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Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize

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This paper describes the first validated method for the determination of 39 mycotoxins in wheat and maize using a single extraction step followed by liquid chromatography with electrospray ionization triple quadrupole mass spectrometry (LC/ESI-MS/MS) without the need for any clean-up. The 39 analytes included A- and B-trichothecenes (including deoxynivalenol-3-glucoside), zearalenone and related derivatives, fumonisins, enniatins, ergot alkaloids, ochratoxins, aflatoxins and moniliformin. The large number and the chemical diversity of the analytes required the application of the positive as well as the negative ion ESI mode in two consecutive chromatographic runs of 21 min each. The solvent mixture acetonitrile/water/acetic acid 79 + 20 + 1 (v/v/v) has been determined as the best compromise for the extraction of the analytes from wheat and maize. Raw extracts were diluted 1 + 1 and were injected without any clean-up. Ion-suppression effects due to co-eluting matrix components were negligible in the case of wheat, whereas significant signal suppression for 12 analytes was observed in maize, causing purely proportional systematic errors. Method performance characteristics were determined after spiking blank samples on multiple levels in triplicate. Coefficients of variation of the overall process of <5.1% and <3.0% were obtained for wheat and maize, respectively, from linear calibration data. Limits of detection ranged from 0.03 to 220 μg/kg. Apparent recoveries (including both the recoveries of the extraction step and matrix effects) were within the range of 100 ± 10% for approximately half of the analytes. In extreme cases the apparent recoveries dropped to about 20%, but this could be compensated for to a large extent by the application of matrix-matched standards to correct for matrix-induced signal suppression, as only a few analytes such as nivalenol and the fumonisins exhibited incomplete extraction. For deoxynivalenol and zearalenone, the trueness of the method was confirmed through the analysis of certified reference materials.

Mycotoxins are toxic secondary metabolites produced by various mold species growing on many agricultural commodities and processed food, either in the field or during storage.[1,2] They have been ranked as the most important chronic dietary risk factor, higher than synthetic contaminants, plant toxins, food additives or pesticide residues.[3] Another area of concern is the exposure to airborne mycotoxins produced by indoor molds.

Approximately 300 to 400 substances are recognized as mycotoxins, comprising a broad variety of chemical structures. The list of the most important target analytes includes trichothecenes (sesquiterpenes including an epoxide ring exhibiting a wide range of polarity), zearalenone and its derivatives (apolar resorcylic acid lactones), fumonisins (polar aliphatic amino alcohols exhibiting acidic side chains), enniatins (apolar cyclic hexadepsipeptides), ergot alkaloids (lysergic acid derivatives, linked to a peptide ring system in the case of ergopeptides), ochratoxins (L-β-phenylalanine-linked dihydroisocoumarin derivatives), aflatoxins (difuranocoumarin derivatives), moniliformin (a cyclobutene derivative with a pK_a of 1.7) and patulin (a bicyclic hydroxylactone).

Many analytical methods dealing with single classes of mycotoxins including a limited number of target analytes have been developed.[4,5] Lately, additive and synergistic effects have been observed concerning the health hazard posed by mycotoxins,[6–8] resulting in the search for multi-toxin methods for the simultaneous screening of different classes of mycotoxins. Recently, the natural occurrence of masked mycotoxins,[9] where the toxin is conjugated, has been reported.[10,11] This requires a more universal, selective and

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sensitive detection principle, which explains why liquid chromatography/mass spectrometry (LC/MS) and particularly LC coupled to tandem mass spectrometry (LC/MS/MS) have become very popular in recent years for mycotoxin analysis. However, the development of such a multi-toxin method is impeded by the chemical diversity of the analytes. This requires compromises to be made on the conditions during sample preparation. A major difficulty arises from the incompatibility between the acidic conditions suitable for the extraction and the chromatographic separation of the fumonisins and the conditions favorable for ionization of the B-trichothecenes,[12,13] that tend to form adducts with the acid anions. Even within a given class of compounds, significant losses of certain target analytes may occur during extraction or clean-up, as has often been observed for the most polar trichothecene, nivalenol.[14–17]

Considering the wide range of polarities of the analytes, it would be advantageous to keep sample preparation to a minimum and to inject the crude extract. Indeed, the seemingly highly selective MS/MS detection may lead wrongly to the perception that all matrix interferences are effectively eliminated and quantitative results may be obtained without any clean-up and with very little chromatographic separation.[18] Unfortunately, co-eluting undetected matrix components often reduce or enhance the ionization efficiency of the analyte, either due to changes in the surface tension of the droplet during electrospray ionization (ESI) or to competition between the compounds for the charge in the liquid phase.[19] This has a negative effect on the repeatability and accuracy of the method and, in extreme cases, the signal is completely suppressed.[20] As a consequence, only a few groups[17,21–23] have described the successful injection of crude extracts. The majority of the publications have described a clean-up of the sample, either by solid-phase extraction (SPE) using Mycosep® columns,[14–16,20,24–26] C18-materials,[12,13] strong anion exchangers[26] and graphitized carbon black,[12] or by extraction with hexane for de-fatting and protein precipitation.[13,27,28] However, matrix effects were still observed after clean-up of the samples in these studies, as has been shown by post-column addition of analytes to the chromatographic effluent.[25] In addition, the applicability of these SPE procedures to the simultaneous analysis of enniatins, ergot alkaloids and moniliformin has yet to be proven and is probably not feasible.

The best approach to overcome quantification problems related to the described matrix effects is the use of isotope labeled internal standards. As they are not present in real world samples and have identical chemical properties to the analytes, they can be spiked to the sample before extraction to correct both for losses during the sample preparation and for ion suppression/enhancement effects. Although there are a few reports on deuterated standards,[26,29–32] only one study deals with the use of fully ^{13}C-substituted deoxynivalenol, which has recently become commercially available.[33] Another option is the use of analogue chemicals (compounds which are structurally related to the target toxins) as internal standards. Whereas zearalanone (a dihydro derivative) seems to be an acceptable internal standard for zearalenone[16,20,34] due to similar behavior in reversed-phase LC, the results obtained with analogues that do not co-elute with the analytes (such as verrucarol or deepoxy-deoxynivalenol)[17,25] may still over- or underestimate the concentration of the target substance.[26] Matrix calibration with matrix-matched standards (i.e. blank sample extracts fortified with an appropriate amount of a multi-analyte standard) are preferred by many authors[12–14,20,22,23,27,28] to compensate separately for the matrix effects for every single analyte. Due to the severe matrix effects for aflatoxins, ergot alkaloids and some other substances, this approach has been chosen for the developed multi-toxin method, as described later.

The aim of this paper is to present the development and the validation of a LC/MS/MS-based method for the quantitative analysis of 39 mycotoxins and related metabolites as well as some mycotoxin conjugates in wheat and maize. Some of these are included in a multi-toxin method for the first time. No other workers have described the validation of a method for such a wide range of mycotoxins. Due to the chemical diversity of the target substances, crude sample extracts were injected without any purification. Matrix effects are discussed in detail as well as the optimization of the extraction solvent. Method performance characteristics such as extraction efficiency, limits of detection, precision and trueness are presented. Although some of the analyte/matrix combinations are of limited practical relevance (e.g. verrucarol is a semisynthetic compound produced by hydrolysis of macrocyclic trichothecenes and will hardly be encountered in naturally contaminated wheat or maize), they were studied systematically as our long-term goal is the establishment of a unified method for many types of analyte/matrix combinations in the field of mycotoxin analysis.

