and amino acid conjugates by LC/MS when they studied the binding of FB_1 to matrix components, such as saccharides and proteins [230] while other groups used ESI MS to monitor the formation of non-covalent complexes of FB_1 with DNA analogue oligonucleotides [231] and to investigated ozone-induced degradation products of FB_1 [232].

5.3.2. Sample preparation

Usually, solid matrices are extracted with methanol, acetonitrile or mixtures of both solvents with water. To enhance solubility of acidic fumonisins in organic solvents some authors applied pressure during extraction [71] or added acids, such as hydrochloric acid [209,210,225], sulphuric acid [70] or formic acid [213,229], to support protonation of fumonisin analytes. For further purification and analyte enrichment, liquid samples and extracts of solid samples – sometimes defatted with an intermediate hexane extraction step [212] – are predominantly submitted to SPE protocols with a wide variety of different sorbent materials, as RP [62,203,208,218,220,221,226,228], strong anion exchanger [62,71,206,210,212,219,220], IAC [205,207,216], carbograph [213] or polymeric materials with RP and anion exchange functionalities [70]. Addressing the needs of high through-put analysis, Newkirk et al. demonstrated that SPE is relatively easily implemented in an automated on-line SPE–LC/MS approach even when several fumonisins and their degradation products have to be analysed simultaneously (Fig. 7) [207].

Due to the structural complexity of naturally occurring fumonisins, special attention has to be paid to a careful selec-

Table 7
Overview on LC–MS methods in fumonisin analysis

Analytes	Matrix	Sample preparation	Liquid chromatography		Mass spectrometry		Ref.
			Column	Mobile phase/additives	Ionisation/ion selection	Scan mode	
FB_1, FB_2, FB_3, FB_4, HFB_1, $PHFB_1$	Corn, corn products	Extraction with ACN/H_2O—50:50, SPE with RP-18 columns	RP-18	Gradient: H_2O/MeOH each with 0.35% AcOH	ESI ± ion trap	Full scan, SIM	[200]
FB_1	Standard solution, corn culture extract		Direct inlet	Isocratic: H_2O/ACN—50:50	ESI + triple quadrupole	Full scan, product ion scan	[195]
FB_1	Liquid cell culture	SPE with RP-18 columns	RP-18	Isocratic: H_2O/ACN/AcOH—44.5:54.5:1	ESI + triple quadrupole	Full scan, product ion scan	[198]
FB_1, FB_2, FB_3	Corn meal	Extraction with ACN/H_2O—50:50, SPE with RP-18 columns	Hydrophobic polymer	Gradient: H_2O with 25 mM NH_4OAc/ACN + AcOH, pH 3.7	ESI + single quadrupole	Full scan, SIM	[218]
$PHFB_1$	Solid corn culture	Extraction with 0.1 M $Ca(OH)_2$, SPE with Amberlite XAD-2, 3× silica gel chromatography, SPE with strong anion exchange and RP-18 materials	Flow-injection	H_2O/MeOH—60:40	ESI + single quadrupole	Full scan	[222]
FA_1, FA_2, FA_3, FB_1, FB_2, FB_3, (FB_4, HFB_1, $PHFB_1$)	Wheat	Extraction with ACN/H_2O—50:50, SPE with strong anion exchange columns (or with RP-18 columns)	RP-18	Gradient: H_2O/MeOH each with 0.01% AcOH	ESI + ion trap	Full scan, SIM	[62,233]
FB_1, FA_1	Solid corn culture	Extraction with MeOH/H_2O—75:25, 5 subsequent preparative HPLC purification steps, with RP-18, cyano, and RP-8 columns	RP-8	Gradient: 0.1% AcOH/ACN	ESI + single quadrupole	Full scan	[178]
FB_1, FB_2	Corn	Extraction with MeOH/H_2O—75:25 SPE with strong anion exchange columns	Loop injection	Isocratic: aqueous 1% HCOOH/MeOH—50:50	ESI ± triple quadrupole	Product ion scan	[206]
FB_1	Standard solution, corn	Extraction with ACN/H_2O—50:50, SPE with RP-18 columns	Capillary electrophoresis	Aqueous 1% AcOH/ACN—95:5 (uncoated columns) or aqueous 0.5% AcOH/ACN—65:35 (C_1-coated columns)	ESI + sector field instrument	Full scan, SIM	[203]
FB_1, $PHFB_1$, HFB_1	Aqueous solution		RP-18	Gradient: H_2O/ACN/HCOOH	ESI + triple quadrupole	Full scan	[224]
FB_1, FB_1 methyl ester, $PHFB_1$	Standard solution		RP-18	Gradient: H_2O/ACN each with 0.2% HCOOH	ESI + triple quadrupole, ion trap	Full scan, product ion scan, precursor ion scan	[204]
FP_1, FP_2, FP_3	Solid corn culture	Extraction with ACN/H_2O—75:25, SPE with RP-18 columns	Loop injection	No data given	ESI + triple quadrupole	Full scan, product ion scan	[179]
FB_1, FB_2, FB_3	Rodent feed	Extraction with ACN/H_2O—50:50, IAC	RP-18	Isocratic: 0.1% aqueous HCOOH/ACN—55:45	ESI + single quadrupole	Full scan, SIM	[205]
FA_1, FA_2, FA_3, FB_1, FB_2, FB_3, FB_4, FB_5, FP_1, FP_2, FP_3, FAK_1, FBK_1, FC_1, $PHFB_1$	Solid corn culture	Extraction with ACN/H_2O—75:25	RP-18	Gradient: H_2O/ACN each with 40 mM HCOOH	ESI + triple quadrupole	Full scan	[217]
FB_1 and ozone induced degradation products	Standard solution		RP-18	Gradient: H_2O/MeOH each with 0.01% AcOH	ESI + triple quadrupole	Full scan	[232]
FB_1	Liquid cell culture	SPE with RP-18 columns	Loop injection	Isocratic: H_2O/MeOH—50:50	ESI − triple quadrupole	Full scan	[221]
FB_1, *N*-carboxymethyl-FB_1	Aqueous sugar solution, corn	Aqueous sugar solutions: extraction with ACN/25 mM HCOOH—50:50, SPE with RP-18 columns, corn samples: liquid/liquid extraction with $CHCl_3$, SPE with RP-18 columns	RP-18	Gradient: 25 mM HCOOH/ACN	ESI + single quadrupole	Full scan	[229]
FB_1, FB_2, FB_3, $PHFB_1$	Rodent feed	Extraction with ACN/H_2O—50:50, IAC on-line coupled to LC–MS	RP-18	Isocratic: 0.1% HCOOH/MeOH—55:45	ESI + single quadrupole	SIM	[207]
FB_1, HFB_1	Corn products	Extraction with ACN/MeOH/H_2O—25:25:50, SPE with RP-18 columns	RP-18	Gradient: H_2O/MeOH each with 0.05% TFA	ESI + triple quadrupole	Full scan, SIM product ion scan	[208]
FB_1	Corn products	Extraction with MeOH/0.1 M HCl	RP-18	Gradient: H_2O/MeOH each with 0.05% TFA	ESI + triple quadrupole	Full scan, SIM	[209]
FB_1 + non-covalent complexes with oligonucleotides	Aqueous solution of oligonucleotides	–	Loop injection	Isocratic: H_2O/MeOH each with 0.1% NH_3—50:50	ESI − single quadrupole	Full scan	[231]
FB_1, FB_2, FB_3	Hair	Reflux with MeOH, SPE with strong anion exchange columns and RP-18 columns	RP-18	No data given	ESI + triple quadrupole	Product ion scan, SRM	[220]
FB_1, $PHFB_1$, HFB_1, *N*-(carboxymethyl)-FB_1, *N*-(1-desoxy-D-fructos-1-yl)-FB_1	Fried corn products, corn	Extraction with ACN/H_2O—50:50, pH 4.5 with 6N HCl	RP-18	Gradient: H_2O/MeOH with 0.1% AcOH/MeOH	ESI + ion trap	Full scan, SIM	[225]
FB_1, HFB_1, *N*-(carboxymethyl)-FB_1	Corn, corn products	Extraction with ACN/MeOH/H_2O—25:25:50, SPE with RP-18 columns	RP-18	Gradient: H_2O/ACN each with 0.05% TFA	ESI + triple quadrupole	Full scan, product ion scan, SIM, SRM	[226]

Table 7 (*Continued*)

Analytes	Matrix	Sample preparation	Liquid chromatography		Mass spectrometry		Ref.
			Column	Mobile phase/additives	Ionisation/ion selection	Scan mode	
FB_1, *N*-(carboxy-methyl)-FB_1, *N*-methyl-FB_1, *N*-(hydroxyacetonyl)-FB_1, *N*-(1-desoxy-D-fructos-1-yl)-FB_1, FB_1-glucose Schiff's base, *N*-(2-hydroxy,2-carboxyethyl)-FB_1, amino acid conjugates	Aqueous solutions of sugars and amino acids	SPE with RP-18 columns	Loop injection	Isocratic: MeOH	ESI + triple quadrupole	Full scan	[228]
FB_1	Asparagus spears, garlic bulbs	Extraction with MeOH/0.1 M HCl, SPE with strong anion exchange columns	RP-18	Gradient: H_2O/MeOH each with 0.05% TFA	ESI + triple quadrupole	SIM	[210]
FB_1, FB_2	Corn-based food	Extraction with MeOH	RP-18	Gradient: aqueous 1% HCOOH/5 mM NH_4OAc in MeOH	ESI + single quadrupole	SIM	[211]
FB_1, $PHFB_1$, HFB_1 *N*-(carboxymethyl)- FB_1, *N*-(1-desoxy-D-fructos-1-yl)-FB_1, FB_1 methylester and degradation products	Aqueous sugar solution	SPE with RP-18 and strong anion exchange columns	RP-18	Gradient: H_2O/MeOH with 1% AcOH/MeOH	ESI + ion trap	Full scan	[227]
FB_1, FB_2, FB_3, FB_4, FC_4	Maize	Extraction with MeOH/H_2O—70:30, SPE with strong anion exchange column	RP-18	Gradient: H_2O/MeOH each with 1% AcOH	ESI + ion trap	SIM	[214,215]
FB_1	Lung, liver, kidney, muscle, heart, brain spleen, pancreas, fat, serum, eye and bile of pigs	Lung, liver, kidney, muscle, brain, spleen, pancreas, eye: extraction with MeOH/H_2O—3:1, defatting with hexane, SPE with strong anion exchange columns, fat: extraction with H_2O, defatting with hexane, SPE with strong anion exchange columns, bile: defatting with hexane, SPE with strong anion exchange column, serum: deproteinising with MeOH, defatting with hexane, SPE with strong anion exchange columns	RP-18	Gradient: H_2O/HCOOH/ACN	ESI + single quadrupole	SIM	[212]
FB_1, glyco- and amino acid conjugates of FB_1	Aqueous solutions of sugars and amino acids	–	RP-18	Gradient: H_2O/ACN each with 0.05% TFA	ESI + triple quadrupole	Full scan, product ion scan	[230]
FB_1	Maize	Accelerated solvent extraction with ACN/H_2O—75:25, SPE with strong anion exchange columns	RP-18	Gradient: H_2O/ACN with 5 mM NH_4OAc and HCOOH, pH 4	APCI + ion trap	Product ion scan, SRM	[71]
FB_1, FB_2, FB_3, FB_4	Maize, maize-based products	Extraction with ACN/50 mM HCOOH—75:25, SPE with RP-18 columns and carbograph-4 column	RP-18	Gradient: H_2O/MeOH each with 25 mM HCOOH	ESI + QTrap	Full scan, product ion scan, neutral loss, SRM	[213]
FB_1, FB_2, FB_3	Cornflakes	Extraction with MeOH/H_2O—70:30 with 0.1 M HCl, IAC	RP-18	Isocratic: ACN/H_2O—60:40 with 0.3% HCOOH	ESI + triple quadrupole	Product ion scan, SRM	[216]
FB_1, FB_2	Milk	Enzymatic deglucuronidation, addition of sulphuric acid, extraction with ACN/hexane—61:39, SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Gradient: H_2O/MeOH with 0.02% AcOH	ESI + triple quadrupole	Product ion scan, SRM	[70]
FB_1, FB_2, FB_3, FC_1, FC_2, FC_3, FC_4	Maize, rice	Extraction with MeOH/H_2O—75:25, SPE with strong anion exchange columns	RP-18	Gradient: H_2O/ACN each with 0.1% HCOOH	ESI + ion trap	Full scan, product ion scan	[219]
FA_1, FA_2, FA_3, FB_1, FB_2, FB_3, FB_4, FB_5, $PHFB_1$, $PHFB_2$, $PHFB_4$, FBK_1, FBK_4, FC_1, FC_2, FC_3, FC_4, $PHFC_4$, FD and other fumonisin like compounds	Fungal culture in rice	Extraction with ACN/H_2O—75:25	RP-18	Gradient: ACN/H_2O each with 0.1% HCOOH	ESI + ion trap	Full scan, SRM	[202]

FA_1: fumonisin A_1, FA_2: fumonisin A_2, FA_3: fumonisin A_3, FB_1: fumonisin B_1, FB_2: fumonisin B_2, FB_3: fumonisin B_3, FB_4: fumonisin B_4, FB_5: fumonisin B_5, FC_1: fumonisin C_1, FC_2: fumonisin C_2, FC_3: fumonisin C_3, FC_4: fumonisin C_4, FD: fumonisin D, FP_1: 3-hydroxypyridinium-FB_1, FP_2: 3-hydroxypyridinium-FB_2, FP_3: 3-hydroxypyridinium-FB_3, $PHFB_1$: partially hydrolyzed fumonisin B_1, $PHFB_2$: fully hydrolyzed FB_1, $PHFB_4$: fully hydrolyzed FB_4, $PHFC_4$: fully hydrolyzed FC_4, FBK_1: fumonisin BK_1, FBK_4: fumoninsin BK_4; FA_1: fumonisin A_1, FA_2: fumonisin A_2, FA_3: fumonisin A_3, FB_1: fumonisin B_1, FB_2: fumonisin B_2, FB_3: fumonisin B_3, FB_4: fumonisin B_4, FB_5: fumonisin B_5, FP_1: 3-hydroxypyridinium-FB_1, FP_2: 3-hydroxypyridinium-FB_2, FP_3: 3-hydroxypyridinium-FB_3, $PHFB_1$: partially hydrolysed fumonisin B_1, HFB_1: fully hydrolysed FB_1, FC_1: fumonisin C_1, FAK_1: fumonisin AK_1.

