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► **To cite this version:**

José Diana Di Mavungu, Sofie Monbaliu, Marie-Louise Scippo, Guy Maghuin-Rogister, Yves-Jacques Schneider, et al.. LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. Food Additives and Contaminants, 2009, 26 (06), pp.885-895. 10.1080/02652030902774649 . hal-00573883

HAL Id: hal-00573883

<https://hal.science/hal-00573883>

Submitted on 5 Mar 2011

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

Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2008-393.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	20-Jan-2009
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Methods/Techniques:	Chromatography - LC/MS
Additives/Contaminants:	Mycotoxins
Food Types:	Dietary supplements



Abstract

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-B₁, aflatoxin-B₂, aflatoxin-G₁ and aflatoxin-G₂), *Alternaria* toxins (alternariol, alternariol methyl ether and altenuene), fumonisins (fumonisin-B₁, fumonisin-B₂ and fumonisin-B₃), ochratoxin A, zearalenone, beauvericin and sterigmatocystin. Optimization of the simultaneous extraction of these toxins and the sample pre-treatment 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 HLBTM 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 and 1-100 ng/g, respectively. Recovery yields were above 60 % for most of 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 analyzed none of the 23 mycotoxins investigated was detected. Positive samples contained at least one of the toxins fumonisin-B₁, fumonisin-B₂, fumonisin-B₃ 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 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 foodborne 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 years, there is a progressive increase of interest in food supplements as they are now consumed more and more. Some are used daily by consumers for various reasons. Raw materials for plant based food 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

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3 could result in the presence of mycotoxins in food supplements, leading to diverse human
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5 health problems. It is therefore necessary to have suitable analytical methods for mycotoxin
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7 determination in food supplements.
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13 Currently analytical methods used for mycotoxin analysis include thin layer chromatography
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15 (TLC) (Betina 1993; Krska *et al.* 2001), enzyme-linked immunosorbent assay (ELISA) (Ware
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17 *et al.* 1999; Thirumala-Devi *et al.* 2001; Heber *et al.* 2001), gas chromatography (GC) with
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19 electron capture (Langseth and Rundberget 1998) or mass spectrometric (Schwadorf and
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21 Müller 1992; Langseth and Rundberget 1998; Valenta 1998; Shephard 1998; Tanaka *et al.*
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23 2000; Nielsen and Thrane 2001; Soleas *et al.* 2001) detection, liquid chromatography with
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25 fluorescence detection (LC-FLD) (Valenta 1998; Shephard 1998; Krska and Josephs 2001),
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27 and liquid chromatography with tandem mass spectrometry (LC-MS/MS) (Young and
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29 Lafontaine 1993; Thakur and Smith 1994; Biselli *et al.* 2005). LC-MS/MS appears to be most
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31 promising as a highly specific, broadly applicable detection method that provides both
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33 qualitative and quantitative data. Considering the possible contamination of foodstuffs by
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35 several mycotoxin producing fungal species and the production of different types of
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37 mycotoxins by one mould, a trend is to develop methods suitable for the determination of
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39 several mycotoxins in a single run (Sewram *et al.* 1999; Monti *et al.* 2000; Rundberget and
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41 Wilkins 2002; Royer *et al.* 2004; Berthiller *et al.* 2005; Cavalière *et al.* 2005; Kokkonen *et al.*
42
43 2005; Sorensen and Elbaek 2005; Abbas *et al.* 2006; Delmulle *et al.* 2006; Monbaliu *et al.*;
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45 Sulyok *et al.* 2006; Spanjer *et al.* 2008). Currently published protocols, however, have been
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47 developed and optimized for different foods and feeds. To the best of our knoweldge, LC-
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49 MS/MS multi-mycotoxin methods for food supplements have not been published yet.
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3 The aim of this study was to develop a multi-component analytical methodology, based on
4 LC-MS/MS for the simultaneous determination of an extended list of 23 mycotoxins in food
5 supplements. Target compounds include A and B trichothecenes [nivalenol (NIV),
6 deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-
7 ADON), neosolaniol (NEO), fusarenon-X (F-X), diacetoxyscirpenol (DAS), HT-2 toxin (HT-
8 2) and T-2 toxin (T-2)], aflatoxins [aflatoxin-B₁ (AF-B1), aflatoxin-B₂ (AF-B2), aflatoxin-G₁
9 (AF-G1) and aflatoxin-G₂ (AF-G2)], *Alternaria* toxins [alternariol (AOH), alternariol methyl
10 ether (AME) and altenuene (ALT)], fumonisins [fumonisin-B₁ (F-B1), fumonisin-B₂ (F-B2)
11 and fumonisin-B₃ (F-B3)], OTA, ZEN, beauvericin (BEAU) and sterigmatocystin (STERIG).
12 The method consists of a single step of extraction for all target compounds, considerably
13 simplifying sample preparation. The multi-analyte method was evaluated in terms of
14 precision, linearity, recovery, limits of detection and quantification on maca (*Lepidium*
15 *meyenii*) food supplements. The general applicability of the LC-MS/MS conditions as well as
16 the extraction and clean-up procedure was explored with a number of food supplements such
17 as soy (*Glycine max*) isoflavones, St John's wort (*Hypericum perforatum*), garlic (*Allium*
18 *sativum*), *Ginkgo biloba* and black radish (*Raphanus niger*).
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43 **Materials and Methods**