EXPERIMENTAL

Chemicals and materials

Methanol and acetonitrile (both LC gradient grade) were purchased from J.T. Baker (Deventer, The Netherlands) and ammonium acetate (MS grade) and glacial acetic acid (p.a.) were obtained from Sigma-Aldrich (Vienna, Austria). Water was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

Mycotoxin standards were purchased from different sources and were dissolved in acetonitrile (ACN) if not stated otherwise. Stock solutions of nivalenol (NIV), deoxynivalenol (DON), fusarenon X (FUSX), 3-acetyldeoxynivalenol (3ADON), deepoxy-deoxynivalenol (DOM), neosolaniol (NEO), diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), T-2 toxin (T-2), zearalenone (ZON), alpha-zearalenol (α-ZOL), beta-zearalenol (β-ZOL), ochratoxins A and B (OTA, OTB), ochratoxin alpha (OTα, in ACN/H_2O 1 + 1, v/v), fumonisins B_1 and B_2 (FB_1, FB_2, in ACN/H_2O 1 + 1), hydrolyzed fumonisin B_1 (HFB_1, in ACN/ H_2O 1 + 1), aflatoxins B_1, B_2, G_1 and G_2 (AFB_1, AFB_2, AFG_1, AFG_2) and patulin (PAT) were obtained from Biopure Referenzsubstanzen GmbH (Tulln, Austria). 15-Monoacetoxyscirpenol (MAS), verrucarol (VOL), verrucarin A (VER), beauvericin (BEA), moniliformin (MON, dissolved in MeOH), ergocornine (ERC, dissolved in MeOH/H_2O 1 + 1) and ergotamine-D-tartrate (ERA, dissolved in MeOH/H_2O 1 + 1) were received from Sigma-Aldrich. A stock solution of enniatin

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A, A_1, B and B_1 (ENN A, ENN A_1, ENN B, ENN B_1) was provided by Dr. Marika Jestoi (EELA Helsinki, Finland). Agroclavine (AGR, dissolved in MeOH) was received from Dr. Miroslav Flieger (Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague). Dihydroergosinmethane sulfonate (DHE, dissolved in MeOH) was purchased from Dr. Danka Pericic (Ruder Boscovik Institute, Zagreb, Croatia). Ergovaline (ERV, dissolved in MeOH) was purchased from Prof. Forrest Smith (Auburn University, AL, USA). Deoxynivalenol-3-glucoside (D3G) was isolated from wheat treated with DON,[11] zearalenone-4-glucoside (Z4G, dissolved in MeOH) was synthesized according to a modified protocol from Grabley *et al.*,[35] and zearalenone-4-sulfate (Z4S, dissolved in MeOH/H_2O 1 + 1) was extracted in our laboratory from rice inoculated with *Fusarium graminearum*. Four combined working standard solutions were prepared weekly by dilution of the stock solutions of the analytes in the related solvents, i.e. MeOH (for Z4G, AGR, MON, EV and DHE), MeOH/H_2O 1 + 1 (for Z4S, ERA and ERC), ACN/H_2O 1 + 1 (for OTα, FB_1, FB_2 and HFB_1) and ACN (for all other analytes), respectively. All solutions were stored at −20°C and were brought to room temperature before use.

Reference materials were purchased from IRMM (Geel, Belgium; BCR 378, BCR 379 and BCR 717), Trilogy Analytical Laboratory (Washington, MO, USA; D-105 and D-107) and Biopure Referenzsubstanzen GmbH (Tulln, Austria). Blank wheat and maize samples were collected from fields in Austria.

Instrumental conditions

Detection and quantification was performed with a QTrap 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with a TurboIonSpray ESI source and a 1100 Series HPLC system (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini® C_{18} column, 150 × 4.6 mm i.d., 5 μm particle size, equipped with a C_{18} 4 × 3 mm i.d. security guard cartridge (all from Phenomenex, Torrance, CA, USA). Both eluents contained 5 mM ammonium acetate and were composed of methanol/water/acetic acid 10 + 89 + 1 (v/v/v; eluent A) or 97 + 2 + 1 (eluent B), respectively. After an initial time of 2 min at 100% A, the proportion of B was increased linearly to 100% within 12 min, followed by a hold time of 3 min at 100% B and 4 min column re-equilibration at 100% A. The flow rate was 1 mL/min. The column effluent was transferred via a six-port valve (VICI Valco Instruments, Houston, TX, USA) either to the mass spectrometer (between 2 and 17 min; no flow splitting was used) or to the waste.

ESI-MS/MS was performed in multiple reaction monitoring (MRM) mode both in positive and negative polarity in two separate chromatographic runs per sample with the following settings: source temperature 550°C, curtain gas 10 psi (69 kPa of max. 99.5% nitrogen), ion source gas 1 (sheath gas) 50 psi (345 kPa of nitrogen), ion source gas 2 (drying gas) 50 psi (345 kPa of nitrogen), ion spray voltage −4000 V and +4000 V, respectively, collision gas (nitrogen) high, MRM dwell time 100 ms (with few exceptions, see 'Results and Discussion'), pause between mass ranges 5 ms. The optimization of the analyte-dependent MS/MS parameters was performed via direct infusion of standards (dissolved in 1 mL MeOH/H_2O 1 + 1 containing 5 mM NH_4Ac) into the mass spectrometer using a 11 Plus syringe pump (Harvard Apparatus, Holliston, MA, USA) at a flow rate of 10 μL/min – see 'Results and Discussion' for the related numerical values.

Procedure

Calibration solutions

For external calibration, a multi-analyte stock solution was freshly prepared by mixing the four combined working solutions (200 μL each) and 800 μL of mobile phase A. This solution was further diluted with mobile phase A to obtain appropriate concentrations (for the method validations experiments. For the validation experiments, the concentrations were matched on each level to the expected analyte concentration in the final diluted extract).

Spiking

Ground wheat or maize kernels (0.5 g) were spiked by consecutively adding the appropriate amounts of the four combined working solutions. The samples were subsequently stored for 3 days at 40°C to allow evaporation of the solvent and to establish equilibration between the analytes and the matrix.

Extraction

A volume of 2 mL of extraction solvent (CH_3CN/H_2O/HAc 79 + 20 + 1) was added to 0.5 g of ground wheat or maize kernels. The samples were extracted for 90 min using a GFL 3017 rotary shaker (GFL, Burgwedel, Germany) and subsequently centrifuged for 2 min at 3000 rpm (radius: 15 cm) on a GS-6 centrifuge (Beckman Coulter Inc., Fullerton, CA, USA). The extracts were transferred into glass vials using Pasteur pipettes and aliquots of 350 μL were diluted with the same amount of a mixture containing CH_3CN/H_2O/HAc 20 + 79 + 1. After appropriate mixing, 5 μL of the diluted extract were injected into the LC/MS/MS system without further pre-treatment. To perform the optimization of the extraction solvent, samples were spiked at one concentration level in duplicate or triplicate and extraction was performed by adding 2 mL of the investigated solvent mixture. Crude extracts were diluted 1 + 9 with eluent A prior to analysis in this experiment to reduce signal suppression/enhancement due to matrix effects.