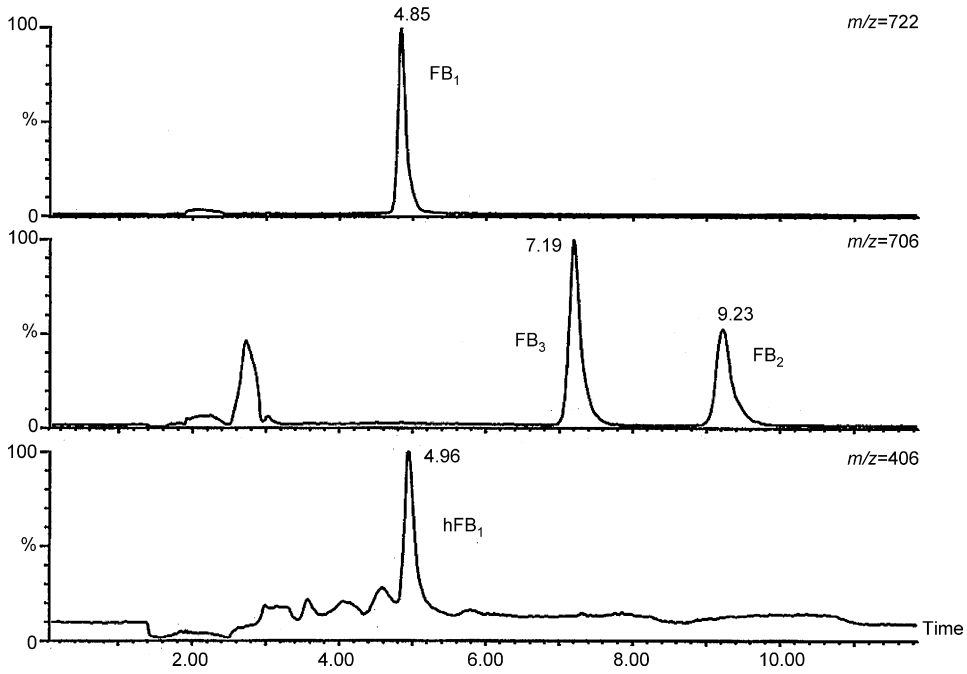


Fig. 7. LC–(SIM)MS chromatograms of a fumonisin mixture (FB_1, FB_2, FB_3 and HFB_1) obtained with on-line IAC/LC–MS clean-up and detection. (Reproduced with permission from Journal of Agricultural and Food Chemistry 46 (1998) 1677. Copyright 1998 American Chemical Society Publications [207].)

tion of SPE sorbent materials. Strong anion exchange resins, for example, cannot be used for hydrolysed fumonisins, since the carboxyl groups responsible for the interaction with the anion exchanger are absent [62,208]. In addition, hydrolysed FBs and other types of fumonisins are also not well retained on FB selective IACs [225]. These observations explain the frequent use of non-selective RP-18 materials when a wide range of different fumonisin species has to be analysed.

To achieve sufficient sensitivities, also two consecutive SPE steps have been considered applying sorbent materials of different selectivities [213,220,227]. Besides, Howard et al. purified raw extract with liquid/liquid extraction followed by RP-18 SPE sample clean up [229] and Musser et al. even applied preparative scale HPLC [178]. All these time-consuming multi-step purification protocols are of special importance for preparative-scale purification, when sufficient analyte material is needed for a detailed structural elucidation of unknown fumonisin species by NMR spectroscopy and MS.

In contrast, but obviously depending on the complexity of the matrix, other authors even omitted any kind of sample preparation and achieved overall method sensitivities in the low ppm range by direct injection of raw extracts into the LC–MS system [202,209,211,225]. However, corresponding method validation data are limited (Table 8).

5.3.3. Typical LC conditions for LC/MS analysis

HPLC separation prior to MS detection is of major importance in this field, because a lot of methods deal with more or less complex fumonisin mixtures. LC almost exclusively relies on RP materials (RP-18) either with methanol/water or acetonitrile/water mixtures as mobile phases in both, the isocratic and gradient mode. In general, fumonisins do not elute well in the RP mode in neutral and unbuffered mobile phase systems. For this reason, formic acid [71,202,204,205,207,212,213,216,217,219,224,229], acetic acid [62,70,178,198,214,215,218,225,232] or TFA [208–210,226,230] are usually added to the mobile phase to enhance analyte retention and also to improve the peak shape and consequently the chromatographic separation efficiency by protonation of the carboxyl groups [213]. A further reduction of peak tailing was observed when LC columns were pre-treated with EDTA to remove traces of metal ions that might form stable complexes with deprotonated fumonisin molecules [213]. Typical LC/MS run times range between 6 and 30 min. Sufficient separation efficiency is seldom a critical issue and baseline separation can easily be achieved even for positional isomers (Fig. 7). Memory effects between consecutive analytical runs have been reported indicating reversible adsorption of fumonisins to the stationary phase when too large amounts of fumonisins are injected onto the LC column [62].

5.3.4. Mass spectrometry

With one exception [71] fumonisins are exclusively analysed with ESI. Stable and high abundant deprotonated $[M-H]^-$ and protonated molecules $[M+H]^+$ with no or a low degree of fragmentation are formed in the negative and positive ion mode (Fig. 8) [62,204–206,218,219,233]. In addition, the formation of doubly charged molecular ions in the negative ion mode [206,231] and sodium and potassium adduct ions in the positive ion mode have been reported [62,209,218,229]. Adduct formation in the positive ion mode seems sometimes a critical issue for the detection sensitivity. Thus, ion recording in the negative ion mode has been recommended [206] though Doerge et al. found the protonated molecule $[M+H]^+$ to be three times more abundant than the deprotonated molecule $[M-H]^-$ [218].

Table 8
Validation data of LC–MS methods in fumonisin analysis

Analytes	Matrix	Recovery (%)	LOD/LOQ (μg/kg) or (μg/L)	Linear range (μg/kg) or (μg/L), calibration procedure	Accuracy/precision (%)	Ref.
FB_1	Corn	–	156/–	500–5000, internal calibration in matrix: tetramethylated FB_1	<30%/6.2–35.6	[203]
FB_1, FB_2, FB_3	Rodent feed	69 (FB_1), 60 (FB_2), 74 (FB_3)	0.3/1.1 (determined in standard solution)	10–200, standard addition in matrix	7.7–16.0/5.8–16.2	[205]
FB_1, FB_2, FB_3, $PHFB_1$	Rodent feed	–	–/250 pg FB_1 standard on column	250–5000 pg FB_1 on column	–/–	[207]
FB_1, FB_2, FB_3, FB_4, $PHFB_1$	Wheat	–	10/–	–	–/–	[62]
FB_1, HFB_1	Corn products	97 (FB_1), 55–88 (HFB_1)	5/– (FB_1), 8/– (HFB_1)	50–150, internal calibration in matrix: [D_6]-FB_1	–/<7.0	[208]
FB_1	Corn products	84	–/20	20–1000, internal calibration in matrix: [D_6]-FB_1	–/–	[209]
FB_1, $PHFB_1$, HFB_1, *N*-(carboxymethyl)-FB_1, *N*-(1-desoxy-D-fructos-1-yl)-FB_1	Fried corn products, corn	–	–/2	2–150, external calibration	–/–	[225]
FB_1, HFB_1, *N*-(carboxymethyl)-FB_1	Corn, corn products	50–60	10/–	50–150 (FB_1, HFB_1), 10–60 (*N*-(carboxymethyl)-FB_1), internal calibration, [D_6]-FB_1	12–15/4–40	[226]
FB_1	Asparagus spears, garlic bulbs	75–92 (asparagus), 96–104 (garlic)	–/–	10–60, internal calibration in matrix: [D_6]-FB_1	–/–	[210]
FB_1, FB_2	Corn-based food	75 (FB_1), 68 (FB_2)	–/5	5–1000, external calibration	–/5–8	[211]
FB_1	Lung, liver, kidney, muscle, heart, brain spleen, pancreas, fat, serum, eye and bile of pigs	37 (fat), 54–92 (all others)	1–2/5–10	5–100 (muscle, heart, brain), 10–100 (serum, eye), 10–500 (bile, spleen, pancreas, lung, liver, fat), 10–4000 (liver), external calibration in matrix	–/3.7–11.1	[212]
FB_1	Maize	90	20/50	50–1000, internal calibration in matrix: [D_6]-FB_1	7.5–14.7/–	[71]
FB_1, FB_2, FB_3, FB_4	Maize, maize-based food	91–105	2.0/– (FB_1), 1.0/– (FB_2)	5–5000 (FB_1, FB_2), internal calibration in matrix: diclofenac (added before LC–MS)	7.7–16.0/<9.0	[213]
FB_1, FB_2	Milk	76–90	CCα/CCβ 0.04/0.09 (FB_1), 0.02/0.04 (FB_2)	–, external calibration in matrix	10/4.9–12 (FB_1), 20/4.0–12 (FB_2)	[70]
FB_1, FB_2, FB_3	Cornflakes	78–85	20/40 (FB_1), 7.5/15 (FB_2), 12.5/25 (FB_3)	25–500 (FB_1), 15–250 (FB_2, FB_3), internal calibration in matrix: (2S, 3R)-2-aminododecane-1,3-diol	4.0–12.0/11–17 (FB_1), 9–16 (FB_2), 7–13 (FB_3)	[216]

FB_1: fumonisin B_1, FB_2: fumonisin B_2, FB_3: fumonisin B_3, FB_4: fumonisin B_4, $PHFB_1$: partially hydrolysed fumonisin B_1, HFB_1: fully hydrolysed FB_1.

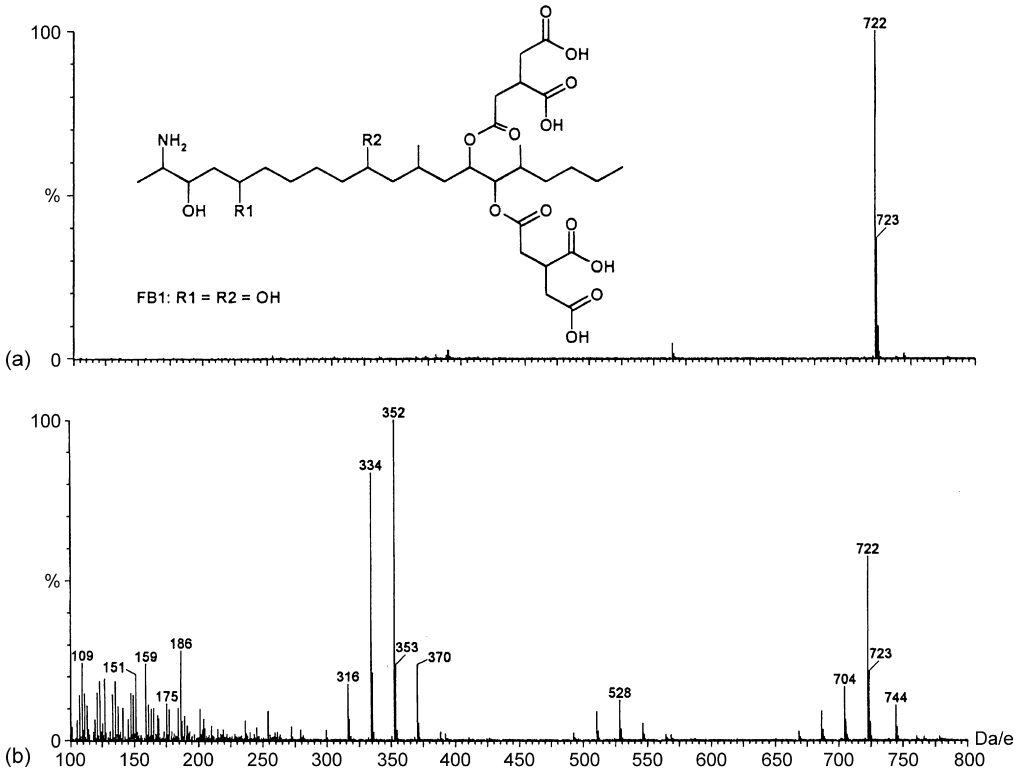


Fig. 8. ESI positive mass spectrum and in-source fragmentation of FB_1 at different cone-skimmer potentials: (a) 40 V and (b) 70 V, obtained on a single quadrupole instrument. (Reproduced with permission from Journal of Agricultural and Food Chemistry 45 (1997) 2573. Copyright 1997 American Chemical Society Publications [205].)

