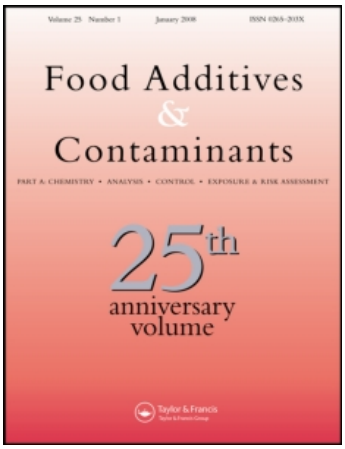

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LC-MS/MS multi-analyte method for mycotoxin determination in food supplements

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LC-MS/MS multi-analyte method for mycotoxin determination in food supplements

José Diana Di Mavungu[a]*, Sofie Monbaliu[a], Marie-Louise Scippo[b], Guy Maghuin-Rogister[b], Yves-Jacques Schneider[c], Yvan Larondelle[c], Alfons Callebaut[d], Johan Robbens[e], Carlos Van Peteghem[a] and Sarah De Saeger[a]

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A multi-analyte method for the liquid chromatography-tandem mass spectrometric determination of mycotoxins in food supplements is presented. The analytes included A and B trichothecenes (nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, neosolaniol, fusarenon-X, diacetoxyscirpenol, HT-2 toxin and T-2 toxin), aflatoxins (aflatoxin-B_1, aflatoxin-B_2, aflatoxin-G_1 and aflatoxin-G_2), *Alternaria* toxins (alternariol, alternariol methyl ether and altenuene), fumonisins (fumonisin-B_1, fumonisin-B_2 and fumonisin-B_3), ochratoxin A, zearalenone, beauvericin and sterigmatocystin. Optimization of the simultaneous extraction of these toxins and the sample pretreatment procedure, as well as method validation were performed on maca (*Lepidium meyenii*) food supplements. The results indicated that the solvent mixture ethyl acetate/formic acid (95:5, v/v) was the best compromise for the extraction of the analytes from food supplements. Liquid–liquid partition with *n*-hexane was applied as partial clean-up step to remove excess of co-extracted non-polar components. Further clean-up was performed on Oasis HLB^{TM} cartridges. Samples were analysed using an Acquity UPLC system coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer equipped with an electrospray interface operated in the positive-ion mode. Limits of detection and quantification were in the range of 0.3–30 ng g^{-1} and 1–100 ng g^{-1}, respectively. Recovery yields were above 60% for most of the analytes, except for nivalenol, sterigmatocystine and the fumonisins. The method showed good precision and trueness. Analysis of different food supplements such as soy (*Glycine max*) isoflavones, St John's wort (*Hypericum perforatum*), garlic (*Allium sativum*), *Ginkgo biloba*, and black radish (*Raphanus niger*) demonstrated the general applicability of the method. Due to different matrix effects observed in different food supplement samples, the standard addition approach was applied to perform correct quantitative analysis. In 56 out of 62 samples analysed, none of the 23 mycotoxins investigated was detected. Positive samples contained at least one of the toxins fumonisin-B_1, fumonisin-B_2, fumonisin-B_3 and ochratoxin A.

Keywords: mycotoxins; multi-target analysis; liquid chromatography; tandem mass spectrometry; food supplements; validation; standard addition

Introduction

Mycotoxins are secondary metabolites produced by a wide range of fungi known to contaminate a variety of food and agricultural commodities worldwide (Shephard et al. 1996). Their occurrence in food, beverages and feed has been recognized as a potential threat to humans and animals, either by direct contamination of plant materials or products thereof (Fink-Gremmels 1999), or by 'carry over' of mycotoxins and their metabolites into animal tissues, milk and eggs after intake of contaminated feed (Galtier 1998; Fink-Gremmels 2008). They are mainly produced by fungi in the *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* genera. Over 400 mycotoxins are known and the food-borne toxins of most interest are aflatoxins, trichothecenes, fumonisins, ochratoxin A (OTA), zearalenone (ZEN) and *Alternaria* toxins, due to their frequent occurrence and their severe effects on animal and human health (Hussein and Brasel 2001). Reports from the literature indicate that these toxins can also be found in a variety of botanicals (Sewram et al. 2006; Trucksess and Scott 2008).

Numerous botanical products enter markets around the world as food supplements. In the last few years there has been a progressive increase of interest in food supplements as they are now increasingly consumed. Some are used daily by consumers for various reasons. Raw materials for plant-based food

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supplements can be contaminated with fungi in the field, during harvesting and storage. Indeed, several surveys of toxigenic moulds in botanicals have found high levels of *Aspergillus*, *Penicillium* and *Fusarium* species (Abeywickrama and Bean 1991; Halt 1998; Rizzo et al. 2004). While the presence of mould might not be correlated with the presence of mycotoxins, there are reports of aflatoxins (Rizzo et al. 1999; Tassaneeyakul et al. 2004; Yang et al. 2005; Ali et al. 2005; D'Ovidio et al. 2006; Trucksess et al. 2007), OTA (Thirumala-Devi et al. 2001; Trucksess et al. 2007), ZEN (Gray et al. 2004). and fumonisins (Martins et al. 2001; Omurtag and Yazicioğlu 2004; Sewram et al. 2006) in medicinal plants, tea and other botanicals. Contamination of these raw materials could result in the presence of mycotoxins in food supplements, leading to diverse human health problems. It is therefore necessary to have suitable analytical methods for mycotoxin determination in food supplements.