Validation

For the final validation of the method, the whole procedure was carried out at different concentration levels (each in triplicate) for wheat (7 levels with a relative concentration of 1:2:7:8:14:20:80) and maize (10 levels with a relative concentration of 1:4:7:10:40:70:100:400:700:1000). These experiments included the spiking of blank extracts (also performed in triplicate) on one concentration level after extraction to differentiate between extraction efficiency and matrix-induced signal suppression/enhancement.

Data evaluation

Standard calibration curves (linear, 1/x weighted) for each analyte were constructed by plotting the signal intensity

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versus the analyte concentration using the Analyst® software version 1.4.1. In the same manner, the MS signal intensities obtained from spiking the samples before and after extraction were plotted against the actual spiking levels. The slopes of the resulting linear, 1/x weighted functions were compared with the related slopes of the calibration functions to calculate the apparent recovery (R_A), the signal suppression/enhancement (SSE) due to matrix effects, and the recovery of the extraction step (R_E) as follows (modified after Matuszewski *et al.*[18]):

$$R_A(\%) = 100 * \text{slope}_{\text{spiked sample}}/\text{slope}_{\text{liquid standard}} \quad (1)$$

$$SSE(\%) = 100 * \text{slope}_{\text{spiked extract}}/\text{slope}_{\text{liquid standard}} \quad (2)$$

$$R_E(\%) = 100 * R_A/SSE \quad (3)$$

IUPAC distinguishes between recovery and apparent recovery.[36] The apparent recovery (abbreviated here as R_A) denotes the ratio of an observed value obtained from a calibration graph divided by a reference known or theoretical value. This is also often referred to as overall or total recovery of a method. The term recovery itself is used to express the yield of a preconcentration or extraction stage (expressed as R_E in this paper) for an analyte divided by the amount of analyte in the original sample. In the present case, R_A is composed multiplicative by R_E and SSE. This way of expressing the 'recovery' according to IUPAC nomenclature was chosen by us to distinguish between incomplete extraction of the analytes on the one hand and effects/losses arising from ion suppression/enhancement on the other. The coefficients of variation (CVs) of the end determination were calculated from linear, 1/x weighted calibration curves using Validata, a Microsoft Excel macro developed by Wegscheider *et al.*[37] Limits of detection (LODs) were calculated based on signal-to-noise (S/N) ratios of 3:1 using the Analyst® software.

RESULTS AND DISCUSSION

Development of the analytical method

MS/MS detection

MS and MS/MS parameters (such as the selection of the most abundant MRM transitions, declustering potentials, collision energies, cell exit potentials) were optimized for all analytes in both the positive and the negative ESI mode. Most of the compounds exhibited precursor ions and product ions with reasonably high signal intensities in both modes. Unfortunately, the list of analytes includes some substances (MON, NIV, Z4S) which gave no or very weak signals in the positive ion mode, whereas it was not possible to apply the negative mode for the enniatins and A-trichothecenes. As polarity switching during the chromatographic run was not possible due to the large number of analytes, it was decided to use the ESI+ and the ESI− mode in two separate chromatographic runs per sample to guarantee optimal MS conditions for all analytes. For each analyte, the polarity giving the most abundant product ions was selected; a second product ion was monitored for confirmation of the identity (with the exception of MON, which showed only one product ion). Table 1 summarizes the parameters of the optimized MRM transitions.

Another point that has to be taken into consideration concerns the formation of adducts. The addition of 5 mM ammonium acetate to the chromatographic eluent was found to be necessary to suppress the formation of stable sodium adducts, which are caused by the presence of traces of alkali ions in the eluents and in the glassware and which often do not yield detectable product ions for aflatoxins and some A-trichothecenes. Under the chosen conditions, A-trichothecenes and enniatins formed $[M+NH_4]^+$ adducts, that gave higher MS/MS signal intensities than the related $[M+H]^+$ species. Similarly, the B-trichothecenes formed the $[M+CH_3COO]^-$ adduct, which yielded upon fragmentation either the acetate ion (*m/z* 59) or the $[M–H]^-$ ion as the most abundant species. As these two MRM transitions are non-analyte specific, a third transition yielding a specific product ion of the respective trichothecene was monitored for secure identification of these analytes, whereas quantification was performed using the most abundant signals. This way at least three identification points (retention time, molecular mass, one characteristic product ion) are included in the method, which is the minimum requirement for the confirmation of substances listed in group B of Annex I of Directive 96/23/EC.[38]

Chromatographic separation

When developing a method dealing with analytes having diametrically opposed polarity or acidity, it has to be accepted that certain conditions may be far from optimal for some of the analytes. In the present case, the choice of the pH of the mobile phase was found to be critical for the fumonisins: These compounds exhibited symmetrical peak shapes only under acidic conditions (as already described[12,13]) due to the presence of four carboxylic groups in their molecular structure. It was therefore decided to add 1% acetic acid to both eluents. However, acidic conditions promote the epimerization of ergot alkaloids at the 8-position of the lysergic ring, which can cause problems in quantification due to differences in the intensities of the MRM transitions of the two epimers.[39] Indeed, ergotamine exhibited two well-resolved peaks (see Fig. 1) under the chosen conditions, but the sum of the areas of these two peaks remains constant for repeated injections of a standard diluted with mobile phase for at least 3 days (this was also checked for all the other analytes).

The Gemini® reversed-phase C_{18} column was chosen as the stationary phase because it exhibited reasonable peak shapes for all the analytes despite their chemical diversity. In particular, a retention factor of 1.20 for MON (corresponding to a retention time of 3.28 min and a column dead time of 1.49 min, see Fig. 2) was obtained with this column, which was far better than we had expected for such a low molecular mass, acidic compound. The injection of a standard prepared in 75% ACN hardly influenced the peak shape of this analyte, whereas the peak shape of NIV (that elutes next to MON) was heavily distorted. Therefore, it can be concluded that probably a different retention mechanism is involved, either ion-pair formation with ammonium as counter-ion or ionic interactions with the silica. Figures 1 and 2 show that it was

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Table 1. Optimized ESI-MS and ESI-MS/MS conditions