Besides, the positive ion mode is less suitable for type A fumonisins due to the reduced proton affinity of the acetylated amino group [62,206].

Multi-stage MS experiments have been either used for structural elucidation or trace quantification. The fragmentation pathways of low energy collision activated product ion scans reflect consecutive cleavages of the tricarballylic acid moieties along with losses of water molecules due to the anhydride formation within the tricarballylic acid residues [198,204,206,216,219]. Information can be derived on modifications of the amino and hydroxyl groups, e.g. glycosylation [230], the number of hydroxyl and carballylic groups but not on their position in the alkyl backbone [179,202,204,206,229]. Unambiguous structural elucidation is consequently only possible in combination with NMR spectroscopy [178,179,220,227,229]. Fumonisin product ion mass spectra are independent of the collision gas or the applied ion separation principle, predominantly ion trap [62,71,202,204,219,225,227] and triple quadrupole instrumentation [70,179,204,206,208,226,228,230]. Faberi et al. used a QTrap instrument to perform both, ion trap and triple quadrupole experiments on the same instrument and obtained almost identical product ion mass spectra [213]. Josephs [204] and Bartok et al. [202] demonstrated the usefulness of ion trap technology in trace structural elucidation of fumonisins. In this respect, automatic gain control and a data-dependent set up of MS experiments enable a highly efficient approach without any space charging phenomena of the ion trap, even when a large amount of analyte material is injected onto the LC-column.

Fumonisins offer an in-source fragmentation behaviour which is very similar to collision activated MS/MS fragmentation [205,206]. Thus, single stage MS users can easily obtain a comparable amount of structural information when they systematically increase parameters, like the cone voltage (Fig. 8) [205,207,212,218,229]. In this context, it was demonstrated by Churchwell et al. that rapid switching of the cone voltage can even be used to reach simultaneously high sensitivity (quantification) and selectivity (structural confirmation) for fumonisins with single stage MS instrumentation (Fig. 8) [205]. Besides, Seefelder et al. reported that SIM is almost as sensitive as SRM since the high molecular masses of fumonisins move their molecular ions far away from any disturbing LC background noise in the low mass region [226].

5.3.5. *Method validation and matrix effects*

Until now, LC/MS analysis of fumonisins is focused on structural elucidation or confirmation of fumonisin species, of their biosynthetic intermediates and degradation products during food processing. On the other side, quantification still relies predominantly on fluorescence detection after derivatisation with *o*-phthaldialdehyde though this approach is not usable for FA and FP fumonisins. Consequently, validation data of quantitative LC/MS methods are limited and often incomplete and only a few sufficiently validated quantitative LC/MS methods have been published (Table 8) [70,71,205,208,213,216,226]. Only one method implemented current EU validation criteria [40,41] and reports the decision limit CCα and the detection capability

CCβ [70]. Cross-validation of LC/MS methodology with LC-FL was only once reported with good agreement of both techniques [62].

Typically, LODs (Table 8) lie in the low ppb range, due to the high molecular mass of fumonisins even with single-stage instruments [211,212]. Recovery rates range predominantly between 50 and 105% depending on matrix environment and analyte polarity and only Meyer et al. reported a recovery value below 50% in pig fat [212]. The more polar hydrolysed fumonisins offer usually lower recovery rates, indicating analyte losses during sample clean up [208]. Calibration curves ranging over one to three orders of magnitude were established in standard solutions or in matrix, with [71,203,208–210,213,216,226] or without an internal standard [211,212,225] and also by a standard addition protocol [205]. Only three compounds were reported as internal standards. Diclofenac applied by Faberi et al. and 2-amino-dodecane-1,3-diol applied by Paepens et al. are, however, only capable to compensate for performance variations of the LC–MS detector since chemical and physical properties are either rather different from those of fumonisins [213] or sample preparation is performed by fumonisin selective IAC that does not retain the internal standard [216]. For these reasons, both internal standards were added after sample preparation and do not compensate for analyte losses during sample clean up [213,216]. On the other side, Humpf and co-workers introduced as isotope labelled internal standard [D_6]-FB_1 which is able to compensate efficiently for any matrix effects and analyte losses during sample clean up [71,208–210,226].

Matrix related phenomena, as changing ionisation efficiency [205] and missing linearity of calibration curves [209] have been mentioned in several papers. None of the authors performed detailed investigations, though the necessity to use suitable (if possible labelled) internal standards was repeatedly pronounced for matrix effect compensation [210,216]. Interestingly, Faberi et al. reported the absence of any matrix effects in corn-based food as they observed similar calibration curves for type B fumonisins in standard and matrix loaded calibration solutions [213].

6. Aflatoxins

Aflatoxins (Fig. 9) are produced by different *Aspergillus* species growing on agricultural commodities predominantly in hot and humid regions. They were the first mycotoxins that were identified as potential health hazard, when 100.000 turkeys died from an acute necrosis of the liver after consuming groundnuts infected by *Aspergillus flavus* and contaminated by aflatoxins (X-disease) [6]. The International Agency for Research on Cancer has classified aflatoxin B_1 (AFB_1) as a human carcinogen and aflatoxins B_2, G_1 and G_2 (AFB_2, AFG_1 and AFG_2) as possible carcinogens to humans [234,235]. In fact, it was shown that they belong to the most potent nephrotoxic natural compounds and liver carcinogens and, consequently, attracted considerable attention since their discovery in the early 60s [1]. Due to carry-over in food and feed they are considered nowadays to have the most severe impact of all mycotoxins on human health. Maximum residue levels have been set down to the ppt range in a wide variety of agricultural commodities, food, feed and milk, as e.g. 0.01 μg/kg of aflatoxin M_1 (AFM_1) in milk for infants [12].

6.1. Conventional aflatoxin analysis

In general, fast and easy-to-use ELISA based aflatoxin screening kits are commercially available for all major types of aflatoxins. Quantification is predominantly done with LC-FL [236]. Due to the excellent native fluorescence activity of aflatoxins detection limits in the low ppt range can easily be achieved when iodine is added post-column to enhance method sensitivity. In addition, immunoaffinity sample clean up has been shown to have a great potential to increase method specificity and sensitivity by selective enrichment and isolation of the target aflatoxins [236].

6.2. Modern LC/MS analysis of aflatoxins

6.2.1. Investigated aflatoxin analytes and typical matrices

LC/MS has been repeatedly used for structural elucidation in metabolism studies (Table 9). Oligonucleotide adducts [237–239], glutathione adducts [240] and mercapturic acid adducts [238,241] along with other urinary biomarkers could be identified in order to improve the understanding of the *in vivo* aflatoxin mode of action and to find suitable biomarkers for future investigations [237,238,241]. Just a limited number of quantitative methods have been published to determine major aflatoxins and the structurally related sterigmatocystin in food [68,69,117,242–246], milk [70,247], herbs [248], urine [238,239,241,249], airborne dust [249] and cigarette smoke [250]. In this field, LC/MS seems to be just a minor alternative or confirmation technique for the already well established, reliable and robust LC-FL methodology [214,215,244,249] though it should be useful to confirm positive results of TLC and ELISA-based screening analysis.

6.2.2. Sample preparation

Except for two methods, where dip fluids of peanuts were directly injected into the LC/MS system [245] or corn extracts were submitted to a liquid/liquid extraction step followed by IAC [214,215], sample clean up of sample extracts or liquid samples consists of one to three SPE steps. Despite the excellent MS selectivity several authors applied highly selective immunoaffinity absorbent materials [214,215,238,241,249,250] while others demonstrated sufficient sample preparation efficiency with RP-18 [237,244], polymeric absorbent materials [70,248] or graphitised carbon black material [247].

6.2.3. Typical LC conditions for LC/MS analysis

Actually, almost all LC separations were performed on RP materials applying methanol/water and acetonitrile/water mixtures as mobile phase in the gradient as well as isocratic mode. In some cases acetic acid, formic acid, TFA and ammonium salts are added in order to support analyte ionisation and to improve the chromatographic separation efficiency [70,117,214,215, 238,240,241,245,247]. Typically, AFB_1, AFB_2, AFG_1, AFG_2

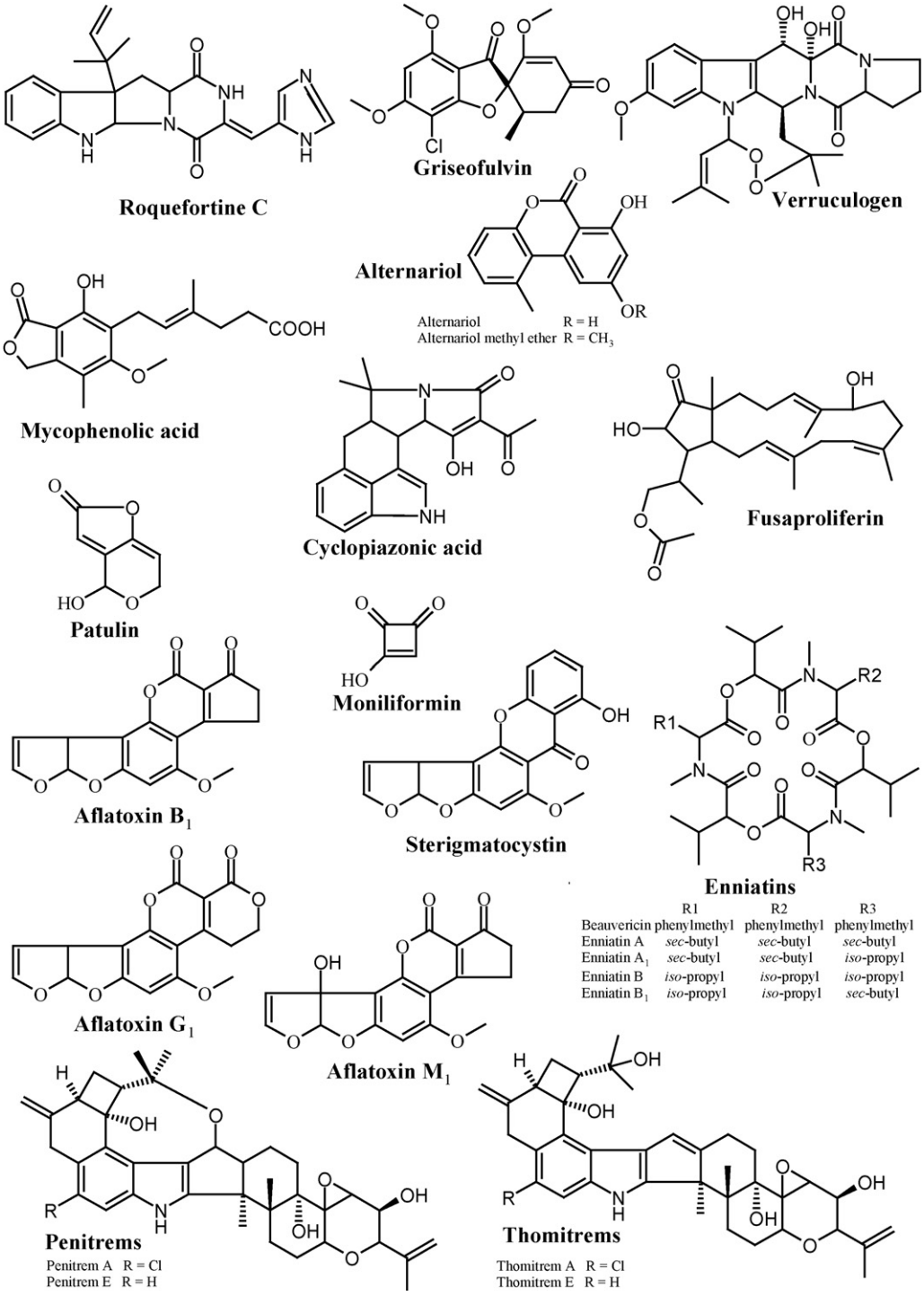


Fig. 9. Structures of different groups of other mycotoxins.