Currently, analytical methods used for mycotoxin analysis include thin-layer chromatography (TLC) (Betina 1993; Krska et al. 2001), enzyme-linked immunosorbent assay (ELISA) (Ware et al. 1999; Thirumala-Devi et al. 2001; Heber et al. 2001), gas chromatography (GC) with electron capture (Langseth and Rundberget 1998) or mass spectrometric (Schwadorf and Müller 1992; Langseth and Rundberget 1998; Shephard 1998; Valenta 1998; Tanaka et al. 2000; Nielsen and Thrane 2001; Soleas et al. 2001) detection, liquid chromatography with fluorescence detection (LC-FLD) (Shephard 1998; Valenta 1998; Krska and Josephs 2001), and liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Young and Lafontaine 1993; Thakur and Smith 1994; Biselli et al. 2005). LC-MS/MS appears to be most promising as a highly specific, broadly applicable detection method that provides both qualitative and quantitative data. Considering the possible contamination of foodstuffs by several mycotoxin-producing fungal species and the production of different types of mycotoxins by one mould, a trend is to develop methods suitable for the determination of several mycotoxins in a single run (Sewram et al. 1999; Monti et al. 2000; Rundberget and Wilkins 2002; Royer et al. 2004; Berthiller et al. 2005; Cavalière et al. 2005; Kokkonen et al. 2005; Sorensen and Elbaek 2005; Abbas et al. 2006; Delmulle et al. 2006; Sulyok et al. 2006; Spanjer et al. 2008; Monbaliu et al. 2009). Currently published protocols, however, have been developed and optimized for different foods and feeds. To the best of our knowledge, LC-MS/MS multi-mycotoxin methods for food supplements have not been published yet.

The aim of this study was to develop a multi-component analytical methodology based on LC-MS/MS for the simultaneous determination of an extended list of 23 mycotoxins in food supplements. Target compounds include A and B trichothecenes [nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), neosolaniol (NEO), fusarenon-X (F-X), diacetoxyscirpenol (DAS), HT-2 toxin (HT-2) and T-2 toxin (T-2)], aflatoxins [aflatoxin-B_1 (AF-B1), aflatoxin-B_2 (AF-B2), aflatoxin-G_1 (AF-G1) and aflatoxin-G_2 (AF-G2)], *Alternaria* toxins [alternariol (AOH), alternariol methyl ether (AME) and altenuene (ALT)], fumonisins [fumonisin-B_1 (F-B1), fumonisin-B_2 (F-B2) and fumonisin-B_3 (F-B3)], OTA, ZEN, beauvericin (BEAU) and sterigmatocystin (STERIG). The method consists of a single step of extraction for all target compounds, considerably simplifying sample preparation. The multi-analyte method was evaluated in terms of precision, linearity, recovery, limits of detection and quantification on maca (*Lepidium meyenii*) food supplements. The general applicability of the LC-MS/MS conditions as well as the extraction and clean-up procedure was explored with a number of food supplements such as soy (*Glycine max*) isoflavones, St John's wort (*Hypericum perforatum*), garlic (*Allium sativum*), *Ginkgo biloba* and black radish (*Raphanus niger*).

Materials and methods

Standards

Mycotoxin standards NIV, DON, 3-ADON, 15-ADON, NEO, F-X, AF-B1, AF-B2, AF-G1, AF-G2, HT-2, AOH, AME, ALT, OTA, ZEN, F-B1, F-B2, BEAU, STERIG as well as the internal standard zearalanone (ZAN) were purchased from Sigma-Aldrich (Bornem, Belgium). DAS and T-2 were purchased from Biopure (Tulln, Austria). F-B3 was obtained from Promec Unit (Tygerberg, South Africa). NIV, NEO and DAS were obtained as solutions ($100\,\mu g\,ml^{-1}$) in acetonitrile. From the solid standards, individual stock solutions were prepared at a concentration of $1\,mg\,ml^{-1}$. Stock solutions of DON, 3-ADON, 15-ADON, F-X, AF-B1, AF-B2, AF-G1, AF-G2, HT-2, T-2, ALT, OTA, ZEN, BEAU, F-B1, F-B2, STERIG and ZAN were prepared in methanol. AOH and AME stock solutions were prepared in methanol/dimethylformamide (60 : 40, v/v), whereas F-B2 and F-B3 stock solutions were prepared in acetonitrile/water (50 : 50, v/v). All stock solutions were stored in the freezer at –18°C, except for FB-2 and FB-3, which were stored at 4°C. Spanjer et al. (2008) reported that under these conditions these stock solutions were stable for at least two years. From the individual stock standard solutions, a standard mixture was prepared at the following concentrations: NIV, 3-ADON, 15-ADON and AME ($60\,ng\,\mu l^{-1}$); DON and ZEN ($40\,ng\,\mu l^{-1}$); NEO, T-2, BEAU and

STERIG (20 ng µl^{-1}); F-X and AOH (50 ng µl^{-1}); AF-B1, AF-B2, AF-G2 and AF-G1 (8 ng µl^{-1}); ALT (24 ng µl^{-1}); F-B1, F-B3 and HT-2 (12 ng µl^{-1}); DAS, OTA and F-B2 (4 ng µl^{-1}). The standard mixture was stored at –18°C and renewed monthly.