Compound	Precursor ion (m/z)	Declustering potential (V)[a]	Product ions (m/z)[a]	Collision energy (V)[a]	Cell exit potential (V)[a]
Neosolaniol	400.2 $[M+NH_4]^+$	46	**215.0/185.0**	25/29	12/14
Verrucarol	267.0 $[M+H]^+$	56	249.1/**219.0**	11/15	15/14
Monoacetoxyscirpenol	342.2 $[M+NH_4]^+$	41	**265.0**/307.0	13/13	26/8
Diacetoxyscirpenol	384.2 $[M+NH_4]^+$	51	**307.2/105.1**	17/61	9/7
HT-2 toxin	442.2 $[M+NH_4]^+$ 447.4 $[M+Na]^+$	46/101	263.1/345.1	21/27	19/20
T-2 toxin	484.3 $[M+NH_4]^+$	56	**215.2/185.1**	29/31	18/11
Verrucarin A	520.2 $[M+NH_4]^+$	51	**249.1/457.1**	25/19	14/14
Nivalenol	371.1 $[M+CH_3COO]^-$	−45	59.1/**281.1**/311.1	−42/−22/−16	−7/−15/−15
Deoxynivalenol	355.1 $[M+CH_3COO]^-$	−40	59.2/295.2/**264.9**	−40/−16/−16	−8/−14/−14
Deoxynivalenol-3-glucoside	517.3 $[M+CH_3COO]^-$	−50	**427.1**/59.1/457.1	−30/−85/−20	−11/−7/−13
Fusarenon X	413.3 $[M+CH_3COO]^-$	−40	59.1/353.6/**262.9**	−44/−14/−22	−9/−5/−16
Deepoxy-deoxynivalenol	339.1 $[M+CH_3COO]^-$	−40	59.1/**248.9**	−20/−18	−9/−17
3-Acetyldeoxynivalenol	397.3 $[M+CH_3COO]^-$	−40	59.2/**307.1**/337.1	−38/−18/−14	−8/−14/−7
Aflatoxin G_2	330.9 $[M+H]^+$	76	**245.1/189.0**	45/59	12/13
Aflatoxin G_1	329.1 $[M+H]^+$	106	**243.0/200.0**	37/59	18/12
Aflatoxin B_2	315.2 $[M+H]^+$	101	**259.1/287.1**	43/41	6/20
Aflatoxin B_1	313.0 $[M+H]^+$	136	**128.1/241.1**	91/51	10/10
Agroclavin	239.2 $[M+H]^+$	56	**183.0/168.2**	29/51	16/14
Ergovalin	534.2 $[M+H]^+$	76	**223.2/208.0**	45/63	12/10
Dihydroergosin	550.2 $[M+H]^+$	96	**270.1/253.0**	47/43	16/16
Ergotamin	582.2 $[M+H]^+$	66	**223.2/208.1**	47/59	12/14
Ergocornin	562.2 $[M+H]^+$	81	544.2/**223.1**	23/49	20/12
Beauvericin	806.5 $[M+Na]^+$ 801.5 $[M+NH_4]^+$	161/86	**384.4**/784.3	73/31	10/12
Enniatin B	657.5 $[M+NH_4]^+$	51	**196.3**/640.3	45/29	18/10
Enniatin B_1	671.1 $[M+NH_4]^+$	81	**196.0**/654.4	41/29	12/10
Enniatin A_1	668.4 $[M+H]^+$ 685.4 $[M+NH_4]^+$	141/66	**210.1**/668.4	37/27	12/10
Enniatin A	699.4 $[M+NH_4]^+$	76	**210.1**/682.4	43/29	12/10
Hydrolyzed fumonisin B_1	406.3 $[M+H]^+$	86	370.3/388.3	29/27	10/20
Fumonisin B_1	722.5 $[M+H]^+$	91	**334.4**/704.4	57/43	4/4
Fumonisin B_2	706.5 $[M+H]^+$	96	**336.3**/688.4	51/41	4/10
Ochratoxin α	254.9 $[M-H]^-$	−60	210.9/**166.9**	−24/−36	−11/−11
Ochratoxin B	370.1 $[M+H]^+$	56	**205.0/103.2**	33/77	12/6
Ochratoxin A	404.0 $[M+H]^+$	61	**239.0/102.0**	37/105	16/14
Zearalenone-4-glucoside	479.2 $[M-H]^-$	−65	**317.1/175.0**	−24/−56	−17/−9
$\alpha+\beta$-Zearalenol	319.2 $[M-H]^-$	−85	**160.0/130.0**	−44/−50	−13/−20
Zearalenone-4-sulfate	397.1 $[M-H]^-$	−75	**317.1/175.0**	−32/−48	−15/−13
Zearalenone	317.1 $[M-H]^-$	−80	**131.1/175.0**	−42/−34	−8/−13
Moniliformin	96.9 $[M-H]^-$	−70	**41.2**	−24	−5
Patulin	152.9 $[M-H]^-$	−20	108.9/135.0	−12/−12	−9/−9

[a] Numerical values are given in the order quantifier/qualifier (/2nd qualifier); specific product ions are given in bold.

not possible to avoid the co-elution of some analytes, but this is of minor importance as the related compounds showed different MRM transitions.

Initially, a dwell time of 25 ms was chosen, leading to a repeatability of 10% for the injection of a calibrant if the MRM transitions of all 26 analytes in the positive ion mode were included in one MS/MS cycle. This relatively poor repeatability may be because it was not possible to obtain enough data points per chromatographic peak if the MRM transitions of all analytes were scanned consecutively with a dwell time of 25 ms and a pause time of 5 ms between two MRM transitions, as the MS/MS cycle time should not exceed 1 s (assuming a peak width of 0.25 min). Therefore, the chromatogram was segmented into different retention time periods (5 for the positive ion mode and 3 for the negative ion mode, see Figs. 1 and 2) and only a limited number of MRM transitions were scanned within these periods. Further experiments showed that the repeatability could be further improved by increasing the dwell time to 100 ms (results not shown). As nine analytes are included in the time window between 10.8 and 12.2 min in the positive ion mode, the dwell time of the related qualifier MRM transitions had to be again reduced to 25 ms.

Evaluation of matrix effects

In order to evaluate the influence of the matrix on the mass spectrometric detection, diluted extracts of blank wheat and maize were spiked with the four combined working solutions to yield analyte concentrations covering two orders of magnitude. The resulting linear calibration functions were compared with that of a calibrant containing no matrix. The

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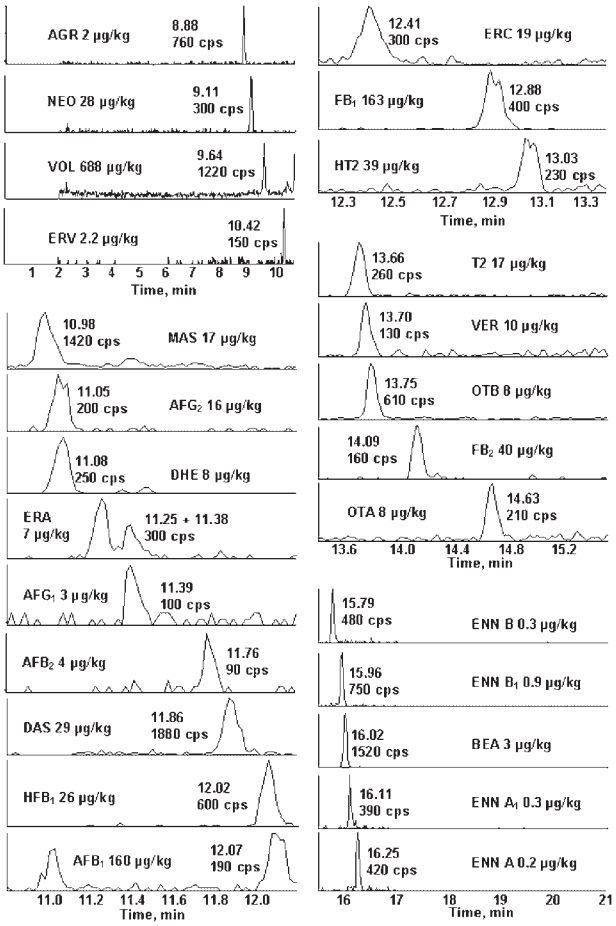

Figure 1. LC/ESI(+)-MS/MS MRM chromatograms of spiked maize samples; peak heights of the 26 quantifier transitions are given in cps (counts per second); note the different time scales of the five retention time periods.