[214,215,238,242,244,248,249], AFM_1 [117] and AFB_1 metabolites and adducts [237,238] are well separated under these conditions with chromatographic run times between 10 and 25 min (Fig. 10). When samples are dissolved in pure methanol or acetonitrile some peak broadening could be observed due to the higher elution power of the organic injection solution compared to the aqueous mobile phase [117]. Besides, Cavaliere et al. applied normal-phase LC with a diol stationary phase and toluene and isopropanol as mobile phase though they did not observe any distinct advantage over RP chromatography regarding separation efficiency and the ionisation efficiency of an atmospheric pressure photoionisation interface (APPI) [247].

6.2.4. Mass spectrometry

In general, all aflatoxins exhibit good ESI ionisation efficiency in the positive ion mode with abundant protonated molecules $[M+H]^+$ and sodium adduct ions, but practically no fragmentation in the full scan spectra [244,248,249]. In this

Table 9
Overview on LC–MS methods in aflatoxin analysis

Analytes	Matrix	Sample preparation	Liquid chromatography		Mass spectrometry		Ref.
			Column	Mobile phase/additives	Ionisation/ion selection	Scan mode	
AFB_1, AFB_2, AFG_1, AFG_2	Airborne dust, urine	Airborne dust: extraction with ACN, IAC, urine: IAC	RP-18	Isocratic: H_2O/ACN/MeOH—60:20:20	ESI + triple quadrupole	Full scan, product ion scan, SIM, SRM	[249]
Sterigmatocystin	Bread, maize, cheese	Extraction with ACN/aqueous 4% KCl—90:10 (bread, maize) or with MeOH/aqueous 4% KCl—90:10 (cheese), defatting with *n*-hexane, liquid/liquid extraction with $CHCl_3$	RP-18	Isocratic: aqueous 10 mM NH_4OAc/ACN/MeOH—20:40:40	APCI + single quadrupole	Full scan, SIM	[243]
AFB_1 mercapturic acid	Rat urine	SPE with RP-18 columns, IAC	RP-18	Gradient: aqueous 1% AcOH/ACN with 40 mM HCOOH	ESI + triple quadrupole	Full scan, product ion scan, SIM	[241]
AFB_1, AFB_2, AFG_1, AFG_2	Peanuts, spices, peanut butter, figs	Extraction with MeOH, column chromatography with cellulose and silica gel	RP-18	Isocratic: H_2O/ACN/MeOH—60:28:12	APCI + triple quadrupole	SRM	[242]
Oligonucleotide adducts of AFB_1	Standard solution in water or aqueous 10 mM sodium phosphate	Preparative LC with an anion exchange columns, SPE with RP-18 columns (desalting)	Infusion via a syringe	Isocratic: H_2O/ACN—50:50	ESI − ion trap	Full scan, product ion scan	[237]
AFB_1 N^7-guanine, AFP_1, AFP_1 glucuronide, AFM_1, AFQ_1, 8,9-dihydro-8,9-dihydroxy AFB_1, AFB_1 mercapturic acid, AFB_1 diol	Rat urine	SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns, IAC, SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Gradient: aqueous 1% AcOH/ACN/MeOH	ESI + ion trap	Full scan, product ion scan	[238]
AFB_1	Peanuts	Dipping with MeOH/H_2O—60:40	Direct injection	Isocratic: H_2O/MeOH—40:60 each with 1% AcOH	APCI + ion trap	Full scan, SRM, product ion scan	[245]
AFB_1, AFB_2	Maize	Extraction with MeOH/H_2O—70:30, IAC	RP-18	Gradient: H_2O/MeOH each with 1% AcOH	APCI + ion trap	SIM	[214,215]
AFB_1, AFB_2, AFG_1, AFG_2	Peanuts	Matrix solid-phase dispersion with RP-18 material	RP-18	Isocratic: H_2O/MeOH—55:45	ESI + single quadrupole	SIM	[244]
AFM_1, AFB_1, N^7-guanine adduct of AFM_1 and AFB_1	Rat urine and liver	SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns, IAC, SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Isocratic: 1% aqueous AcOH/MeOH/ACN—60:38:2	ESI + ion trap	Full scan, product ion scan	[239]
AFB_1, AFB_2, AFG_1, AFG_2	Medicinal herbs	Extraction with MeOH/H_2O—80:20, SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Isocratic: H_2O/MeOH—70:30	ESI + single quadrupole	SIM	[248]
AFB_1, AFB_2, AFG_1, AFG_2	Peanut, corn, nutmeg, red pepper	No data available	RP-18	No data available	ESI+, APPI +	Full scan, product ion scan, SRM	[246]
AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2	Cereal based food and feed	Extraction with ACN/H_2O—85:15, clean up with MycoSep 226	RP-18	Gradient: H_2O/MeOH	ESI − triple quadrupole	SRM	[68,69]
AFB_1, AFB_2, AFG_1, AFG_2, AFM_1	Cheese	Extraction with 0.1% HCOOH in ACN/hexane—55:45	RP-18	Gradient: 0.1% AcOH in H_2O/ACN	ESI + triple quadrupole	SRM	[117]
AFM_1	Milk	Enzymatic deglucuronidation, extraction with ACN/hexane—61:39 SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Gradient: H_2O/MeOH each with 0.02% AcOH	ESI + triple quadrupole	Product ion scan, SRM	[70]
Glutathione adduct of AFB_1	–	–	RP-5	RP-Gradient: H_2O/ACN each with 0.1% HCOOH and 0.005% TFA	ESI + ion trap	Full scan, product ion scan	[240]
AFB_1	Cigarette smoke	Extraction of filter with propan-2-ol, IAC	Phenyl	Isocratic: aqueous 10 mM NH_4OOCH/ACN each with 0.05% HCOOH—67:33	ESI + triple quadrupole	SIM, SRM	[250]
AFM_1	Milk	Proteine precipitation with acetone, SPE with carbograph-4 columns	RP-18, diol	RP-gradient for ESI: H_2O/ACN each with 2 mM NH_4OAc, RP-gradient for APPI: H_2O/MeOH each with 13% acetone, NP-gradient: toluene/isopropanol	ESI+, APPI+, QTrap	Product ion scan, SRM	[247]

AFB_1: aflatoxin B_1, AFB_2: aflatoxin B_2, AFG_1: aflatoxin G_1, AFG_2: aflatoxin G_2, AFP_1: aflatoxin P_1, AFM_1: aflatoxin M_1, AFQ_1: aflatoxin Q_1.

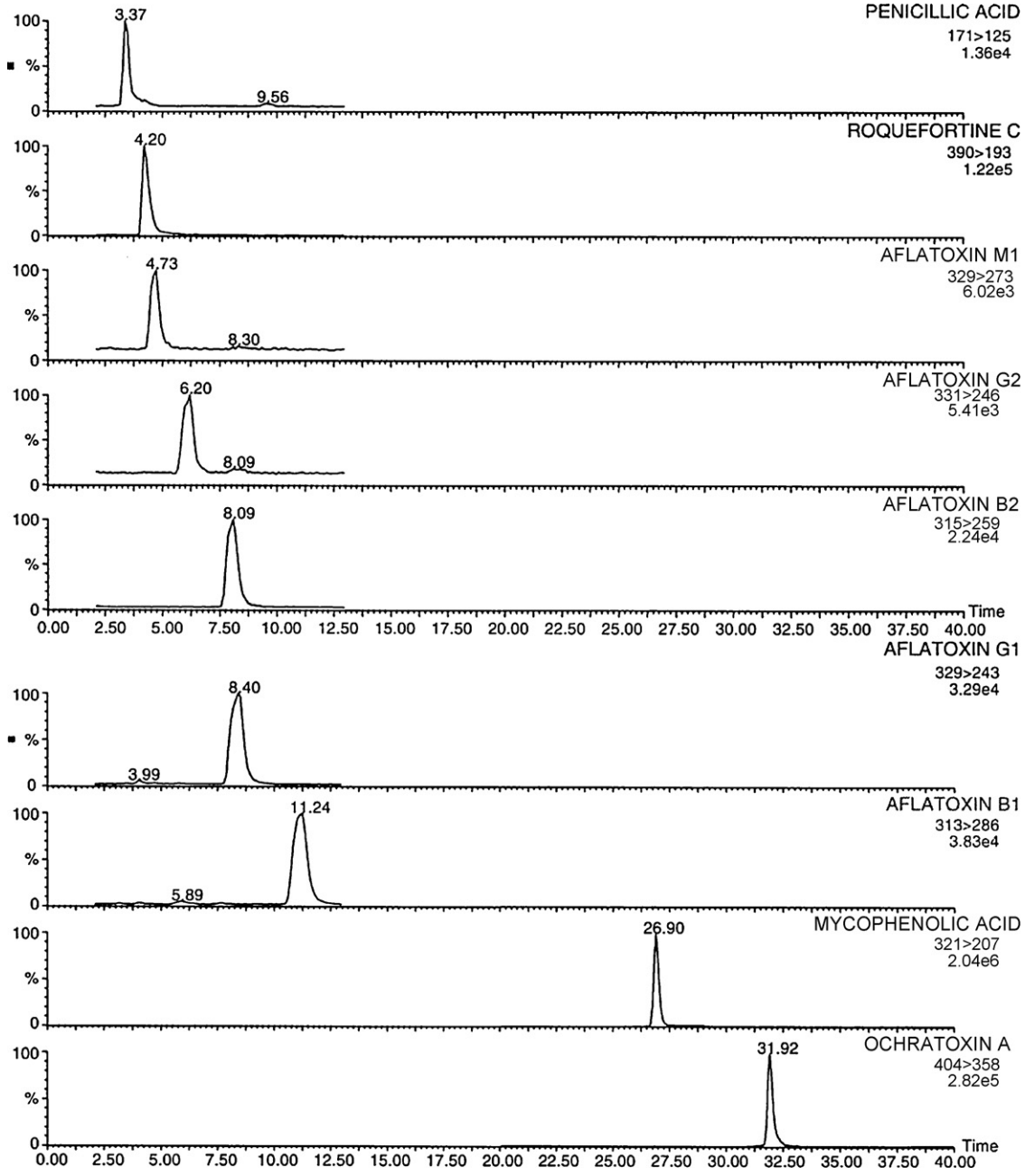


Fig. 10. LC–(SRM)MS/MS chromatogram of a mixture of penicillic acid, roquefortine C, AFM_1, AFG_2, AFB_2, AFG_1, AFB_1, MPA and OTA in cheese obtained with a triple quadrupole instrument (each analyte 50 μg/kg, AFM_1 5 μg/kg). (Reproduced with permission from Food Additives and Contaminants 22 (2005) 449. Copyright 2005 Taylor & Francis [117].)

context, the formation of sodium adduct ions can easily be suppressed by the addition of ammonium ions to the mobile phase leading to a better MS sensitivity [247]. Reports about the utility of APCI interfaces are inconsistent and ionisation efficiencies in this mode seem to be highly dependent on the aflatoxin subgroup and the APCI interface geometry [214,215]. In this respect, only the structurally related sterigmatocystin offers strikingly better sensitivity with an APCI interface in the positive ion mode than with ESI [243], and consequently only Abbas et al. applied APCI for the detection of AFBs in the low ppb range [214,215]. According to recent investigations, APPI seems to be a more reliable alternative to ESI. Since this interface offers strikingly lower levels of chemical noise and ion suppression than ESI it was found to be two to three times more sensitive [246,247].