Reagents and materials

HPLC-grade methanol and acetonitrile as well as *n*-hexane were purchased from VWR International (Zaventem, Belgium). Ethyl acetate, dichloromethane and dimethylformamide were purchased from Acros Organics (Geel, Belgium). A Milli-Q purification system (Millipore, Brussels, Belgium) was used to purify demineralized water. Hydrochloric acid, acetic acid and formic acid from Merck (Darmstadt, Germany) were used. Ammonium acetate was supplied by Grauwmeer (Leuven, Belgium). Trifluoroacetic acid was from Fluka (Buch, Switzerland). The cartridges used for solid-phase extraction (SPE) were Oasis HLB^{TM} (200 mg, 6 ml) from Waters (Milford, MA, USA). Other cartridges tested were Carbograph from LARA (Rome, Italy) and Bakerbond aminopropyl (NH_2) from Achrom (Zulte, Belgium). Ultrafree®-MC centrifugal filter devices (0.22 µm) from Millipore (Bedford, MA, USA) were used.

Samples

The 62 samples analysed consisted of different maca (*Lepidium meyenii*), soy (*Glycine max*) isoflavones, St John's wort (*Hypericum perforatum*), garlic (*Allium sativum*), *Ginkgo biloba* and black radish (*Raphanus niger*) based food supplements. These food supplements were obtained commercially from drugstores, specialized shops or through the internet. Capsules (dried products and oils) were opened and the content was released and homogenized before use according to the sample preparation procedure. Tablets were ground to obtain a fine and homogenized powder prior to the extraction step.

Method optimization and method validation were performed using commercial food supplement samples purchased from drugstore and which were tested free from mycotoxins. The absence of mycotoxins was confirmed as follows: a portion of sample was analysed as such and another portion was spiked with the target analytes before analysis. By comparing with a solution of standards, no peaks corresponding to the target analytes were found in the non-spiked sample, whereas they were found in the spiked sample.

Sample preparation

One gram of sample was extracted with 25 ml ethyl acetate/formic acid (95 : 5, v/v) during 30 min on an Agitelec overhead shaker (J. Toulemonde & Cie, Paris, France). The sample extract was centrifuged and 20 ml of the extract were evaporated until dryness. For defatting, the residue was reconstituted in 5 ml of methanol/water (50 : 50, v/v) and 10 ml of *n*-hexane were added. The mixture was shaken and centrifuged. The aqueous fraction was transferred into a test tube using a Pasteur pipette. Fresh solutions (2 × 5 ml) of methanol/water (50:50, v/v) were added to the hexane fraction and the procedure was repeated as mentioned above. After evaporation of the combined aqueous fraction, the residue was reconstituted in 400 µl methanol/water (50 : 50, v/v) and centrifuged in a Ultrafree®-MC centrifugal device for 10 min at 14,000*g*. Afterwards, a 250 µl aliquot of the filtrate was diluted to 25 ml with water. The obtained solution was further cleaned-up using Oasis HLB^{TM} SPE cartridges. Firstly, the SPE cartridges were conditioned with 10 ml of dichloromethane/methanol (80 : 20, v/v) containing 50 mM formic acid, followed by 5 ml methanol, 20 ml acidified water (10 mM hydrochloric acid in water) and finally 10 ml water. After the conditioning step, the sample extract was quantitatively brought onto the SPE cartridge, which was then washed by passing 10 ml of water. Elution of mycotoxins was performed by passing consecutively 1 ml methanol and 4 ml dichloromethane/methanol (80 : 20, v/v) containing 50 mM formic acid. The eluate was evaporated under a gentle stream of nitrogen and reconstituted with 100 µl of injection solvent, which consisted of methanol/water/acetic acid (57.2:41.8 : 1, v/v/v) containing 5 mM ammonium acetate. The resulting solution was centrifuged in a Ultrafree®-MC centrifugal device for 10 min at 14 000*g* before LC-MS/MS analysis.