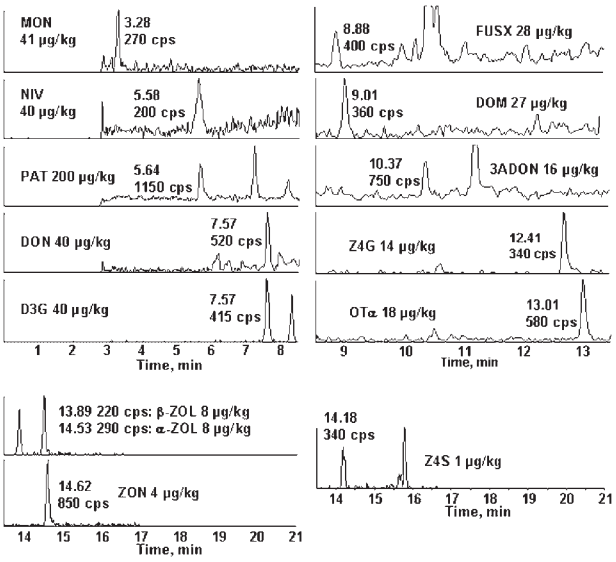

Figure 2. LC/ESI(−)-MS/MS MRM chromatograms of spiked maize samples; peak heights of the 13 quantifier transitions are given in cps (counts per second); note the different time scales of the three retention time periods.

signal suppression/enhancement (SSE) was calculated according to Eqn. (2).

As can be seen in Table 2, the extent of SSE was quite different for the two matrices. Wheat exhibited only minor matrix effects when injected as a 1 + 1 diluted extract. The related SSE was inside the range 100 ± 15% for all analytes except the two glucosides and MON. In contrast to that, the maize matrix had a larger influence on the signal intensity, even if diluted tenfold. Matrix effects were mainly observed for the mycotoxins eluting between 10.4 and 12.5 min (ergot alkaloids, aflatoxins, DAS, HFB_1, HT-2, OTα; MAS and FB_1 are the exceptions) due to the co-elution of one or more major matrix component(s). Interestingly, the signal of the qualifier transition of HT-2 (which uses a different precursor ion than the quantifier transition) was suppressed by a factor of 3 in maize. It seems that the use of sodium adducts is particularly

Table 2. Signal suppression/enhancement in spiked sample extracts. The crude extracts were prepared by extracting 10 g of blank sample with 40 mL of ACN/H_2O 84 + 16 and were diluted with eluent A

Matrix	Wheat		Maize			
Dilution	1 + 2	1 + 1	1 + 9	1 + 4	1 + 2	1 + 1
NEO	101	98	100	96	97	101
VOL	100	90	91	91	84	88
MAS	97	92	104	97	99	105
DAS	101	99	97	93	76	74
HT-2	106	95	99	89	94	85
T-2	103	97	105	98	96	97
VER	n.d.	n.d.	103	n.d.	n.d.	n.d.
NIV	103	102	96	96	99	101
DON	108	109	99	97	93	103
D3G	125	123	97	121	105	141
FUSX	109	111	100	103	101	107
DOM	105	107	99	98	102	110
3ADON	102	100	99	93	103	96
AFG_2	91	91	101	99	65	58
AFG_1	93	95	86	76	76	67
AFB_2	91	95	92	63	60	54
AFB_1	102	96	34	31	22	25
AGR	99	96	96	90	93	94
ERV	93	85	86	80	77	68
DHE	102	97	85	74	63	55
ERA	107	101	78	62	59	53
ERC	101	96	69	50	46	37
BEA	101	89	101	104	101	107
ENN B	111	109	103	103	106	109
ENN B_1	112	109	102	104	103	106
ENN A_1	110	111	101	105	107	113
ENN A	107	106	104	106	108	112
HFB_1	100	96	81	75	69	67
FB_1	100	96	105	106	106	106
FB_2	105	98	102	113	113	117
OTα	100	94	80	82	82	86
OTB	98	96	98	100	98	95
OTA	107	97	98	102	103	108
Z4G	111	125	112	129	134	148
β-ZOL	115	108	91	92	85	95
Z4S	109	112	89	95	79	93
α-ZOL	106	104	92	95	92	96
ZON	112	114	99	99	90	102
PAT	102	96	100	93	95	96
MON	125	129	99	100	125	120

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critical in the case of A-trichothecenes. Similar to wheat, the signal intensities of DON- and ZON-glucoside and MON were significantly enhanced also in maize.

In the course of these experiments, it was observed that the sample turned turbid upon dilution, probably due to precipitation of co-extracted fats and proteins. This dilution step is necessary to reduce the content of ACN in order to avoid distortion of the early eluting peaks, i.e. NIV, DON and D3G. It cannot be ruled out that interactions with colloidal particles emerging from dilution are partially responsible for the reduced signal intensities of aflatoxins and ergot alkaloids in maize, particularly as the former substance class is known to adsorb on particulate matter.[40]

These investigations revealed that the injection of diluted crude extracts without any prior clean-up is feasible for both wheat and maize. Even for the few critical analyte/matrix combinations, the relative changes in signal intensities remained stable over the concentration range investigated. This LC/MS/MS performance is a result of the changes in design of the modern generation of ESI interfaces compared with earlier models where there was signal suppression by a factor of 3 for ZON in maize despite the application of a clean-up procedure.[16] For the final method, it was decided to dilute the raw extracts 1 + 1, since the decrease in matrix effect in more diluted maize extracts does not compensate for the loss in sensitivity.