The product ion spectra of the protonated aflatoxin species contain a number of abundant product ions reflecting bond cleavages and rearrangement reactions of the polycyclic ring system along with loss of water, carbon monoxide and carbon dioxide [247,249]. Despite this favourable fragmentation behaviour, only the quantitative LC/MS/MS approaches of Kokkonen et al. [117], Sorensen and Elbaek [70] and Cavaliere et al. [247] and none of the quantitative single stage LC/MS methods met the EU criteria concerning unambiguous compound identification in residue analysis [40,41] (Table 10). In this respect, Cavaliere et

Table 10
Validation data of LC–MS methods in aflatoxin analyis

Analytes	Matrix	Recovery (%)	LOD/LOQ (μg/kg) or (μg/L)	Linear range (μg/kg) or (μg/L), calibration procedure	Accuracy/precision (%)	Ref.
AFB_1, AFB_2, AFG_1, AFG_2	Airborne dust, urine	–	0.05/–	0.05–2, external calibration	–/–	[249]
Sterigmatocystin	Bread, maize, cheese	118 (bread) 96 (maize), 55 (cheese)	1.9/– (bread), 1.7/– (maize), 2.4/– (cheese)	5–200, external calibration	–/9 (bread), 11 (maize), 20 (cheese)	[243]
AFB_1, AFB_2, AFG_1, AFG_2	Peanuts, spices, peanut butter, fig	42–276 (AFB_1), 68–270 (AFB_2), 74–20 (AFG_1), 49–147 (AFG_2)	0.1/–	–, internal calibration: AFM_1	–/–	[242]
AFB_1 N^7-guanine, AFP_1, AFP_1 glucuronide, AFM_1, AFQ_1, 8,9-dihydro-8,9-dihydroxy AFB_1, AFB_1 mercapturic acid, AFB_1diol	Rat urine	85–90 (AFB_2)	0.013 (AFM_1 and AFQ_1)/–	–, internal calibration: AFB_2	–/12.5 (AFB_1 N^7-guanine) 12.8 (AFB_1 mercapturic acid), 5.8 (AFM_1)	[238]
AFB_1, AFB_2, AFG_1, AFG_2	Peanut	78–86	0.07/0.2 (AFB_1), 0.2/0.6 (AFB_2), 0.07/0.2 (AFG_1), 0.2/0.6 (AFG_2)	–	–/–	[244]
AFB_1, AFB_2, AFG_1, AFG_2	Medicinal herbs	77–110	10 ng on column/25 ng on column	10–5000 ng absolute, external calibration	–/1–19	[248]
AFB_1, AFB_2, AFG_1, AFG_2, AFM_1	Cheese	129–143	0.8/5.0 (AFB_1, AFB_2, AFG_1, AFG_2) 0.3/0.6 (AFM_1)	5–1000 (AFB_1, AFB_2, AFG_1, AFG_2); 0.6–120 (AFM_1), external calibration in matrix	–/2.3–12.1	[117]
AFM_1	Milk	80–98	0.01 (CCα)/0.02 (CCβ)	External calibration in matrix	10/5.5–12	[70]
AFB_1	Cigarette smoke	82–96	3.75 pg on column/11.25 pg on column	11.25–150 pg on column, internal calibration without matrix: [$^{13}CD_3$]-AFB_1	1.3/4.2	[250]
AFM_1	Milk	92–98	–/0.012 (ESI), 0.006–0.035 (APPI)	0.012–1200 (ESI), 0.006–600 and 0.035–3500 (APPI), external calibration without matrix	–/3–8 (ESI), 10 (APPI)	[247]

AFB_1: aflatoxin B_1, AFB_2: aflatoxin B_2, AFG_1: aflatoxin G_1, AFG_2: aflatoxin G_2, AFP_1: aflatoxin P_1, AFM_1: aflatoxin M_1, AFQ_1: aflatoxin Q_1.

al. demonstrated that the QTrap technology opens a new dimension of MS analyte confirmation and quantification. Its operation in the quadrupole linear ion trap configuration (enhanced product ion scans) produces complete product ions mass spectra even close to the LOQ which guarantees accurate analyte quantification simultaneously to unambiguous analyte confirmation [247].

6.2.5. Method validation and matrix effects

Validation data of a couple of methods are excellent regarding method sensitivity, linear range, precision, accuracy and recovery despite the fact that only three groups used internal standards, AFB_2 [238], AFM_1 [242] and [$^{13}CD_3$]-AFB_1 [250], and calibration curves were predominantly set up in standard solutions (Table 10). From this point of view matrix effects seem to be absent in most cases. This is in agreement with two detailed investigations about ion suppression [247,250]. Cavaliere et al. compared the calibration curves set up in standard solution and in sample matrix and found close similarity of both slopes proving that the influence of matrix components on the analyte signal was negligible and matrix effects could be excluded [247]. Alternatively, Edinboro and Karnes infused post-column the aflatoxin analyte into a blank sample injection. As they did not find any dips in the baseline they concluded that ion suppression was absent in the analyte elution zone [250]. The observation of the same authors that the use of a labelled internal standard improved strikingly method accuracy, can, therefore, only be explained by a reliable compensation for MS performance variations.

Direct comparison of LC/MS and LC-FL revealed in most cases good correlation of quantitative results [242,244,249] though LC/MS method robustness and sensitivity seem to be inferior to LC-FL. In this context, Vahl and Jorgensen reported large variations of the recovery rates in different spices. They attributed this observation to severe matrix effects that are not compensated by the applied internal standard AFM_1 and by a calibration curve set up in standard solution [242]. Besides, Blesa et al. demonstrated in peanut samples that LC/MS is less sensitive than LC-FL [244] though this can be partly explained by the use of single quadrupole instrumentation in the SIM mode [244] that is inferior to a tandem MS and SRM recording [242,247,249].

7. LC/MS analysis of other mycotoxins

Though some hundred different mycotoxin species have been discovered so far, analytical efforts have been focused on the five groups of mycotoxins discussed in the previous chapters, since most of the other mycotoxins exhibit either a distinctly lower toxic potential or their presence in agricultural commodities, food and feed is limited in frequency and concentration levels.

For this reason all remaining mycotoxins are discussed in alphabetical order only in a limited style just to complete the overall picture of LC/MS technology in mycotoxin analysis. For more details about mycotoxins with only one or two published LC/MS references, as alternariol and its methyl ether [251], cyclopiazonic acid [252], roquefortine C [117,253,254,292], fusaproliferin [231,256], penicillic acid, chaetoglobsin, verruculogen, penitrems and others [253,254,255], the interested reader is referred to the cited literature and partially to Tables 13 and 14.

7.1. Enniatins and beauvericin

Enniatins (EA) and structurally related beauvericin (BEA) (Fig. 9) are produced by different *Fusarium* species on agricultural commodities, as grains. They possess a cyclic hexadepsipeptide structure differing in the *N*-methylamino acid substitution (Fig. 9). Though they have not been associated with acute mycotoxicoses, several toxicological studies clearly demonstrate that they are cytotoxic [257], have insecticidal properties [258] and may induce apoptosis and DNA fragmentation in mammalian cells [259].

Typically, BEA and EAs are analysed with LC–UV detection with a detection limit of 50 μg/kg in maize being achieved at a detection wavelength of 192 nm [260]. LC/MS based methods (Table 11) offer distinctly less time-consuming sample clean up and avoid also other limitations of the non-selective UV detection. In this respect, methanol, acetonitrile/water and supercritical fluid extracts, predominantly of grain samples, are either directly injected into the LC–MS system [256,261–263] or purified with a further simple RP-SPE step [259,264,265]. In general, chromatographic LC separation of mixtures of BEA and EAs can easily be achieved on RP-18 LC columns within 10–15 min, either with acetonitrile/water [263,266] or methanol/water mixtures [256] in the gradient mode or with isocratic mixtures of acetonitrile/methanol/aqueous ammonium formiate [259,261,262].

With two exceptions [261,262], EAs have been exclusively analysed with ESI (Table 11). The negative ion mode has been reported to be 100 times less sensitive than the positive ion mode [261] and has only been applied for the investigation of non-covalent interactions between BEA and oligonucleotides [231,267]. In the positive ion mode EAs form readily protonated molecular species but also abundant ammonium, sodium and potassium adduct ions [256,263,264]. The MS sensitivity can be strikingly enhanced when ammonium salts are added to the mobile phase to support formation of ammonium adduct ions [259,261,262,264,265] which are transformed into the respective protonated molecular species by adjusting MS parameters, as the cone voltage [264] or collision energy [261].

Collision activated product ion spectra of EAs indicate cleavages of the amide bonds and losses of one or two monomer units [256,261,264] followed by the elimination of water [264] and carbon monoxide [261]. The charged monomer and dimer units are usually used as product ions for SRM detection. Only one LC/MS/MS method is in agreement with the EU criteria on unambiguous identification in residue analysis [40,41] and offers detection limits for all analytes well below the one ppb level [264] (Table 12). This is strikingly more sensitive than any of the published LC–UV methods. Interestingly, Sewram et al. observed higher MS sensitivity for BEA in the SIM mode (0.5 μg/kg in grain) than in the SRM mode which can presumably be attributed to the applied ion trap instrumentation [256].

Investigations on matrix effects and method validation data are sometimes inconsistent or incomplete (Table 12), e.g. Jestoi

Table 11
Overview on LC–MS methodology of other mycotoxins

Analytes	Matrix	Sample preparation	Liquid chromatography		Mass spectrometry		Ref.
			Column	Mobile phase/additives	Ionisation/ion selection	Scan mode	
Enniatins							
BEA and its non-covalent oligonucleotide adducts	Aqueous standard solution	–	Loop injection	Gradient: H_2O/MeOH each with 0.1% NH_3, isocratic: H_2O/MeOH each with 0.1% NH_3—50:50	ESI − single quadrupole	Full scan	[267,231]
BEA	Fungal cultures, maize	Extraction with MeOH	RP-18	Gradient: H_2O/MeOH each with 0.1% HCOOH	ESI + ion trap	Full scan, product ion scan, SIM, SRM	[256]
BEA, EA_1, EB, EB_1	Water	Liquid/liquid extraction with $CHCl_3$	RP-18	Gradient: H_2O/ACN	ESI + single quadrupole	Full scan	[266]
BEA, EA, EA_1, EB, EB_1	Grain, grain-based food	Extraction with ACN/H_2O—84:16, SPE with RP-8 column	RP-18	Isocratic: 10 mM aqueous NH_4OOCH/ACN/MeOH—10:45:45	ESI + triple quadrupole	SRM	[259,265]
BEA, EA, EA_1, EB, EB_1	Grains	Extraction with ACN/H_2O—84:16	RP-18	Isocratic: H_2O/ACN/MeOH each with 15 mM NH_4OOCH—15:45:40	APCI+/(-), ion trap	SRM	[261,262]
BEA	Maize	SFE with CO_2	RP-18	Gradient: H_2O/ACN	ESI + single quadrupole	Full scan, SIM	[263]
BEA, EA, EA_1, EB, EB_1	Grain	Extraction with ACN/H_2O—84:16, SPE with RP-8 column	RP-18	Isocratic: aqueous 10 mM NH_4OOCH/ACN/MeOH—10:45:45	ESI + triple quadrupole	SRM	[264]
Moniliformin							
MON	Maize, fungal culture	Extraction with ACN/H_2O—95:5, liquid/liquid extraction with hexane, SPE with RP-18 column	RP-18	Isocratic: aqueous 100 mM NH_4OOCH/MeOH/triethyl amine—89.95:9.95:0.1	APCI − ion trap	Full scan, SIM	[268]
MON	Aqueous standard solution	Derivatisation with 1,2-diamino-4,5-dichlorobenzene	RP-18	Isocratic: H_2O/ACN each with 0.01% TFA—50:50	ESI + triple quadrupole	Full scan, product ion scan, SIM	[274]
MON	Grain	Extraction with ACN/H_2O—84:16	RP-18	Isocratic: aqueous 100 mM NH_4OOCH/MeOH/triethyl amine—89.95:9.95:0.1	ESI − triple quadrupole	Product ion scan, SRM	[275]
MON	Grain, grain-based food	Extraction with ACN/H_2O—84:16, SPE with RP-8 column	RP-18	Isocratic: aqueous 100 mM NH_4OOCH/MeOH/triethyl amine—89.95:9.95:0.1	ESI − triple quadrupole	Product ion scan SRM	[259,265]
Mycophenolic acid							
MPA	Plasma	Protein precipitation	RP-18	Isocratic: H_2O/ACN each with 0.1% AcOH—57:43	ESI − single quadrupole	Full scan, SIM	[283]
MPA, MPA glucuronide	Aqueous standard solution	–	RP-18	Isocratic: H_2O/ACN each with 0.5% HCOOH—57:43 + post-column addition of NH_3	ESI − single quadrupole, triple quadrupole	Full scan, product ion scan, SIM, SRM	[282]
MPA glucosides, MPA carboxyl-linked glucuronides	Plasma	Protein precipitation, preparative HPLC, SPE with RP-18 columns (desalting)	Direct infusion	Isocratic: H_2O/MeOH—50:50	ESI (+)/-, triple quadrupole	Full scan, product ion scan	[276]
MPA	Plasma	Ultrafiltration, SPE with RP-18 columns	RP-18	Isocratic: H_2O with 2 mM NH_4OOCH/MeOH—45:55	APCI − triple quadrupole	Product ion scan, SRM	[277]
MPA, mycophenolate mofetil	Human skin	Extraction with MeOH, ultrafiltration	RP-18	Isocratic: H_2O/MeOH—20:80 each with 0.02% AcOH	ESI + single quadrupole	SIM	[278]
MPA	Food, vegetable, meat, coffee, feed	Extraction with ACN/H_2O—9:1 + 0.1% HCOOH, defatting with hexane	RP-18	Gradient: H_2O/MeOH each with 50 mM NH_4OAc	APCI + ion trap	Full scan, SIM, SRM	[253]
MPA	Fungal cultures in food waste	Extraction with ACN/H_2O—9:1 + 0.1% HCOOH, defatting with hexane	RP-18	Gradient: H_2O/MeOH each with 5 mM NH_4OAc	APCI + ion trap	SRM	[255]
MPA, MPA glucuronides	Human plasma	Ultrafiltration, SPE with RP-18 column	Pentafluorophenylpropyl	Isocratic: aqueous 40 mM NH_4OOCH/MeOH—25:75	ESI + triple quadrupole	SRM	[279]
MPA, MPA glucuronide, MPA acyl glucuronide	Plasma	Addition of HOCl and Na-tungstate, Ultrafiltration, on-line SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Isocratic: 1% aqueous AcOH/MeOH/ACN—16:63:21	ESI + triple quadrupole	SRM	[281]
MPA, MPA glucuronide, MPA acyl glucuronide	Plasma	Ultrafiltration, SPE with RP-18 columns	RP-8	Gradient: aqueous 0.05% HCOOH/ACN	ESI − triple quadrupole	SRM	[284]
MPA	Cheese	Extraction with 0.1% HCOOH in ACN/hexane—55:45	RP-18	Gradient: 0.1% aqueous AcOH/ACN	ESI + triple quadrupole	SRM	[117]
Patulin							
^{13}C-labelled Patulin	Standard solution in MeOH/H_2O—98:2 or ethyl acetate	–	RP-18	Isocratic: H_2O/ACN—90:10	ESI−, APCI−, ion trap	Full scan, product ion scan	[291]
Patulin	Apple juice, fruit products, wheat bread	Extraction with ethyl acetate, washing with aqueous 1.5% Na_2CO_3	RP-18	Gradient: H_2O/ACN	ESI (+)/−, APCI −, ion trap	Full scan, SIM	[287]
Patulin	Apple juice	Extraction with ethyl acetate, washing with aqueous 1.4% Na_2CO_3	RP-18	Isocratic: H_2O/ACN—90:10	APCI (+)/−, ion trap	Full scan, product ion scan, SRM	[288]
Patulin	Apple juice	On-line SPE with RP-18 columns by column switching technique	RP-18	Isocratic: aqueous 10 mM NH_4OAc/MeOH—98:2	APPI−, APCI−, single quadrupole	Full scan, SIM	[289]
Patulin	Apple juice	SPE with a *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Gradient: H_2O/ACN	ESI−, single quadrupole	SIM	[290]