LC-MS/MS analysis

The LC-MS/MS system consisted of a Waters Acquity UPLC apparatus coupled to a Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray ionization (ESI) interface. The analytical conditions were as previously described (Monbaliu et al. 2009) and are summarized beneath. Chromatographic separation was achieved using a Symmetry C_{18} column (5 µm, 150 × 2.1 mm i.d.) with a Sentry guard column (3.5 µm, 10 × 2.1 mm i.d.) both supplied by Waters (Zellik, Belgium). The column was kept at room temperature. A mobile phase consisting of eluents A [water/methanol/acetic acid (94 : 5 : 1, v/v/v) containing 5 mM ammonium acetate] and B [methanol/water/acetic acid (97:2:1, v/v/v) containing 5 mM ammonium acetate] was used at a flow rate of 0.3 ml min^{-1}. A gradient elution was applied as follows: 0–7 min, 95% A/5% B – 35% A/65% B; 7–11 min, 35% A/65%

B – 25% A/75%B; 11–13 min, 25% A/75% B – 0% A/100% B; 13–15 min, 0% A/100% B; 15–16 min, 0% A/100% B – 40% A/60% B; 16–22 min, 40% A/60% B – 60% A/40% B; 22–23 min, 60% A/40% B – 95% A/5% B; 23–25 min 95% A/5% B. The injection volume was 20 µl.

The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. MS parameters for the analysis were as follows: ESI source block and desolvation temperatures: 150 and 350°C, respectively; capillary voltage: 3.2 kV; argon collision gas: 4.12×10^{-3} mbar; cone nitrogen and desolvation gas flows: 20 and 500 l h^{-1}, respectively. In order to identify the target mycotoxins in the food supplement samples, a diagnostic MS 'fingerprint' was built up firstly based on the MS spectra of solutions of standards. After selection of the precursor ions for each analyte, product ions were obtained with a combination of cone voltages and collision energies, parameters that were previously optimized. For increased sensitivity and selectivity, data acquisition was performed working in multiple reaction monitoring (MRM) mode. For part of the mycotoxins investigated, more than two MRM transitions were initially monitored. The two transitions that resulted in higher sensitivity and better selectivity were selected in the final method. MRM transitions, the optimum cone voltages and collision energies selected for each transition are given in Table 1, as well as the indicative retention times on the column. The first transition, which corresponds to the most abundant product ion was used for quantification, and the second one for confirmation purposes. Masslynx and Quanlynx software (Micromass, Manchester, UK) was used for data acquisition and processing.

Standard addition technique

When mycotoxin concentrations had to be confirmed in suspect positive samples, the standard addition approach was applied. Each sample was divided into four portions. One portion was analysed as such, whereas the other three portions were spiked prior to analysis with the target analytes at increasing levels of concentrations, corresponding to two-, three- and four-fold the initial level, which was previously assessed by external standard. All four fractions were submitted to the sample preparation procedure as described above, and the internal standard was added before determination by LC-MS/MS. The ratios of the peak areas of the target analytes and the internal standard were plotted versus the concentration and the intercept of this regression line with the x-axis gave the initial analyte concentration in the sample.

Method validation

Since food supplements with certified concentrations of mycotoxins are not available, artificially fortified mycotoxin-free samples were analysed during the development of the method as well as in the validation study to verify the recovery, trueness, precision, linearity, limits of detection and limits of quantification. The use of a triple quadrupole provides high selectivity and specificity. Two MRM transitions were monitored, which improves specificity. For identification, the ratios of the two MRM transitions were compared with those of the standards. The trueness was evaluated by recovery experiments. Standards were added to analyte-free samples prior to the extraction step and the spiked samples were analysed by the standard addition method as described above. Precision of the method was studied by repeated analysis of spiked samples. The experiments were carried out at two concentrations of the analyte in the sample on the same day (intra-day precision) and on three consecutive days (inter-day precision). The precision was calculated as relative standard deviation (RSD) of replicate measurements. The limits of detection (LODs) and limits of quantification (LOQs) were determined from spiked blank samples, as the minimum detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. In detail, analyte-free samples were fortified with decreasing amounts of the target compounds and subsequently subjected to the whole analytical procedure. The fortification level of the target compounds was close to the assumed LODs and LOQs, on the basis of preliminary experiments. Based on these measurements, calibration curves for each analyte were established, which were then utilized to calculate the LODs and LOQs.

Results and discussion

Optimization of the extraction and clean-up procedure

The extraction procedure, as described above, was optimized after evaluating the performance of different mixtures of solvents as well as different clean-up procedures. The diversity and complexity of plant matrix do not facilitate the analysis of organic contaminants and matrix interferences must be carefully considered. Indeed, plants are rich in pigments, essential oils or fatty acids, which may interfere with mycotoxin analysis. A commercial sample of maca (*Lepidium meyenii*) food supplement was used as being representative of the complexity of plant matrix to set up the extraction and clean-up procedures. Besides, the structural diversity of mycotoxins leads to difficulties to recover the different types of mycotoxins during sample preparation and therefore,

Table 1. MS/MS parameters for the analysis of target analytes by MRM ESI-positive ionization mode.