44 **Standards**

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46 Mycotoxin standards NIV, DON, 3-ADON, 15-ADON, NEO, F-X, AF-B1, AF-B2, AF-G1,
47 AF-G2, HT-2, AOH, AME, ALT, OTA, ZEN, F-B1, F-B2, BEAU, STERIG as well as the
48 internal standard zearalanone (ZAN) were purchased from Sigma-Aldrich (Bornem,
49 Belgium). DAS and T-2 were purchased from Biopure (Tulln, Austria). F-B3 was obtained
50 from Promec Unit (Tygerberg, South Africa). NIV, NEO and DAS were obtained as
51 solutions (100 µg.mL⁻¹) in acetonitrile. From the solid standards, individual stock solutions
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3 were prepared at a concentration of $1 \text{ mg}\cdot\text{mL}^{-1}$. Stock solutions of DON, 3-ADON, 15-
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5 ADON, F-X, AF-B1, AF-B2, AF-G1, AF-G2, HT-2, T-2, ALT, OTA, ZEN, BEAU, F-B1, F-
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7 B2, STERIG and ZAN were prepared in methanol. AOH and AME stock solutions were
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9 prepared in methanol/dimethylformamide (60:40, v/v), whereas F-B2 and F-B3 stock
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11 solutions were prepared in acetonitrile/water (50:50, v/v). All stock solutions were stored in
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13 the freezer at $-18 \text{ }^{\circ}\text{C}$, except for FB-2 and FB-3, which were stored at $4 \text{ }^{\circ}\text{C}$. Spanjer *et al.*
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15 2008, reported that under these conditions, these stock solutions were stable for at least 2
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17 years. From the individual stock standard solutions, a standard mixture was prepared at the
18
19 following concentrations: NIV, 3-ADON, 15-ADON and AME ($60 \text{ ng}\cdot\mu\text{L}^{-1}$); DON and ZEN
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21 ($40 \text{ ng}\cdot\mu\text{L}^{-1}$); NEO, T-2, BEAU and STERIG ($20 \text{ ng}\cdot\mu\text{L}^{-1}$); F-X and AOH ($50 \text{ ng}\cdot\mu\text{L}^{-1}$); AF-
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23 B1, AF-B2, AF-G2 and AF-G1 ($8 \text{ ng}\cdot\mu\text{L}^{-1}$); ALT ($24 \text{ ng}\cdot\mu\text{L}^{-1}$); F-B1, F-B3 and HT-2 (12
24
25 $\text{ng}\cdot\mu\text{L}^{-1}$); DAS, OTA and F-B2 ($4 \text{ ng}\cdot\mu\text{L}^{-1}$). The standard mixture was stored at $-18 \text{ }^{\circ}\text{C}$ and
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27 renewed monthly.
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36 Reagents and materials