BEA: beauvericin, EA: enniatin A, EA_1: enniatin A_1, EB: enniatin B, EB_1: enniatin B_1, MON: moniliformin, MPA: mycophenolic acid.

Table 12
Validation data of LC–MS methods of other mycotoxins

Analytes	Matrix	Recovery (%)	LOD/LOQ (μg/kg) or (μg/L)	Linear range (μg/kg) or (μg/L), calibration procedure	Accuracy/precision (%)	Ref.
Enniatins						
BEA	Fungal cultures, maize	94	0.5/–	–	–/7.0	[256]
BEA, EA, EA_1, EB, EB_1	Grain based food	31–82 (BEA), 60–102 (EA), 66–98 (EA_1), 60–124 (EB), 90–180 (EB_1)	–/–	–	–/–	[265]
BEA, EA, EA_1, EB, EB_1	Grains	86 (BEA), 97 (EA), 87 (EA_1), 72 (EB), 88 (EB_1)	3/10 (BEA, EA, EB, EB_1), 4/13 (EA_1)	10–512 (BEA), 10–30 (EA), 13–160 (EA_1), 10–152 (EB), 14–432 (EB_1), external calibration in matrix	–/20 (BEA), 16 (EA), 14 (EA_1), 22 (EB), 15 (EB_1)	[261,262]
BEA, EA, EA_1, EB, EB_1	Grain	76–82 (BEA), 55–66 (EA), 71–80 (EA_1), 57–103 (EB), 68–116 (EB_1)	0.1/0.2 (BEA), 0.1/0.2 (EA), 0.3/0.7 (EA_1), 0.4/0.9 (EB), 0.7/1.5 (EB_1)	10–300 (BEA), 0.6–18 (EA), 4–120 (EA_1), 3.8–114 (EB), 10.8–324 (EB_1), external calibration	–/10 (BEA), 15 (EA), 12 (EA_1), 10 (EB), 14 (EB_1)	[264]
Moniliformin						
MON	Maize, fungal culture	–	10/30	30–700, external calibration	–/2.0	[268]
MON	Grain	105	10/20	20–1000, external calibration	–/3.8	[259,275]
MON	Grain-based food	45–98	10/20	20–1000, external calibration	–/–	[265]
Mycophenolic acid						
MPA	Plasma	–	20 pg (on-column)/9.2	–, internal calibration in matrix	–/1.2	[283]
MPA	Plasma	76	–/–	25–1000, internal calibration without matrix: indomethacin	–/<9.0	[277]
MPA, mycophenolate mofetil	Human skin	92	10/–	25–1000, internal calibration without matrix: *n*-hexadecyl-β-D-glucopyranoside	≤9.8/15.2	[278]
MPA	Food, vegetable, meat, coffee, feed	78–116	10/– (MPA)	10–1000 (MPA), internal calibration in matrix: $[D_3]$-T-2	–/2.5–12.5	[253]
MPA, MPA glucuronides	Huma plasma	82	–/1.0	1–200, internal calibration without matrix: indomethacin	<6.0/<7.3	[279]
MPA, MPA glucuronide, MPA acyl glucuronide	Plasma	98–109 (MPA)	–/0.5	0.5–1000, internal calibration in matrix: carboxybutoxy ether MPA	–/1.8–8.1	[281]
MPA, MPA glucuronide, MPA acyl glucuronide	Plasma	99–106 (MPA)	–/1.0	1.0–1000, internal calibration in matrix: indomethacin	–/2.3–10.6	[284]
MPA	Cheese	96–135	0.3/0.6 (MPA)	5–1000, external calibration in matrix	–/3.1–10.9	[117]
Patulin						
Patulin	Apple juice, fruit products, wheat bread	94	20 (apple juice)/63	–, internal calibration in matrix: $[^{13}C_2]$-Patulin	–/28	[287]
Patulin	Apple juice	96	4/10	10–400, external calibration	–/< 7.1	[288]
Patulin	Apple juice	94–103	0.13/– (APPI), 0.20/– (APCI)	0.2–100, external calibration	6.0/2.1 (APPI), 6.5 (APCI)	[289]
Patulin	Apple juice	97–100	2.5/5	5–500, internal calibration: $[^{13}C_2]$-Patulin	–/<10.8	[290]

BEA: beauvericin, EA: enniatin A, EA_1: enniatin A_1, EB: enniatin B, EB_1: enniatin B_1, MON: moniliformin, MPA: mycophenolic acid.

et al. reported severe matrix effects and poor recovery rates varying from grain to grain [265]. Contrary to that, in another paper with an almost identical clean up protocol the same authors could not observe any matrix effects at all, since they found standard calibration curves and matrix matched curves nearly identical though recovery rates were still not satisfactory [264]. Uhlig and Ivanova also observed striking differences of standard calibration curves and matrix matched calibration curves in grains. Besides, they reported matrix effects varying from LC/MS to LC/MS run which indicates poor method robustness [261]. To overcome this problem they recommend a recovery experiment as part of the analytical routine. Another problem might be the non-linear calibration plots as reported by Sewram et al. [256] at concentrations above 0.25 μg/mL in the injection solution. Though this might be attributed to typical saturation phenomena in the applied ion trap, it seems to be in general advisable to eliminate MS related problems and to improve method performance by the use of more elaborated analytical protocols, as isotope labelled internal standards, standard addition calibration, improved sample clean up or improved chromatographic separation prior to MS detection [126].

7.2. Moniliformin

The mycotoxin moniliformin (MON, Fig. 9) is produced by *Fusarium* fungi growing on different grains [2] where it was repeatedly detected on the ppb to ppm level [259,265,268]. Its acute and long-term toxicity for humans has not yet been fully investigated, however, it has been shown that MON causes pathological changes in animals, including myocardial degeneration and necrosis. In this respect, the oral toxicity of MON is considered to be at the same level as the most toxic trichothecenes [269]. Furthermore, MON is suspected to be involved in Keshan disease, a myocardial impairment of humans reported in China and South Africa [270].

Apart of few TLC and GC/MS methods, MON is predominantly analysed by LC–UV [271,272]. Due to its high polarity and low molecular mass, retention on RP column is insufficient and, consequently, either ion pair RP-LC or ion exchange LC has to be used. Filek and Lindner introduced precolumn derivatisation of MON with 1,2-diamino-4,5-dichlorobenzene followed by conventional RP-LC separation, on-line post-column basification of the LC effluent and FL detection. With this method a detection limit of 20 μg/kg could be achieved in maize [273]. This method was also combined with MS/MS detection with the advantage that derivatisation increases the molecular mass to such an extent that the protonated molecule is moved out of the noisy low mass range of the mass spectrum. Unfortunately, this study was focused on structural elucidation of the derivatisation product and did not provide validation data or further quantitative analytical results [274].

Underivatised MON can be efficiently ionised with ESI [259,265] or APCI [268] (Table 11). Due to its acidic properties and the low chemical background noise, the negative ion mode provides an abundant deprotonated molecule $[M-H]^-$ and is always preferred. The product ion spectrum of the deprotonated molecule $[M-H]^-$ is dominated by losses of carbon monoxide [275], and SRM detection seems not to be more sensitive than SIM. Both monitoring techniques afford LODs of 10 μg/kg and LOQs of 20–30 μg/kg which are well below the detection limits of UV and FL detectors [268,275] (Table 12). Sewram et al. compared quantitative LC/MS and LC–UV data and found a good correlation between both data sets [268].

To achieve sufficient RP chromatographic retention/separation prior to MS detection triethylamine is added as ion pairing reagent to the mobile phase which in addition supports the formation of the deprotonated molecule $[M-H]^-$ in the negative ion mode [259,265,268,275]. Due to the high MS selectivity, sample clean up of grain extracts is either omitted [275] or reduced to one SPE step with RP absorbent material [259,265,268]. Consequently, matrix effects have repeatedly been reported especially when sample clean up was completely omitted [265,275]. In this context Jestoi et al. reported differences between standard and matrix matched calibration curves [275] and also found varying recovery values depending on the matrix under investigation [265] (Table 12).

7.3. Mycophenolic acid

The mycotoxin mycophenolic acid (MPA, Fig. 9) is produced by several *Penicillium* species [2]. It has also been identified as the active metabolite of the registered prodrug mycophenolate mofetil, which is used in renal patients for the prophylaxis of acute rejections [276]. When taken orally mycophenolate mofetil is rapidly hydrolysed to MPA and therefore, most of the published LC/MS [276–279] and LC/UV methods (e.g. [280]) are focused on pharmaceutical analyses of MPA in human plasma and skin while only three LC/MS approaches directly cover aspects of mycotoxin analysis in food and feed [117,253,255].

Typically, MPA plasma samples are either purified with ultrafiltration [277–279] and RP SPE or protein precipitation [276] followed by RP SPE. To measure the total MPA content in plasma samples protein bound MPA should be released with perchloric acid and sodium tungstate prior to further sample clean up [281]. Solid samples, as cheese and other food stuff are extracted with acidified acetonitrile/water mixtures that are afterwards defatted with hexane [117,253,255]. Alternatively, human skin was extracted with methanol followed by ultrafiltration but without any further sample clean up [278].

MPA, its glucuronidated and/or glycosylated metabolites and its pharmaceutical precursor mycophenolate mofetil are readily separated on RP LC columns within 5 [281] to 20 min [276] using methanol/water and acetonitrile/water mixtures with formic acid, acetic acid or their respective ammonium salts to improve the retention characteristics of acidic MPA. Besides, it was demonstrated that a pentafluorophenylpropyl stationary phase provides similar separation efficiency as RP materials at a shorter time scale. This material allows increased organic strength of the mobile phase which is reported by Atcheson et al. to enhance significantly MPA ionisation efficiency in the ESI interface [279]. Furthermore, Plätzer et al. recommend the use of

isocratic LC-elution to achieve a higher degree of MS robustness [278].