Optimization of extraction solvent

The composition of the solvent applied for extraction is a crucial parameter during the development of a multi-toxin method, particularly in view of the chemical diversity of the analytes. Different mixtures of water and organic solvents (ACN and MeOH, respectively) with and without the addition of acetic acid were tested. Table 3 summarizes the results obtained for wheat (note that the apparent recoveries are given; however, as the extract was diluted 1 + 9 prior to injection, SSE should be negligible). It can be seen that mixtures containing a large fraction of ACN are appropriate for the extraction of most of the analytes, with the exception of FB_1, FB_2 and PAT (the large values obtained for Z4G in the three most polar mixtures cannot be explained). The addition of 1% acetic acid to the solvent mixture improved the extraction of the two former compounds, whereas the results for the latter remained unsatisfactory. The extraction of other analytes was hardly affected by the change in pH, only MON and OTα exhibited a somewhat lower apparent recovery. Due to the more pronounced matrix effects in maize, a direct comparison

Table 3. Dependence of the apparent recovery of spiked wheat samples on the composition of the water/solvent mixture applied for extraction (BEA and the enniatins are not included due to the contamination of the blank wheat)

Solvent % (v/v)	MeOH 50 (n = 2)	MeOH 75 (n = 2)	ACN 50 (n = 2)	ACN/HAc 74 + 1 (n = 3)	ACN 75 (n = 3)	ACN/HAc 79 + 1 (n = 3)	ACN 80 (n = 3)	ACN 84 (n = 3)
NEO	82 ± 4	81 ± 13	101 ± 3	88 ± 11	104 ± 3	109 ± 1	99 ± 5	101 ± 4
VOL	95 ± 17	111 ± 16	108 ± 18	115 ± 28	99 ± 6	107 ± 12	96 ± 3	98 ± 8
MAS	70 ± 18	66 ± 2	76 ± 7	96 ± 7	101 ± 4	106 ± 3	94 ± 5	94 ± 6
DAS	74 ± 2	78 ± 7	80 ± 4	86 ± 4	105 ± 3	106 ± 6	99 ± 6	101 ± 5
HT-2	80 ± 7	71 ± 10	69 ± 3	76 ± 0	108 ± 6	106 ± 6	102 ± 9	100 ± 9
T-2	59 ± 1	55 ± 1	51 ± 3	89 ± 1	104 ± 3	107 ± 6	99 ± 7	98 ± 8
NIV	94 ± 11	68 ± 3	109 ± 1	83 ± 4	91 ± 8	87 ± 3	78 ± 2	80 ± 9
DON	83 ± 5	54 ± 9	90 ± 20	77 ± 3	114 ± 4	112 ± 7	107 ± 3	106 ± 9
D3G	n.d.	n.d.	n.d.	n.d.	72 ± 2	78 ± 1	71 ± 1	69 ± 4
FUSX	89 ± 24	65 ± 1	105 ± 3	78 ± 26	100 ± 7	99 ± 8	95 ± 4	101 ± 2
DOM	n.d.	n.d.	n.d.	n.d.	105 ± 1	99 ± 2	94 ± 11	98 ± 6
3ADON	57 ± 12	50 ± 5	70 ± 3	91 ± 25	91 ± 4	90 ± 4	84 ± 3	87 ± 9
AFG_2	73 ± 4	85 ± 13	82 ± 18	106 ± 14	94 ± 7	106 ± 6	91 ± 13	106 ± 14
AFG_1	30 ± 18	34 ± 6	31 ± 2	103 ± 16	92 ± 3	104 ± 4	93 ± 16	100 ± 6
AFB_2	31 ± 5	50 ± 9	47 ± 7	93 ± 13	92 ± 7	99 ± 1	86 ± 6	87 ± 4
AFB_1	40 ± 0	34 ± 8	65 ± 20	104 ± 1	85 ± 13	83 ± 13	75 ± 7	90 ± 10
AGR	25 ± 5	46 ± 8	63 ± 2	97 ± 6	94 ± 4	102 ± 5	96 ± 4	86 ± 3
ERV	18 ± 0	38 ± 5	30 ± 3	80 ± 3	78 ± 3	88 ± 6	86 ± 10	87 ± 16
DHE	19 ± 5	27 ± 0	28 ± 0	79 ± 4	84 ± 2	93 ± 5	87 ± 2	90 ± 9
ERA	15 ± 4	27 ± 1	22 ± 1	87 ± 6	85 ± 4	102 ± 4	87 ± 3	89 ± 4
ERC	8 ± 0	10 ± 2	12 ± 1	96 ± 9	97 ± 0	102 ± 3	96 ± 4	94 ± 3
HFB_1	46 ± 2	58 ± 3	57 ± 0	116 ± 13	96 ± 6	85 ± 9	73 ± 1	58 ± 5
FB_1	89 ± 14	72 ± 3	83 ± 5	105 ± 16	17 ± 2	34 ± 5	4 ± 1	1 ± 0
FB_2	117 ± 40	69 ± 8	89 ± 6	85 ± 49	35 ± 6	49 ± 12	18 ± 5	10 ± 1
OTα	106 ± 1	109 ± 22	108 ± 17	79 ± 8	100 ± 3	70 ± 7	96 ± 12	77 ± 2
OTB	63 ± 1	65 ± 6	77 ± 2	91 ± 1	95 ± 1	103 ± 3	89 ± 3	83 ± 7
OTA	66 ± 3	71 ± 10	97 ± 23	103 ± 12	102 ± 3	106 ± 5	93 ± 9	91 ± 3
Z4G	177 ± 1	204 ± 11	378 ± 1	98 ± 10	109 ± 5	98 ± 3	104 ± 8	98 ± 10
β-ZOL	45 ± 1	59 ± 13	77 ± 12	91 ± 9	94 ± 2	97 ± 6	92 ± 7	96 ± 3
Z4S	41 ± 6	41 ± 0	41 ± 2	87 ± 26	87 ± 7	88 ± 4	92 ± 6	91 ± 5
α-ZOL	47 ± 3	50 ± 3	60 ± 2	77 ± 8	105 ± 1	108 ± 3	94 ± 17	102 ± 10
ZON	51 ± 4	39 ± 12	42 ± 23	107 ± 2	111 ± 2	113 ± 1	98 ± 17	106 ± 5
PAT	4 ± 0	9 ± 1	9 ± 1	23 ± 16	28 ± 4	29 ± 3	20 ± 15	18 ± 15
MON	113 ± 44	92 ± 22	133 ± 11	84 ± 13	109 ± 2	82 ± 3	102 ± 13	105 ± 2

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of the extraction efficiencies of different solvent mixtures is not straightforward. Apparent recoveries indicated a similar trend as for wheat (data not shown). It was therefore decided to use ACN/H_2O/HAc 79 + 20 + 1 (v/v/v) for extraction of both wheat and maize in the final method as the best compromise for the extraction of the 39 mycotoxins to be determined.

Method validation

The method was validated for wheat and maize by spiking blank samples on multiple levels in triplicate. The results as well as the number of replicates and the concentration levels are summarized in Tables 4 and 5. CVs of the overall process of <5.1% and <3.0% for wheat and maize were obtained from linear (confirmation through Mandel test) calibration data for all analytes with two exceptions (PAT and MON, see below). The repeatability at the highest concentration level was generally below 10% (except for D3G and MON in wheat). For the B-trichothecenes, the MRM transitions using acetate (*m*/*z* 59) as the product ion were compared with the transitions using analyte-specific product ions, as there is a lack of agreement among the working groups involved in mycotoxin analysis on the use of non-specific product ions such as acetate. The transitions to *m*/*z* 59 exhibited smaller values for the CV, probably due to their higher abundance (however, it should be mentioned that the LODs based on the S/N ratio were higher – up to a factor of 3 for NIV – due to an increased baseline noise). PAT exhibited a wide range of apparent recoveries for different concentration levels without a visible trend and therefore had to be omitted from the final method. The peak areas obtained for MON showed a non-linear dependence on the concentration both for the liquid standard and for the spiked samples (Fig. 3). This seems to be a consequence of a decrease in the ionization efficiency at higher concentrations.