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38 HPLC-grade methanol and acetonitrile as well as *n*-hexane were purchased from VWR
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40 International (Zaventem, Belgium). Ethyl acetate, dichloromethane and dimethylformamide
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42 were purchased from Acros Organics (Geel, Belgium). A Milli-Q purification system
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44 (Millipore, Brussels, Belgium) was used to purify demineralized water. Hydrochloric acid,
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46 acetic acid and formic acid from Merck (Darmstadt, Germany) were used. Ammonium acetate
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48 was supplied by Grauwmeer (Leuven, Belgium). Trifluoroacetic acid was from Fluka (Buch,
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50 Zwitserland). The cartridges used for solid phase extraction (SPE) were Oasis HLBTM (200
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52 mg, 6 mL) from Waters (Milford, MA, USA). Other cartridges tested were Carbograph from
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54 LARA (Rome, Italy) and Bakerbond aminopropyl (NH_2) from Achrom (Zulte, Belgium).
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3 Ultrafree[®]-MC centrifugal filter devices (0.22 µm) from Millipore (Bedford, MA, USA) were
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5 used.
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9 10 **Samples**

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12 The 62 samples analysed consisted of different maca (*Lepidium meyenii*), soy (*Glycine max*)
13 isoflavones, St John's wort (*Hypericum perforatum*), garlic (*Allium sativum*), *Ginkgo biloba*
14 and black radish (*Raphanus niger*) based food supplements. These food supplements were
15 obtained commercially from drugstores, specialized shops or through the internet. Capsules
16 (dried products and oils) were opened and the content was released and homogenised before
17 use according to the sample preparation procedure. Tablets were ground to obtain a fine and
18 homogenised powder prior to the extraction step.
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34 Method optimization and method validation were performed using commercial food
35 supplement samples purchased from drugstore and which were tested free from mycotoxins.
36 The absence of mycotoxins was confirmed as follows: a portion of sample was analyzed as
37 such and another portion was spiked with the target analytes prior to analysis. By comparing
38 with a solution of standards, no peaks corresponding to the target analytes were found in the
39 non-spiked sample, whereas they were found in the spiked sample.
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51 **Sample preparation**

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53 One gram of sample was extracted with 25 mL ethyl acetate/formic acid (95:5, v/v) during 30
54 minutes on an Agitelec overhead shaker (J. Toulemonde & Cie, Paris, France). The sample
55 extract was centrifuged and 20 mL of the extract was evaporated until dryness. For defatting,
56 the residue was reconstituted in 5 mL of methanol/water (50:50, v/v) and 10 mL of *n*-hexane
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3 were added. The mixture was shaken and centrifuged. The aqueous fraction was transferred
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5 into a test tube using a pasteur pipette. Fresh solutions (2 times 5 mL) of methanol/water
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7 (50:50, v/v) were added to the hexane fraction and the procedure was repeated as mentioned
8
9 above. After evaporation of the combined aqueous fraction, the residue was reconstituted in
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11 400 μ L methanol/water (50:50, v/v) and centrifuged in a Ultrafree[®]-MC centrifugal device for
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13 10 min at 14000 g. Afterwards, a 250- μ L aliquot of the filtrate was diluted to 25 mL with
14
15 water. The obtained solution was further cleaned-up using Oasis HLB[™] SPE cartridges.
16
17 Firstly, the SPE cartridges were conditioned with 10 mL of dichloromethane/methanol (80:20,
18
19 v/v) containing 50 mM formic acid, followed by 5 mL methanol, 20 mL acidified water (10
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21 mM hydrochloric acid in water) and finally 10 mL water. After the conditioning step, the
22
23 sample extract was quantitatively brought onto the SPE cartridge, which was then washed by
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25 passing 10 mL of water. Elution of mycotoxins was performed by passing consecutively 1 mL
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27 methanol and 4 mL dichloromethane/methanol (80:20, v/v) containing 50 mM formic acid.
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29 The eluate was evaporated under a gentle stream of nitrogen and reconstituted with 100 μ L of
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31 injection solvent, which consisted of methanol/water/acetic acid (57.2:41.8:1, v/v/v)
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33 containing 5 mM ammonium acetate. The resulting solution was centrifuged in a Ultrafree[®]-
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35 MC centrifugal device for 10 min at 14000 g prior to LC-MS/MS analysis.
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45 **LC-MS/MS analysis**