MPA exhibits excellent ESI [117,276,278,279,282] and APCI ionisation properties [253,255,277] in the positive [117,253,255,278,279,281] and negative ion mode [276,277,282–284] (Table 11) which is reflected by abundant protonated molecules $[M+H]^+$, ammonium adduct ions, sodium adduct ions and deprotonated molecules $[M-H]^-$ in the full scan spectra along with a minor degree of in-source fragmentation in the negative ion mode (decarboxylation) [283]. On the other hand, Atcheson et al. reported lower MS sensitivities for the MPA glucuronides compared to LC–UV. The authors attributed this observation to the elution of these compounds in the matrix loaded aqueous solvent front, which seems to cause severe ion suppression [279]. Besides, MPA glucuronides offer a considerable degree of in-source degradation into MPA presumably further decreasing MS sensitivity [281,284,285]. As a further consequence, chromatographic separation of these compounds from MPA is absolutely necessary to avoid any kind of MS interferences leading to overestimation of free MPA plasma concentrations or false positive results [281,284,285].

Positive and negative product ion spectra of MPA reveal losses of carbon dioxide, methanol and bond cleavages in the alkyl side chain [276,277,282] while glucuronides additionally cleave off the glucuronide moiety prior to MPA typical fragmentation [276]. SRM offers enhanced detection sensitivity compared to SIM [253] with detection limits for MPA in the lowest ppb range (Table 12). This is superior to LC–UV method performance though the results of both techniques showed excellent agreement when MPA concentration levels were high enough for UV detection [281,283]. None of the published LC/MS methods meet the EU criteria on unambiguous identification of target compounds since only the protonated molecule $[M+H]^+$, deprotonated molecule $[M-H]^-$ or one adduct ion ($[M+NH_4]^+$, $[M+Na]^+$) or one precursor/product ion pair was selected for SIM and SRM experiments, respectively.

Several authors reported on ionisation suppression effects in different matrices [117,253]. Streit et al. [281] and Patel et al. [284] investigated matrix effects in detail by continuous post-column infusion of MPA into a LC/MS run of a blank plasma sample. Areas of ion suppression were indicated by dips in the baseline and both groups concluded that MPA lies outside of any zone of ion suppression. To compensate for any kind of matrix effects, matrix matched calibration curves were recommended [253]. Several authors additionally applied internal standards though indomethacin [277,279,284], hexadecyl-β-D-glucopyranoside [278] and $[D_3]$-T-2 toxin [253] offer only insufficient chemical and physical similarity to MPA. Consequently, accuracy and precision of most MPA methods are poor and Plätzer et al. recommend stable isotope labelled internal standards to improve overall method performance [278] (Table 12). Alternatively, the use of the structurally similar MPA carboxybutoxy ether as internal standard may improve the overall quality of MPA method validation data as demonstrated by Streit et al. [281]. On the other hand, Patel et al. [284] reported MPA carboxybutoxy ether reference materials to be contaminated with MPA and replaced it for this reason by indomethacin.

7.4. Patulin

Patulin (Fig. 9) is produced by different *Penicillium* fungi especially on apples but also on other fruits and corn [2]. Due to its acute toxic, teratogenic and possibly carcinogenic potency the WHO recommends to limit its content in foods to 50 μg/kg. Consequently, many countries regulate patulin in beverages between 20 and 50 μg/L [12].

RP HPLC, coupled to UV detection has been found most suitable in patulin trace analysis since it exhibits strong UV absorption [286]. Following derivatisation, GC/MS is frequently used for structural confirmation [29]. LC/MS has been predominantly applied for the analysis of apple juices [287–290] and to a lower degree of other fruit and corn products [287]. Typically, sample preparation of liquid samples consists either of liquid/liquid extraction with ethyl acetate or SPE with RP absorbent materials. In this context, Takino et al. [289] achieved excellent through put rates by automated on-line coupling of SPE and LC/MS detection and Ito et al. [290] compared different polymeric SPE absorbent materials for reliable patulin trace analysis by LC/MS. Despite its low molecular weight, patulin is well retained on RP columns and sufficient separation from matrix compounds can easily be achieved within 10 min. APCI temperatures were found to have a tremendous influence on LC/MS peak shapes being strikingly better at higher temperatures [288]. Due to its acidic nature patulin is more efficiently ionised in the negative than in the positive ion mode of ESI and APCI [287,289] (Table 11). Post-column addition of ammonia does not support the deprotonation process and surprisingly abundant radical molecular anions were observed by Takino et al. reflecting a kind of electron capture mechanism [289]. MS sensitivity is enhanced in the negative ion mode since it offers less chemical noise below 200 mass units resulting in enhanced signal-to-noise ratios for the molecular ion [288]. Interestingly, Takino et al. compared APCI with APPI. Their results indicate that APPI in the negative ion mode provides even lower chemical noise and less signal suppression than APCI resulting in an increased overall MS sensitivity [289]. The product ion mass spectra of patulin in the positive and negative ion mode are poor in compound specific fragmentation reflecting only loss of water, carbon monoxide, carbon dioxide and formaldehyde [288,291]. Consequently, tandem MS monitoring did not show a striking increase of sensitivity compared to SIM and only one group applied SRM for trace quantification of patulin [288]. Though detection limits in the low ppb to ppt range could be achieved (Table 12) none of the published methods could meet the EU rules concerning unambiguous compound identification [40,41]. Furthermore, Rychlik and Schieberle compared LC/MS with GC/MS and demonstrated that LC/MS offers less sensitivity, less robustness and lower recovery rates, but on the other hand reduces considerably sample preparation efforts. The authors recommend to use LC/MS rather for structural confirmation than for quantification of patulin [287]. In contrast, other authors used ^{13}C-labelled patulin as internal standard and achieved accurate and reproducible quantitative data without matrix matched calibration curves [287,290]. Besides, Sewram et al. found an excellent correlation of LC–UV

Table 13
Overview on multitoxin LC–MS methodology

Analytes	Matrix	Sample preparation	Liquid chromatography		Mass spectrometry		Ref.
			Column	Mobile phase/additives	Ionisation/ion selection	Scan mode	
OTA, pencillic acid, roquefortine C and 17 further *Penicillium* mycotoxins	Fungal culture	Extraction with $CHCl_3$/MeOH/ethyl acetate/HCOOH—33:16:50:1	Flow injection	Isocratic: H_2O/MeOH—10:90	ESI + single quadrupole	Full scan	[292]
BEA, FUS	Fungal cultures, maize	Extraction with MeOH	RP-18	Gradient: H_2O/MeOH each with 0.1% HCOOH	ESI + ion trap	Full scan, product ion scan, SRM	[256]
BEA, FUS and non-covalent complexes with oligonucleotides	Aqueous solution of oligonucleotides		Loop- injection	Isocratic: H_2O/MeOH each with 0.1% NH_3—50:50	ESI − single quadrupole	Full scan	[231]
BEA, EA_1, EB, EB_1, FUS	Aqueous solution	Liquid/liquid extraction with $CHCl_3$	RP-18	Gradient: H_2O/ACN	ESI + single quadrupole	Full scan	[266]
MPA, verruculogen, griseofulvin, penitrem A roquefortine C, chaetoglobsin B	Food, vegetable, meat, coffee, feed	Extraction with ACN/H_2O—90:10 + 0.1% HCOOH	RP-18	Gradient: H_2O/MeOH	APCI + ion trap	Full scan, SIM, SRM	[253]
DON, ZON	Pure reference material	Dissolution in MeOH	RP-18	Gradient: H_2O/ACN	APCI–/(+), ion trap	Full scan, SIM	[67]
AFB_1, AFB_2, FB_1, FB_2, FB_3, FB_4, FC_4	Maize	Extraction with MeOH/H_2O—3:1 (fumonisins) and 4:1 (aflatoxins)	RP-18	Gradient: H_2O/MeOH each with 1% AcOH	APCI + (aflatoxins), ESI + (fumonisins), ion trap	SIM	[214,215]
474 mycotoxins, aflatoxins, fumonisins, trichothecenes, ochratoxins, ZON and metabolites	Fungal cultures	Extraction with 1% HCOOH in ethyl acetate and isopropanol	RP-18	Gradient: H_2O/ACN	ESI + TOF	Full scan	[293]
Penitrem A-F, thomitrem A and E, roquefortine C	Fungal cultures	Extraction with ACN/H_2O—9:1, defatting with hexane	RP-18	Gradient: H_2O/ACN each with 50 mM NH_4OAc	APCI + ion trap	Full scan	[254]
MPA, verruculogen, griseofulvin, chaetoglobsin B, penitrem A-F, thomitrem A and E	Fungal cultures in food wastes	Extraction with ACN/H_2O—9:1	RP-18	Gradient: H_2O/MeOH each with 5 mM NH_4OAc; penitrems and thomitrems: gradient: H_2O/ACN each with 5 mM NH_4OAc	APCI + ion trap	SRM	[255]
DON, FB_1, ZON	Maize	Accelerated solvent extraction with ACN/H_2O—75:25, SPE with a strong anion exchange column (FB_1), extraction with MycoSep 226 columns (DON, ZON)	RP-18	Gradient: H_2O with 1% ACN, 5 mM NH_4OAc and HCOOH, pH 4/ACN	APCI ± polarity switching, ion trap	Product ion scan, SRM	[71]
BEA, EA, EA_1, EB, EB_1, MON	Grain, grain-based food	Extraction with ACN/H_2O—84:16, SPE with RP-8 column	RP-18	BEA, EAs and EBs: isocratic: 10 mM aqueous NH_4OOCH/ACN/MeOH—10:45:45, MON: isocratic: aqueous 100 mM NH_4OOCH/MeOH/triethyl amine—89.95:9.95:0.1	ESI + triple quadrupole	SRM	[259,265]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, ZON (AFB_1, OTA)	Cereal based food and feed	Extraction with ACN/H_2O—85:15, clean up with MycoSep 226	RP-18	Gradient: H_2O/MeOH	ESI ± (APCI±) triple quadrupole	SRM	[68,69]
AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, MPA, OTA, penicillic acid, roquefortine C	Cheese	Extraction with 0.1% HCOOH in ACN/hexane—55:45	RP-18	Gradient: H_2O/ACN each with 0.1% AcOH	ESI + triple quadrupole	SRM	[117]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, DAS, ZON	Maize	Extraction with ACN/H_2O—84:16, clean up with MycoSep 227 or 226 columns	RP-18	Gradient: H_2O/MeOH each with 5 mM NH_4OAc	APCI ± polarity switching, triple quadrupole	Full scan, product ion scan, SRM	[74]
AFM_1, DON, DOM-1, 3-AcDON, 15-AcDON, HT-2, T-2, T-2 triol, DAS, MAS, FB_1, FB_2, OTA, ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Milk	Enzymatic deglucuronidation, extraction with ACN/hexane—61:39, SPE with *N*-vinylpyrrolidone/divinylbenzene co-polymer columns	RP-18	Gradient: H_2O/MeOH each with 0.02% AcOH (T-2, HT-2, T-2 triol, DAS, MAS, FB_1, FB_2, AFM_1), gradient: H_2O/MeOH (DON, DOM-1, 3-AcDON, 15-AcDON, OTA, ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL)	ESI + (HT-2, T-2, T-2 triol, DAS, MAS, FB_1, FB_2, AFM_1), ESI − (DON, DOM-1, 3-AcDON, 15-AcDON, OTA, ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL), triple quadrupole	Product ion scan, SRM	[70]
DON, NIV, 3-AcDON, 15-AcDON, F-X, ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Maize	Extraction with H_2O/ACN—75:25, SPE with carbograph-4 columns	RP-18	Trichothecenes: gradient H_2O/ACN, ZON/metabolites: isocratic: H_2O/MeOH/ACN-50:15:35	ESI − triple quadrupole	SRM	[73]

AFB_1: aflatoxin F_1, AFB_2: aflatoxin B_2, AFG_1: aflatoxin G_1, AFG_2: aflatoxin G_2, AFM_1: aflatoxin M_1, 3-AcDON: 3- acetyldesoxynivalenol, 15-AcDON: 15-acetyldesoxynivalenol, BEA: beauvericine, DAS: diacetoxyscirpenol, DOM-1: deepoxydesoxynivalenol, DON: desoxynivalenol, FB_1: fumonisin B_1, FB_2: fumonisin B_2, F-X: fusarenon X, FUS: fusaproliferin, HT-2: HT-2 toxin, MAS: monoacetoxyscirpenol, MON: monoliformin, MPA: mycophenolic acid, NIV: nivalenol, OTA: ochratoxin A, T-2: T-2 toxin, α-ZAL: α-zearalanol (zeranol), β-ZAL: β-zearalanol (taleranol), ZAN: zearalanone, α-ZOL: α-zearalenol, β-ZOL: β-zearalenol, ZON: zearalenone.