Target compounds	Retention time (min)	Precursor ion (m/z)	Cone voltage (V)	Product ions (m/z)	Collision energy (eV)
NIV	3.18	313.0 $[M+H]^+$	26	124.9[a]	12
				177.2	13
DON	4.29	297.0 $[M+H]^+$	23	203.3[a]	10
				249.5	15
NEO	5.39	400.1 $[M+NH_4]^+$	20	305.3[a]	13
				365.1	10
F-X	5.26	355.1 $[M+H]^+$	22	175.3[a]	11
				247.3	9
3-ADON	6.47	339.2 $[M+H]^+$	23	231.2[a]	10
				203.2	10
15-ADON	6.49	339.1 $[M+H]^+$	24	137.1[a]	10
				321.2	8
AF-G2	6.99	331.0 $[M+H]^+$	46	313.1[a]	24
				245.3	30
AF-G1	7.31	328.8 $[M+H]^+$	43	311.2[a]	25
				243.4	20
AF-B2	7.68	315.0 $[M+H]^+$	50	287.3[a]	25
				259.4	29
AF-B1	7.98	313.0 $[M+H]^+$	47	285.3[a]	21
				241.4	34
DAS	7.97	384.2 $[M+NH_4]^+$	19	307.3[a]	11
				247.3	15
ALT	8.09	293.1 $[M+H]^+$	24	257.2[a]	14
				275.2	10
FB-1	9.56	722.5 $[M+H]^+$	51	334.4[a]	37
				352.3	36
HT-2	9.26	441.9 $[M+NH_4]^+$	16	263.2[a]	13
				215.3	12
AOH	10.02	259.1 $[M+H]^+$	53	185.2[a]	30
				213.1	25
T-2	10.17	484.3 $[M+NH_4]^+$	12	305.2[a]	12
				245.2	12
FB-3	10.89	706.4 $[M+H]^+$	51	336.3[a]	35
				354.0	29
OTA	11.27	404.0 $[M+H]^+$	24	239.1[a]	22
				358.1	14
ZEN	11.66	319.2 $[M+H]^+$	25	185.4[a]	25
				283.3	13
FB-2	12.30	706.4 $[M+H]^+$	51	336.3[a]	35
				318.0	38
STERIG	11.73	325.0 $[M+H]^+$	44	310.2[a]	35
				281.3	25
AME	12.75	273.0 $[M+H]^+$	54	199.2[a]	26
				258.1	25
BEAU	13.13	801.3 $[M+NH_4]^+$	32	244.3[a]	30
				262.4	30
ZAN	11.36	321.2 $[M+H]^+$	27	303.2[a]	14
				189.2	21

Note: [a]Most abundant product ion.

compromises have to be found. Different proportions of acetonitrile/water and methanol/water were frequently used to extract mycotoxins (Krska et al. 2008). Attempts to apply these solvents to food supplements resulted in the extraction of matrix components that made further clean-up difficult. The use of ethyl acetate and dichloromethane has also been reported (Delmulle et al. 2006). In this study, the best compromise for the simultaneous extraction of the different mycotoxins from food supplements was achieved using the solvent mixture ethyl acetate/formic acid (95 : 5, v/v), which proved to be more efficient than ethyl acetate alone. The latter did not allow the extraction of F-B1, F-B2, F-B3 and OTA. Alternatively, the solvent mixture ethyl acetate/trifluoroacetic acid (97 : 3, v/v) was tested, and drastically

improved the extraction of the fumonisins (F-B1: 59%, F-B2 : 55%, F-B3 : 61%). However, this solvent mixture was not developed further, because it resulted in a dramatic decrease of the recovery for STERIG, AF-B1, and AF-G1. Moreover, the extraction of other toxins was also generally less good.

For the extract clean-up, the use of NH_2 SPE cartridges was first investigated. The results obtained showed a loss of fumonisins. Further, two adsorbents suitable for the extraction of both polar and non-polar compounds, namely Oasis HLB^{TM} and Carbograph, were tested. The best results were obtained using Oasis HLB^{TM} SPE cartridges. Considering the high amount of fatty matrix compounds that were co-extracted with the ethyl acetate-containing solvent, a *n*-hexane defatting step was necessary prior to SPE.

The performance of the extraction and clean-up procedure was evaluated by extraction yield experiments carried out by spiking analyte-free sample, before and after the extraction and clean-up step,

Table 2. Recoveries obtained for the target analytes in different food supplements at two spiking levels.