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48 The LC-MS/MS system consisted of a Waters Acquity UPLC apparatus coupled to a
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50 Micromass Quattro Micro triple quadrupole mass spectrometer (Waters, Milford, MA, USA)
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52 equipped with a Z-spray electrospray ionization (ESI) interface. The analytical conditions
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54 were as previously described (Monbaliu *et al.* 2009) and are summarized beneath.
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57 Chromatographic separation was achieved using a Symmetry C₁₈ column (5 μ m, 150 x 2.1
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59 mm i.d.) with a Sentry guard column (3.5 μ m, 10 x 2.1 mm i.d.) both supplied by Waters
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3 (Zellik, Belgium). The column was kept at room temperature. A mobile phase consisting of
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5 eluents A [water/methanol/acetic acid (94:5:1, v/v/v) containing 5 mM ammonium acetate]
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7 and B [methanol/water/acetic acid (97:2:1, v/v/v) containing 5 mM ammonium acetate] was
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9 used at a flow rate of 0.3 mL.min⁻¹. A gradient elution was applied as follows: 0–7 min, 95%
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11 A / 5% B – 35% A / 65% B; 7–11 min, 35% A / 65% B – 25% A / 75%B; 11–13 min, 25% A
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13 / 75% B – 0% A / 100% B; 13–15 min, 0% A / 100% B; 15–16 min, 0% A / 100% B – 40% A
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15 / 60% B; 16–22 min, 40% A / 60% B – 60% A / 40% B; 22–23 min, 60% A / 40% B – 95% A
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17 / 5% B; 23–25 min 95% A / 5% B. The injection volume was 20 µL.
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23 The mass spectrometer was operated in the positive electrospray ionization (ESI+) mode. MS
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25 parameters for the analysis were as follows: ESI source block and desolvation temperatures:
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27 150 °C and 350 °C, respectively; capillary voltage: 3.2 kV; argon collision gas: 4.12 x 10⁻³
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29 mbar; cone nitrogen and desolvation gas flows: 20 and 500 L.h⁻¹, respectively. In order to
30
31 identify the target mycotoxins in the food supplement samples, a diagnostic MS “fingerprint”
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33 was built up firstly based on the MS spectra of solutions of standards. After selection of the
34
35 precursor ions for each analyte, product ions were obtained with a combination of cone
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37 voltages and collision energies, parameters that were previously optimized. For increased
38
39 sensitivity and selectivity, data acquisition was performed working in multiple reaction
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41 monitoring (MRM) mode. For part of the mycotoxins investigated, more than two MRM-
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43 transitions were initially monitored. The two transitions that resulted in higher sensitivity and
44
45 better selectivity were selected in the final method. MRM transitions, the optimum cone
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47 voltages and collision energies selected for each transition are given in Table 1, as well as the
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49 indicative retention times on the column. The first transition, which corresponds to the most
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51 abundant product ion was used for quantification, and the second one for confirmation
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53 purposes. Masslynx and Quanlynx software (Micromass, Manchester, UK) was used for data
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55 acquisition and processing.
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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 2, 3 and 4-folds the initial level, which was previously assessed by external standard. All four fractions were submitted to the sample preparation procedure as described under section 2.4, 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 analyzed 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 analyzed 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 3 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

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3 quantification (LOQs) were determined from spiked blank samples, as the minimum
4 detectable amount of analyte with a signal-to-noise ratio of 3 and 10, respectively. In detail,
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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, 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),