Table 14
Validation data of multitoxin LC–MS methods

Analytes	Matrix	Recovery (%)	LOD/LOQ (μg/kg) or (μg/L)	Linear range (μg/kg) or (μg/L), calibration procedure	Accuracy/precision (%)	Ref.
BEA, FUS	Fungal cultures, maize	94 (BEA), 71 (FUS)	0.5/– (BEA), 1.0/– (FUS)	–	–/7.0 (BEA), –/7.0 (FUS)	[256]
MPA, verruculogen, griseofulvin, penitrem A, roquefortine C, chaetoglobsin B	Food, vegetable, meat, coffee, feed	78–116	10 (MPA), 5 (griseofulvin), 20 (roquefortine C), 20 (chaetoglobsin), 20 (verruculogen), 5 (penitrem A)/10–20	10–1000 (MPA, griseofulvin, penitrem A), 20–1000 (roquefortine C, chaetoglobsin, verruculogen), internal calibration in matrix: $[D_3]$-T-2	–/2.5–12.5	[253]
DON, FB_1, ZON	Maize	70 (DON), 40 (ZON), 90 (FB_1)	10/50 (DON), 3/10 (ZON), 20/50 (FB_1)	50–2000 (DON) internal calibration in matrix: VER, 50–1000 (ZON), internal calibration in matrix: α-ZAL, 50–1000 (FB_1), internal calibration in matrix: $[D_6]$-FB_1	6.4–16.6/– (DON), 4.1–8.3/– (ZON), 7.5–14.7/– (FB_1)	[71]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, ZON	Cereal based food and feed	54–89 (trichothecenes), 92 (ZON)	0.2 (T-2)/10 (trichothecenes)	10–500 (trichothecenes), 10–200 (ZON), internal calibration in matrix: VER and ZAN	–/–	[68,69]
AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, MPA, OTA, penicillic acid, roquefortine C	Cheese	129–143 (aflatoxins), 135 (MPA), 102 (roquefortine C), 109 (penicillic acid), 105 (OTA)	0.3–0.8/0.6–5 (aflatoxins), 0.3/0.6 (MPA), 0.4/0.8 (roquefortine C), 2.0/4.0 (penicillic acid), 0.3/0.6 (OTA)	5–1000 (AFB_1, AFB_2, AFG_1, AFG_2, MPA, penicillic acid, roquefortine C, OTA), 0.6–120 (AFM_1), external calibration in matrix	–/2.3–12.1 (aflatoxins), –/3.1–10.9 (MPA, penicillic acid, roquefortine C) –/2.5 (OTA)	[117]
DON, NIV, 3-AcDON, 15-AcDON, HT-2, T-2, F-X, DAS, ZON	Maize	50–94 (trichothecenes), 30 (ZON)	0.3–3.8/0.8–18.3 (trichothecenes), 0.9/3.2 (ZON)	30–1000 (trichothecenes), external calibration in matrix 10–1000 (ZON), internal calibration in matrix: ZAN	4.0/7.2 (DON), 5.0/9.6 (ZON)	[74]
AFM_1, DON, DOM-1, 3-AcDON, 15-Ac-DON, FB_1, FB_2, HT-2, T-2, T-2 triol, DAS, MAS, OTA, ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Milk	84–108 (trichothecenes), 103 (OTA), 82–106 (ZON and metabolites), 80–98 (AFM_1), 76–90 (FB_1, FB_2)	CCα/CCβ 0.03–0.1/0.05–0.15 (trichothecenes), 0.01/0.02 (OTA), 0.02–0.06/0.03–0.08 (ZON and metabolites), 0.01/0.02 (AFM_1), 0.02–0.04/0.04–0.05 (FB_1, FB_2)	–, external calibration in matrix	1–16/3.2–15 (trichothecene) 20/3.8–15 (OTA), 2–20/4.4–15 (ZON and metabolites), 10/5.5–12 (AFM_1), 10–20/4.0–12 (FB_1, FB_2)	[70]
DON, NIV, 3-AcDON, 15-AcDON, F-X, ZON, α-ZOL, β-ZOL, α-ZAL, β-ZAL	Maize	79–97 (trichothecenes), 89–106 (ZON/metabolites)	2–12/5, 20 (F-X) (trichothecenes), 3–6/5–10 (ZON/metabolites)	5–5000, 20–5000 (F-X), (trichothecenes), internal calibration in matrix: nafcillin, 5–5000 (ZON/metabolites), internal calibration: alpha-estradiol	–/3–10 (trichothecenes), –/5–10 (ZON/metabolites)	[73]

AFB_1: aflatoxin F_1, AFB_2: aflatoxin B_2, AFG_1: aflatoxin G_1, AFG_2: aflatoxin G_2, AFM_1: aflatoxin M_1, 3-AcDON: 3-acetyldesoxynivalenol, 15-AcDON: 15-acetyldesoxynivalenol, BEA: beauvericine, DAS: diacetoxyscirpenol, DOM-1: deepoxydesoxynivalenol, DON: desoxynivalenol, F-X: fusarenon X, FUS: fusaproliferin, HT-2: HT-2 toxin, MPA: mycophenolic acid, NIV: nivalenol, OTA: ochratoxin A, T-2: T-2 toxin, VER: verrucarol, α-ZAL: α-zearalanol (zeranol), β-ZAL: β-zearalanol (taleranol), ZAN: zearalanone, α-ZOL: α-zearalenol, β-ZOL: β-zearalenol, ZON: zearalenone.

and LC/MS data even without an internal calibration procedure [288].

8. Multitoxin LC/MS analysis

It is well known that *Fusarium*, *Penicillium* and *Aspergillus* fungi produce numerous mycotoxins frequently belonging to different toxin groups [1,2]. To cope with this situation and to enable reliable and fast risk estimation of mycotoxin intake and poisoning, the development of multimycotoxin methods with one common sample preparation and final analysis procedure is highly desirable. In this respect, LC/MS may play an important role, as LC coupled to API MS is more or less independent from compound mass and polarity and can be considered as ideal separation and detection system for almost all types of mycotoxins.

The number of such multitoxin LC/MS methods is still limited due to the complexity of food, feed and biological matrices as well as the wide range of physical and chemical properties of mycotoxins challenging both, sample preparation and LC/MS detection (Tables 13 and 14). Recent assays are focused on typical *Fusarium* toxins as trichothecenes, ZON and its metabolites [68–71,74], sometimes together with fumonisins [70,71], OTA [68–70] and aflatoxins [68–70]. Other mycotoxins analysed together were BEA, FUS, EAs [231,256,259,265,266] and MON [259,265] and to less extent *Penicillium* toxins including MPA, aflatoxins, fumonisins, OTA, roquefortine C, penitrems, thomitrems, verruculogen and chaetoglobsin B [117,214,215,253–255,292]. In this respect Nielsen and Smedsgaard [293] monitored simultaneously up to 474 mycotoxins in fungal cultures in order to compile a data base for pharmaceutical high throughput screening or to identify individual *Penicillium* species by their mycotoxin patterns [292].

The majority of multitoxin LC/MS has been done in fungal cultures and grain and to less extent in cheese, milk and other food stuff. Typically, sample clean up of relatively simple matrices, as fungal cultures and aqueous solutions but also cheese consists of either direct injection of liquid samples into the LC–MS system or one sample extraction step prior to LC/MS analysis (Table 13) [117,231,253,256,266,292,293]. For more complex food matrices, as e.g. grain, some of the published multitoxin LC/MS methods rely basically on multiple but parallel or sequential sample preparation strategies of one sample followed by separate analysis of each isolated class of mycotoxins, partly even without LC/MS [62–73,77,80,214,215,259,265]. Other approaches include a joint sample clean up of food, maize and milk extracts for all target mycotoxins, however, perform final analysis in two separate LC/MS runs. This procedure is required either due to insufficient chromatographic separation of mutually interfering analytes [253,255] or by mycotoxin specific MS sensitivity differences in the positive and negative ion mode [70,71,74]. Positive/negative polarity switching has been shown to be a proper tool to solve this latter problem within one LC/MS run whenever modern MS instrumentation with sufficiently rapid polarity switching capability is available or, alternatively, analytes are sufficiently separated from each other by LC that a limited number of positive and negative mode windows can be set up within one LC/MS run (Fig. 2) [71,74].

Royer et al. isolated and purified FB_1, DON and ZON from maize in two consecutive clean up steps with strong anion exchange SPE and a MycoSep column. Finally, the purified extracts were pooled and all three analytes were quantified in one LC/MS run [71]. As confirmed also by others, the use of MycoSep 226 columns seems to be in general an attractive approach to analyse simultaneously ZON and trichothecenes in grain [68,69,74]. Considerable variation of trichothecene recovery values along with low recovery values of ZON below 40% reveal the inability of these methods to cope in each respect with the whole range of different analytes though ZAN as internal standard was shown to compensate for the loss of ZON during sample clean up [74].

Aside the problematic sample preparation of complex matrices, LC/MS analyses of heterogeneous mixtures of mycotoxins suffer in principal from dramatic differences of analyte ionisation efficiencies [71,74,256]. These are influenced by various parameters, as physical and chemical properties of the analytes, the applied ionisation interface (APCI or ESI), the preferred ionisation polarity, the LC elution solvent and the presence of disturbing matrix components. For this reason, MS sensitivity can hardly be kept stable over a wide LC elution zone and polarity range, especially when one interface with one polarity is used for the whole range of analytes. As a consequence, considerable validation efforts with the implementation of a sufficient number of suitable internal standards are necessary to compensate for ionisation enhancing or suppressing matrix effects which were shown to be strikingly more likely for components eluting early in the chromatograms in the presence of a polar matrix [68,69,71]. Multiple LC/MS runs per sample with adjusted and analyte specific MS conditions [70,73,253,255] or polarity switching within one run are feasible alternatives [74] frequently in combination with improved sample clean up and/or chromatographic separation that, however, decreases considerably sample throughput. Alternatively, combined ESI/APCI sources that are already commercially available from several manufacturers might be an option for the future.

9. Conclusion

Due to their toxicity and frequent occurrence and driven by regulatory authorities worldwide, there is a distinct need for highly selective and accurate methods to identify and quantify mycotoxins in a wide variety of agricultural, biological, food and feed matrices. Numerous different analytical techniques are necessary to cope with this large range of analyte polarities and diversity of matrices along with considerable efforts to enrich analytes and clean up samples to enable sufficiently sensitive and selective detection of mycotoxins. With the advent of API interfaces in the late 1980s, the coupling of LC to MS became accessible on a routine basis. The on-line combination of both techniques is principally rather independent from analyte polarity and molecular mass and offers a universal approach to detect and quantify mycotoxins in complex matrices. From the mid 1990s onwards LC/MS technology spread rapidly into the field

of mycotoxin analysis. Its excellent detection selectivity enabled a dramatic reduction of sample preparation and improved sample throughputs in a manner which met especially the increasing demand of regulatory authorities to monitor mycotoxin contamination in the ppb to ppt range.

Presently, many quantitative LC/MS methods are available for all important mycotoxin groups. MS/MS experiments, as SRM, are frequently used to quantify mycotoxins with enhanced sensitivity and accuracy. In contrast to other methods, multimycotoxin analysis, such as for trichothecenes is easily feasible and also enables the quantitative and qualitative investigation of metabolic pathways or of hitherto unknown mycotoxin species in complex matrices either by SRM or product ion scan experiments.

Despite these tremendous achievements several drawbacks of the technique have also been identified. Ionisation efficiencies are not equal for all analytes and can be strikingly influenced by the instrument performance and especially by co-eluting matrix compounds. Due to the complexity and diversity of food samples these matrix effects vary from sample matrix to sample matrix and were repeatedly shown to reduce considerably quantification accuracy. Improved sample clean up and chromatography but also sufficient dilution of samples are feasible measures to avoid or diminish the amount of co-eluting matrix components. Besides, extensive validation efforts have to be undertaken, as e.g. calibration curves should always be set up in the matrix together with an internal standard. Internal standards should have close chemical and physical similarity to the analytes. In this respect, co-eluting stable isotope labelled compounds should be chosen though they are not commercially available for a lot of mycotoxins. Furthermore, more than one internal standard is recommended for multitoxin methods.

Especially the older literature has considerable deficiencies concerning validation features. Future analytical work with LC/MS should, therefore, not only focus on the detection of new types of mycotoxins and of masked mycotoxins (bioadducts, degradation products and in vivo metabolites) but also provide fully validated assays that are especially in agreement with recent EU regulations about residues analysis and method validation.

A second focus of LC/MS/MS mycotoxin analysis can be expected in the field of multi-analyte methodology, especially when the whole mycotoxin pattern of a mycotoxin producing fungus has to be considered. This field has been touched right now only by a handful papers though there seems to be a great necessity to monitor simultaneously several groups of mycotoxins to enable a more reliable and quicker assessment of mycotoxin contamination. With regard to the already published data, considerable efforts will be necessary to achieve elaborated LC/MS methods due to the complexity of sample matrices along with the diversity of analyte polarities and their different ionisation capabilities. It will be of great interest to see how far the technical and methodological frontiers of LC/MS technology can be moved forward in this challenging field taking into account that more recent MS technology (e.g. QTrap) already enables accurate quantification and reliable structural elucidation and confirmation at trace levels within one LC/MS run.

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