		Per cent recovery ((RSD, %), $n = 5$)					
Compounds	Level (ng g^{-1})	Maca	Soy isoflavones	Garlic	Black radish	St John's wort	Ginkgo biloba
NIV	60	39 (14)	47 (10)	38 (11)	49 (13)	37 (15)	41 (12)
	240	36 (9)	45 (8)	37 (9)	49 (10)	36 (9)	42 (11)
DON	40	65 (8)	72 (9)	66 (13)	71 (10)	68 (12)	66 (9)
	160	66 (6)	71 (6)	65 (9)	70 (8)	67 (7)	67 (7)
NEO	20	64 (8)	72 (10)	68 (10)	69 (13)	71 (11)	66 (12)
	80	62 (6)	74 (8)	67 (9)	69 (8)	70 (7)	67 (9)
F-X	50	66 (12)	73 (10)	75 (16)	72 (10)	69 (13)	71 (11)
	200	68 (11)	72 (7)	75 (8)	73 (8)	68 (9)	72 (9)
3-ADON	60	60 (9)	66 (9)	64 (13)	68 (11)	65 (10)	63 (11)
	240	62 (7)	65 (6)	64 (8)	69 (8)	64 (7)	63 (9)
15-ADON	60	70 (11)	73 (9)	73 (10)	72 (10)	69 (11)	69 (12)
	240	73 (7)	72 (6)	72 (8)	72 (8)	68 (9)	70 (9)
AF-G2	8	61 (12)	75 (11)	73 (15)	67 (13)	73 (15)	64 (12)
	32	63 (8)	76 (9)	74 (10)	65 (9)	74 (10)	65 (9)
AF-G1	8	61 (11)	76 (12)	61 (15)	68 (8)	66 (15)	69 (11)
	32	60 (9)	77 (7)	61 (9)	69 (7)	67 (11)	68 (8)
AF-B2	8	72 (13)	71 (8)	65 (16)	69 (12)	67 (14)	70 (13)
	32	73 (9)	70 (6)	65 (10)	68 (8)	68 (9)	71 (7)
AF-B1	8	70 (13)	85 (10)	74 (16)	71 (14)	74 (13)	72 (14)
	32	68 (8)	83 (8)	73 (8)	72 (10)	74 (8)	73 (10)
DAS	4	87 (10)	87 (8)	89 (9)	90 (9)	89 (10)	86 (9)
	16	86 (7)	89 (5)	88 (6)	90 (5)	90 (6)	87 (7)
ALT	24	77 (8)	81 (9)	84 (12)	76 (13)	80 (10)	78 (11)
	96	78 (6)	81 (6)	84 (8)	77 (7)	79 (8)	79 (9)
FB-1	12	35 (10)	28 (9)	35 (12)	30 (13)	35 (12)	32 (11)
	48	36 (8)	28 (6)	35 (9)	29 (7)	34 (9)	31 (8)
HT-2	12	82 (10)	91 (9)	96 (13)	93 (9)	86 (10)	84 (9)
	48	81 (7)	92 (6)	96 (8)	92 (7)	87 (7)	84 (6)
AOH	50	89 (13)	88 (10)	86 (11)	82 (15)	84 (11)	86 (13)
	200	87 (7)	87 (9)	85 (6)	83 (7)	83 (7)	86 (8)
T-2	20	94 (8)	92 (11)	91 (12)	93 (12)	94 (9)	92 (10)
	80	96 (5)	94 (8)	91 (9)	94 (7)	95 (6)	93 (7)
FB-3	12	33 (11)	34 (10)	29 (14)	32 (13)	30 (13)	32 (14)
	48	34 (8)	34 (8)	30 (11)	30 (8)	29 (10)	32 (8)
OTA	4	87 (9)	89 (10)	90 (10)	92 (11)	89 (10)	88 (9)
	16	87 (6)	90 (7)	88 (7)	92 (7)	88 (8)	89 (6)
ZEN	40	94 (8)	92 (7)	96 (8)	94 (10)	93 (11)	95 (11)
	160	94 (8)	93 (6)	95 (7)	95 (6)	94 (9)	96 (8)
FB-2	4	32 (11)	35 (15)	28 (14)	31 (12)	29 (13)	26 (12)
	16	30 (9)	33 (8)	28 (10)	30 (9)	29 (8)	27 (10)
STERIG	20	41 (20)	45 (18)	39 (21)	40 (18)	42 (20)	43 (17)
	80	44 (13)	44 (12)	35 (15)	38 (10)	41 (14)	42 (13)
AME	60	69 (12)	71 (13)	75 (14)	72 (14)	74 (12)	75 (13)
	240	71 (6)	71 (9)	77 (7)	73 (8)	74 (9)	76 (10)
BEAU	20	60 (17)	70 (15)	63 (15)	66 (17)	62 (14)	62 (15)
	80	61 (10)	69 (9)	61 (11)	65 (9)	61 (11)	63 (10)

and following the rest of the procedure. Calculations were performed by comparing peak areas for the same compound in samples spiked *ante* and *post* extraction and clean-up. Recovery data for different food supplements are shown in Table 2. For the different combinations of matrices and analytes, the recoveries were above 60%, except for NIV, STERIG and the fumonisins. Nevertheless, low recovery was not considered to be an obstacle for a reliable determination, as the other performance data such as trueness, precision, linearity and sensitivity were good.

Validation

When LC-MS was applied to the analysis of mycotoxins in food supplements, significant signal suppression was observed. Variations of signal suppression between different samples were also substantial. Addition of internal standard did not compensate for matrix effect differences between samples. Indeed, the special problem of matrix effects in LC-MS stems from the fact that the sample matrix may be subjected to the chromatographic separation, resulting in a different and in each case unknown matrix effect for each of the analytes in a multi-component analysis. Thus, one internal standard cannot compensate for these effects but a chemically similar and co-eluting compound is required for each analyte. An approach is the addition of isotopically labelled standard (Rychlik and Asam 2008). The use of these substances is useful for the correction of the signal deviation because they have the same chemical properties and the same retention times as the non-labelled substances. However, isotopically labelled internal standards were not available for all analytes. On the other hand, matrix-matched calibration could not be applied due to matrix differences between food supplement samples. Therefore, the standard addition was the only available method to perform correct quantitative analysis.