Signal suppression/enhancement (re-evaluated by spiking three blank extracts at one concentration level) occurred to a similar extent as in preliminary investigations (presented in Table 2) despite the slightly different composition of the extraction solvent. The reduced signal intensities for four of the ergot alkaloids in wheat and maize may be explained by epimerization of the analytes in the acidic extract. For the other analytes, the signal intensities for the two glucosides and MON were enhanced, whereas signal suppression of >10% for aflatoxins, VOL, DAS, HT-2, HFB_1, OTα, β-ZOL and Z4G was observed in maize. In the non-quantitative

Table 4. Overview of apparent recovery (R_A), signal suppression/enhancement (SSE), recoveries (R_E), relative standard deviations at the lowest and the highest concentration levels and coefficients of variations of the overall procedure (CV) of the method validation performed in wheat (n = 3; the enniatins are not included due to contamination of the blank wheat)

	Conc. range [μg/kg]	Evaluable levels	R_A (%)	SSE (%)	R_E (%)	RSD (%) low/high	CV (%)
NEO	20.2–1620	7	96	104	92	8.6/3.5	0.6
VOL	86–6880	7	90	101	89	3.2/5.7	0.5
MAS	21.2–1700	7	93	102	91	6.7/1.2	0.5
DAS	20.8–1660	7	98	105	94	9.8/0.4	0.7
HT-2	20–1580	7	98	104	94	5.1/4.2	0.6
T-2	20.6–1650	7	96	105	92	10.4/3.6	0.5
VER	5–400	7	90	105	86	25.6/4.4	2.6
NIV	20.2–1620	7	66	99	67	18.2/9.4	1.3
DON	20.2–1620	7	95	107	89	7.8/3.2	1.7
D3G	10–800	7	71	119	60	25.2/11.5	3.1
FUSX	20.2–1620	7	90	104	87	2.9/5.2	0.9
DOM	19.4–1560	7	88	103	85	15.0/2.4	4.1
3ADON	20–1600	7	83	96	86	13.1/7.5	0.9
AFG_2	2–160	7	79	106	74	21.4/5.8	3.8
AFG_1	2–160	7	79	100	79	44.8/1.9	5.1
AFB_2	2–160	7	83	93	90	16.8/5.0	3.1
AFB_1	2–160	7	75	92	81	17.5/8.2	4.7
AGR	1.44–114	7	83	101	82	7.2/1.4	2.6
ERV	2.16–173	7	68	73	93	12.4/3.5	2.1
DHE	4.24–340	7	70	81	87	3.0/4.8	2.4
ERA	5.2–418	7	81	87	94	2.9/2.1	3.2
ERC	9.76–778	7	90	86	104	6.3/4.8	1.6
BEA	4–80	6	99	101	98	2.4/3.9	2.0
HFB_1	11.0–880	7	74	102	73	14.2/2.5	1.2
FB_1	10.2–816	7	41	97	43	6.3/2.3	2.6
FB_2	10.1–808	7	55	104	53	22.8/5.4	2.7
OTα	4.46–364	7	72	106	67	32.2/4.4	1.6
OTB	10–800	7	88	104	85	6.5/0.6	0.8
OTA	4.12–328	7	90	104	86	11.4/4.3	1.9
Z4G	10–800	7	87	99	88	14.7/1.4	1.7
β-ZOL	10–800	7	89	94	95	5.8/3.3	0.8
Z4S	1–80	7	92	110	84	11.6/1.0	2.6
α-ZOL	10–800	7	98	103	95	8.9/3.3	1.0
ZON	20.4–1630	7	108	106	102	12.8/2.6	1.1
MON	46.4–9280	7	113	140	81	9.1/13.1	—

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Table 5. Overview of apparent recovery (R_A), signal suppression/enhancement (SSE), recoveries (R_E), relative standard deviations at the lowest and the highest concentration levels and coefficients of variations of the overall procedure (CV) of the method validation performed in maize (n = 3)

	Conc. range [μg/kg]	Evaluable levels	R_A (%)	SSE (%)	R_E (%)	RSD (%) low/high	CV (%)
NEO	4.04–4040	10	98	90	108	21.4/2.5	0.3
VOL	121–17200	8	76	78	97	10.3/3.0	0.2
MAS	4.24–4240	10	95	91	105	19.8/6.4	0.5
DAS	4.16–4160	10	90	89	101	11.7/1.6	0.2
HT-2	15.7–3920	9	80	74	108	8.4/3.3	0.3
T-2	4.16–4160	10	97	92	105	8.6/1.9	0.2
VER	4–1000	9	92	92	100	11.0/1.4	0.8
NIV	16–4000	9	74	92	80	20.2/2.2	0.5
DON	4.04–4040	10	106	108	98	40.4/4.5	0.3
D3G	4–1000	9	103	157	66	16.6/4.8	1.3
FUSX	16.1–4040	9	108	110	98	15.6/0.3	0.4
DOM	15.6–3900	9	106	108	99	14.6/3.7	0.4
3ADON	16–4000	9	95	107	89	9.6/3.6	0.5
AFG_2	2.8–400	8	68	62	110	27.8/2.2	1.7
AFG_1	1.6–400	9	60	56	107	7.4/6.0	1.6
AFB_2	1.6–400	9	49	48	102	17.2/1.4	1.3
AFB_1	16–400	6	17	18	95	32.1/9.9	2.8
AGR	0.286–286	10	79	93	85	9.3/3.3	1.3
ERV	0.860–216	9	62	62	100	11.9/2.4	1.3
DHE	3.40–848	9	43	50	86	16.9/0.8	0.9
ERA	1.04–734	9	45	41	110	28.8/3.5	1.0
ERC	1.94–1360	9	29	27	110	3.6/2.9	0.6
BEA	3.2–800	9	94	109	86	7.3/7.9	1.9
ENN B	0.304–304	10	105	101	103	6.2/3.8	1.1
ENN B_1	0.864–864	10	104	101	103	8.2/3.5	0.6
ENN A_1	0.32–320	10	106	103	103	9.5/1.9	0.7
ENN A	0.048–48	10	102	104	98	15.8/1.8	0.9
HFB_1	2.64–658	9	47	63	75	22.5/2.3	1.4
FB_1	16.3–4080	9	58	101	57	16.7/3.6	0.5
FB_2	16.2–4040	9	70	104	67	5.3/3.0	0.4
OTα	1.92–454	9	71	83	86	24.5/0.9	1.2
OTB	2–2000	10	98	96	103	15.8/3.0	0.4
OTA	3.30–824	9	100	100	100	11.0/2.1	0.5
Z4G	8–2000	9	141	141	100	21.8/5.0	0.7
β-ZOL	2–2000	10	87	81	107	23.1/6.6	0.6
Z4S	0.4–70	8	79	78	101	8.2/2.9	3.0
α-ZOL	2–2000	10	97	95	102	15.6/3.2	0.5
ZON	4.08–2860	9	101	108	93	6.7/0.5	0.7
MON	10.2–1020	10	93	124	75	12.5/5.7	—