When using standard addition as quantification technique, to our knowledge, no guidelines for method validation are available. Nevertheless, it remains necessary to investigate specificity, sensitivity, linearity, trueness and precision.

The specificity of the method was improved by monitoring two MRM transitions for each target mycotoxin. Deviations of relative ion intensities for the MRM transitions were not greater than the maximum permitted tolerances (European Commission 2002). According to the European Commission, the relative ion intensities expressed as a percentage of the intensity of the most abundant ion must correspond to those of the ions in the solutions of standards, with a maximum permitted deviations of 20% (relative ion intensity $>50\%$), 25% (relative ion intensity: 21–50%), 30% (relative ion intensity: 11–20%), 50% (relative ion intensity $\leq 10\%$). In

Table 3. Trueness and standard addition calibration data.

			Standard addition calibration	
Compound	Level of spiking ($ng\,g^{-1}$)	Recovery ((RSD, %), $n=5$)	Regression equation	R^2
NIV	30	102 (19)	$y=0.0053x+0.1627$	0.989
DON	20	95 (12)	$y=0.0286x+0.5440$	0.991
NEO	10	97 (10)	$y=0.0213x+0.2064$	0.992
F-X	25	90 (14)	$y=0.0037x+0.0831$	0.990
3-ADON	30	91 (9)	$y=0.0332x+0.9084$	0.993
15-ADON	30	91 (10)	$y=0.0606x+1.6569$	0.992
AF-G2	2	94 (18)	$y=0.0087x+0.0163$	0.990
AF-G1	2	92 (19)	$y=0.0108x+0.0201$	0.990
AF-B2	2	96 (16)	$y=0.0116x+0.0111$	0.991
ALT	6	89 (8)	$y=0.0417x+0.2224$	0.994
AF-B1	2	97 (17)	$y=0.0144x+0.0282$	0.992
DAS	1	103 (10)	$y=0.0251x+0.0258$	0.997
AOH	25	88 (13)	$y=0.0298x+0.6561$	0.989
HT-2	3	93 (10)	$y=0.0060x+0.0167$	0.997
FB-1	3	96 (9)	$y=0.0657x+0.1893$	0.992
T-2	10	101 (11)	$y=0.2419x+2.4434$	0.996
FB-3	3	89 (9)	$y=0.1437x+0.3837$	0.992
OTA	1	92 (13)	$y=0.0618x+0.0569$	0.994
ZEN	20	98 (11)	$y=0.0814x+1.5953$	0.994
FB-2	1	95 (12)	$y=0.5554x+0.5276$	0.992
AME	30	85 (15)	$y=0.0094x+0.2406$	0.989
BEAU	10	109 (19)	$y=0.0022x+0.0244$	0.980
STERIG	10	106 (17)	$y=0.0094x+0.0993$	0.982

Table 4. Precision data.

	Intra-day precision				Inter-day precision			
	Low level		High level		Low level		High level	
Compound	Level ($ng\,g^{-1}$)	RSD,% ($n=6$)	Level ($ng\,g^{-1}$)	RSD,% ($n=6$)	Level ($ng\,g^{-1}$)	RSD,% ($n=18$)	Level ($ng\,g^{-1}$)	RSD,% ($n=18$)
NIV	60	14	240	8	60	16	240	10
DON	40	8	160	7	40	12	160	10
NEO	20	8	80	6	20	10	80	9
F-X	50	12	200	9	50	18	200	13
3-ADON	60	9	240	6	60	12	240	10
15-ADON	60	10	240	6	60	12	240	9
AF-G2	8	12	32	8	8	14	32	12
AF-G1	8	12	32	8	8	16	32	11
AF-B2	8	13	32	10	8	17	32	13
ALT	24	8	96	6	24	11	96	8
AF-B1	8	14	32	7	8	17	32	12
DAS	4	11	16	7	4	14	16	10
AOH	50	12	200	7	50	18	200	13
HT-2	12	11	48	8	12	17	48	12
FB-1	12	11	48	9	12	13	48	11
T-2	20	8	80	5	20	10	80	8
FB-3	12	12	48	8	12	14	48	10
OTA	4	9	16	7	4	11	16	9
ZEN	40	9	160	5	40	12	160	9
FB-2	4	12	16	9	4	14	16	11
AME	60	12	240	6	60	14	240	10
BEAU	20	17	80	14	20	21	80	17
STERIG	20	20	80	13	20	22	80	16

addition, relative retention times with regard to the internal standard were below the maximum permitted deviation of 2.5%. The trueness of the method was assessed as described above. The results are summarized in Table 3 and are in good agreement with European Commission (2002) performance criteria for quantitative methods of analysis. All trueness values are within 80–110%. The linearity data of the standard addition calibration plots are also shown in Table 3. The calibration curves obtained revealed good linearity for most of analytes, with correlation coefficients R^2 not lower than 0.98 for BEAU and STERIG and not lower than 0.99 for all the other analytes. The results of the method precision are shown in Table 4 and are in agreement with the recommended acceptable RSD values for repeatability for different analyte concentrations (European Commission Regulation 2006/401/EC). LOD and LOQ data are reported in Table 5. The LODs were in the range 0.3–10 $ng\,g^{-1}$, except for NIV (30 $ng\,g^{-1}$), F-X (25 $ng\,g^{-1}$) and AME (30 $ng\,g^{-1}$). The European Commission has established regulatory limits for mycotoxins published in Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in some foodstuffs. Commission Regulation (EC) No. 1126/2007 of 28 September 2007 amends Regulation (EC) No. 1881/2006 setting maximum levels for

Table 5. Method limits of detection and quantification.

Compound	Limit of detection ($ng\,g^{-1}$)	Limit of quantification ($ng\,g^{-1}$)
NIV	30	100
DON	6	20
NEO	3	10
F-X	25	75
3-ADON	10	30
15-ADON	10	30
AF-G2	2	6
AF-G1	2	6
AF-B2	2	6
ALT	2	6
AF-B1	2	6
DAS	0.3	1
AOH	8	25
HT-2	1	3
F-B1	1	3
F-B2	0.3	1
T-2	3	10
F-B3	1	3
OTA	0.3	1
ZEN	6	20
AME	30	100
BEAU	3	10
STERIG	3	10

Table 6. Mycotoxin contamination in food supplements.

Product type	Sample number	Origin	FB_1 ($ng\,g^{-1}$)	FB_2 ($ng\,g^{-1}$)	FB_3 ($ng\,g^{-1}$)	OTA ($ng\,g^{-1}$)
Garlic	22	Specialized shop	<1	<0.3	<1	6
Maca	29	Drugstore	<1	<0.3	<1	2.5
Garlic	36	Drugstore	10	8	3	1
Soy isoflavones	41	Drugstore	4	<1	<1	<0.3
Black radish	53	Internet	4	2	<3	<0.3
Soy isoflavones	62	Internet	<1	<0.3	<1	1

Fusarium toxins in maize and maize products. Although no limits were set for mycotoxins in food supplements, results presented in Table 5 indicated that the developed method was suitable for the detection of mycotoxins according to the existing European regulations.

Mycotoxin analysis in commercial food supplement samples

The optimized and validated method was used to assess mycotoxins in different food supplement samples. In total, 62 samples belonging to six different types of food supplements namely maca, soy isoflavones, St John's wort, garlic, ginkgo biloba and black radish were analysed. Due to different matrix effects observed in different food supplement samples, the standard addition technique was applied to perform accurate quantification.

In 56 out of 62 samples analysed, none of the 23 mycotoxins investigated was detected. Positive samples (Table 6) contained at least one of the toxins F-B1, F-B2, F-B3 and OTA. The European Commission has established maximum levels for mycotoxins in certain contaminants in foodstuffs; no regulatory limits were set for mycotoxins in food supplements. Therefore, limits for other foodstuffs were considered as an indication. In two samples, OTA was found at a level above $2\,ng\,g^{-1}$ (maximum level of OTA in wine and grape juice). The levels of F-B1, F-B2 and F-B3 were largely below $800\,ng\,g^{-1}$ (maximum level for the sum of F-B1 and F-B2 in breakfast cereals) in all positive samples.

In order to evaluate health hazard due to the intake of the contaminated food supplements, a preliminary risk assessment was performed for OTA, on the sample with the highest level of contamination, namely sample number 22 (OTA, $6\,ng\,g^{-1}$). The European Food Safety Authority (EFSA) estimated the tolerable weekly intake (TWI) at 120 ng OTA kg^{-1} body weight $week^{-1}$, which corresponds to a tolerable daily intake (TDI) of $17.14\,ng\,kg^{-1}$ body weight day^{-1}. This means less than 1200 ng OTA day^{-1} for an adult of 70 kg. Considering the recommendations provided by the supplier in terms of daily consumption (approximately 1.5 g), OTA intake from sample number 22 would be $9\,ng\,day^{-1}$, which is 0.75% of the TDI. Consequently, the risk from the occurrence of mycotoxins in the food supplements analysed in the present study is negligible. However, the presence of mycotoxins in some food supplement samples indicates the need for quality control of these products.

Conclusions

An LC-MS/MS method for the simultaneous analysis of 23 mycotoxins (NIV, DON, 3-ADON, 15-ADON, NEO, F-X, DAS, HT-2, T-2, AF-B1, AF-B2, AF-G1, AF-G2, AOH, AME, ALT, F-B1, F-B2, F-B3, OTA, ZEN, BEAU and STERIG) was optimized and validated for food supplements. The method was successfully applied to maca, soy isoflavones, St John's wort, garlic, Ginkgo biloba and black radish samples. Due to different matrix effects observed in different food supplement samples and because labelled internal standards are not available for each individual analyte, we recommend the standard addition approach for correct quantitative analysis. This study is the first report of the analysis of food supplement samples for an extended list of 23 mycotoxins, and four of these toxins were detected.

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