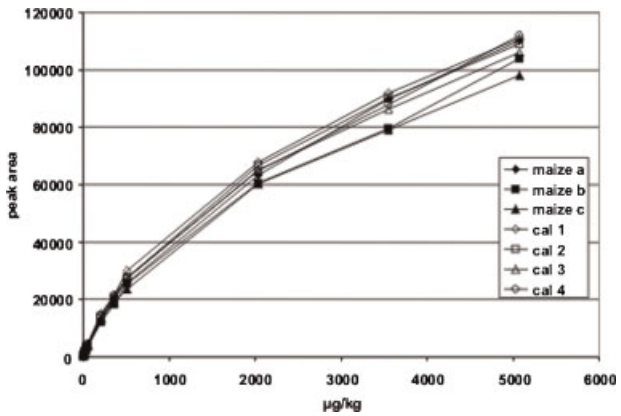

Figure 3. Correlation between the concentration of monoliformin (in the liquid standard solution and the spiked maize samples, respectively) and the related peak areas.

extraction, the most critical compounds are the fumonisins and other polar analytes such as NIV, D3G, OTα and MON.

The apparent recoveries were within the range of 100 ± 10% for 15 out of 35 analytes in wheat and for 20 out of 39 analytes in maize. At first glance, this suggests that the developed method is of limited value for the quantitative determination of all analytes. However, all analyte losses that occurred due to non-quantitative extraction or SSE caused purely proportional systematic errors. If isotopically labelled standards are not available, these errors can be compensated for either by standard addition (which is rather time-consuming, as every individual sample has to be fortified on multiple levels) or by the use of matrix calibration (which, however, does not correct for analyte losses during the extraction step). As already mentioned, the latter approach is preferred by most researchers active in the field of mycotoxin analysis but its applicability depends on the comparability of the apparent recoveries between individual samples. This has yet to be determined for the developed method.

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Significant differences in the LODs between the calibrant and the matrix samples were observed for the analytes showing the most pronounced matrix effects, i.e. aflatoxins and ergot alkaloids (Table 6). The LODs of the developed method ranged between 0.03 μg/kg for Z4S in wheat and 220 μg/kg for VOL in maize, which is far below the European regulations and proposals for maximum levels of mycotoxins in food and feed in the case of trichothecenes, fumonisins and ZON.[41] For OTA, the LOD was comparable with that in the regulations (2–10 μg/kg, 0.5 μg/kg for baby food),[42] whereas the sensitivity for aflatoxins is not adequate, as the regulated values range between 2 and 8 μg/kg for AFB_1 and between 4 and 15 μg/kg for the sum of the aflatoxins in nuts, cereals and spices.[43] The simplest solution to that problem is an increased injection volume, but this disturbs the chromatographic equilibrium, as the elution strength of the initial eluent (containing 10% MeOH) is significantly increased by larger amounts of injected sample (containing 50% ACN). This is reflected by distortions of the early eluting peaks. In addition, larger matrix effects and decreased column life times may be expected due to the increased amount of matrix entering the system.

The developed method was applied to the analysis of reference materials certified for their mass concentrations of DON and ZON. The experimentally determined concentrations showed satisfactory agreement with the certified values, with the exception of BCR 378 (Table 7).

Table 7. Comparison between certified and measured concentrations of certified reference materials

Material	Analyte	Matrix	Certified conc. [μg/kg]	Measured conc. [μg/kg]; (n = 2)
Trilogy D-107	DON	wheat	1100 ± 100	1146 ± 11
Trilogy D-105	DON	wheat	4300 ± 300	4550 ± 70
BCR 379	DON	wheat	670 ± 20	663 ± 26
BCR 378	DON	maize	430 ± 40	348 ± 31
Biopure	DON	maize	474 ± 30	500 ± 19
BCR 717	ZON	maize	83 ± 9	88 ± 12

Table 6. Overview of the estimated LODs in the standard solution and in the two matrices. Numbers in parentheses indicate the maximum levels stated in EU Commission Regulations[41–44]

	Relative sensitivity[a]	Standard solution [μg/kg]	Wheat [μg/kg]	Maize [μg/kg]
NEO	6.8	2	2	4
VOL	1	170	170	220
MAS	48	4	4	4
DAS	11	4	4	4
HT-2	2.5	16	16	16
T-2	9.8	2	2	4
VER	6.2	16	16	16
NIV	3.8	20	20	30
DON	8.4	8	8 (1250)	16 (750[b])
D3G	6.5	4	4	4
FUSX	6.3	16	16	16
DOM	5.9	16	16	16
3ADON	21	12	12	12
AFG_2	6.4	2	3	4
AFG_1	16	1	2	4
AFB_2	15	2	2	4
AFB_1	3.9	4	8 (2)	80 (5)
AGR	220	0.15	0.3	0.3
ERV	40	0.6	0.8	0.8
DHE	27	1.6	1.6	1.6
ERA	68	0.5	1	1
ERC	21	2	4	8
BEA	240	0.08	0.08	0.08
ENN B	450	0.03	n.d.	0.03
ENN B_1	290	0.1	n.d.	0.1
ENN A_1	270	0.1	n.d.	0.1
ENN A	760	0.015	n.d.	0.05
HFB_1	15	2.5	2.5	2.5
FB_1	1.9	16	16	16 (2000[c])
FB_2	2.9	8	8	8 (2000[c])
OTα	24	3.5	6	4.5
OTB	37	2	2	2
OTA	13	3.5	3.5 (5)	3.5
Z4G	11	2	2	2
β-ZOL	14	1	2	2
Z4S	130	0.03	0.03	0.1
α-ZOL	17	1	2	2
ZON	75	0.5	0.5 (100)	0.5 (200[d])
MON	—	20	20	20

[a] Slope of linear calibration function of the standard solution, normalized to VOL.
[b] Maize flour.
[c] Sum of FB_1 and FB_2; this value will be applied to unprocessed maize if no specific level is fixed before October 1st, 2007.
[d] This value will be applied to unprocessed maize if no specific level is fixed before July 1st, 2007.

CONCLUSIONS

The developed LC/ESI-MS/MS method demonstrated that the latest generation of mass spectrometers tolerate the direct injection of crude extracts. This is a prerequisite for a unified method for all the analytes investigated in this study, as the chemical diversity of the analytes requires that the sample pre-treatment be kept as simple as possible. In the accurate quantification of the analytes, non-quantitative extraction and signal suppression/enhancement due to matrix effects were both potential sources of proportional systematic errors. The former was a problem only for a limited number of analytes, however, and the latter may be compensated for by the use of matrix calibration. A thorough investigation of the extents of these two effects is essential for the adaptation of the method to other matrices and analytes.

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