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3 which proved to be more efficient than ethyl acetate alone. The latter did not allow the
4 extraction of F-B1, F-B2, F-B3 and OTA. Alternatively, the solvent mixture ethyl acetate/
5 trifluoroacetic acid (97:3, v/v) was tested, and drastically improved the extraction of the
6 fumonisins (F-B1: 59 %, F-B2: 55 %, F-B3: 61 %). However, this solvent mixture was not
7 developed further, because it resulted in a dramatic decrease of the recovery for STERIG, AF-
8 B1, and AF-G1. Moreover, the extraction of other toxins was also generally less good.
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21 For the extract clean-up, the use of NH₂ SPE cartridges was first investigated. The results
22 obtained showed a loss of fumonisins. Further, two adsorbents suitable for the extraction of
23 both polar and non-polar compounds, namely Oasis HLBTM and Carbograph, were tested. The
24 best results were obtained using Oasis HLBTM SPE cartridges. Considering the high amount
25 of fatty matrix compounds that were co-extracted with the ethyl acetate-containing solvent, a
26 *n*-hexane defatting step was necessary prior to SPE.
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40 The performance of the extraction and clean-up procedure was evaluated by extraction yield
41 experiments carried out by spiking analyte-free sample, before and after the extraction and
42 clean-up step, and following the rest of the procedure. Calculations were performed by
43 comparing peak areas for the same compound in samples spiked *ante* and *post* extraction and
44 clean-up. Recovery data for different food supplements are shown in Table 2. For the
45 different combinations of matrices and analytes, the recoveries were above 60 %, except for
46 NIV, STERIG and the fumonisins. Nevertheless, low recovery was not considered to be an
47 obstacle for a reliable determination, as the other performance data such as trueness,
48 precision, linearity and sensitivity were good.
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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 (Commission Decision 657/2002). According

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3 to the European Commission Decision 657/2002, the relative ion intensities expressed as a
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5 percentage of the intensity of the most abundant ion must correspond to those of the ions in
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7 the solutions of standards, with a maximum permitted deviations of 20% (relative ion
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9 intensity > 50%), 25% (relative ion intensity: 21-50%), 30% (relative ion intensity: 11-20%),
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11 50% (relative ion intensity \leq 10%). In addition, relative retention times with regard to the
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13 internal standard were below the maximum permitted deviation of 2.5 %. The trueness of the
14
15 method was assessed as described above. The results are summarized in Table 3 and are in
16
17 good agreement with the Commission Decision 657/2002 performance criteria for
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19 quantitative methods of analysis. All trueness values are within 80-110 %. The linearity data
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21 of the standard addition calibration plots are also shown in Table 3. The calibration curves
22
23 obtained revealed good linearity for most of analytes, with correlation coefficients R^2 not
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25 lower than 0.98 for BEAU and STERIG and not lower than 0.99 for all the other analytes.
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27 The results of the method precision are shown in Table 4 and are in agreement with the
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29 recommended acceptable RSD values for repeatability for different analyte concentrations
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31 (European Commission Regulation 2006/401/EC). LOD and LOQ data are reported in Table
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33 5. The LODs were in the range 0.3 -10 ng/g, except for NIV (30 ng/g), F-X (25 ng/g) and
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35 AME (30 ng/g). The European Commission has established regulatory limits for mycotoxins
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37 published in Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting
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39 maximum levels for certain contaminants in some foodstuffs. Commission Regulation (EC)
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41 No 1126/2007 of 28 September 2007 amends Regulation (EC) No 1881/2006 setting
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43 maximum levels for *Fusarium* toxins in maize and maize products. Although no limits were
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45 set for mycotoxins in food supplements, results presented in Table 5 indicated that the
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47 developed method was suitable for the detection of mycotoxins according to the existing
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49 European regulations.
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Mycotoxin analysis in commercial food supplement samples

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3 The optimized and validated method was used to assess mycotoxins in different food
4 supplement samples. In total, 62 samples belonging to 6 different types of food supplements
5 namely maca, soy isoflavones, St John's wort, garlic, ginkgo biloba and black radish were
6 analyzed. Due to different matrix effects observed in different food supplement samples, the
7 standard addition technique was applied to perform accurate quantification.
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18 In 56 out of 62 samples analyzed, none of the 23 mycotoxins investigated was detected.
19 Positive samples (Table 6) contained at least one of the toxins F-B1, F-B2, F-B3 and OTA.
20 The European Commission has established maximum levels for mycotoxins in certain
21 contaminants in foodstuffs; no regulatory limits were set for mycotoxins in food supplements.
22 Therefore, limits for other foodstuffs were considered as an indication. In 2 samples, OTA
23 was found at a level above 2 ng/g (maximum level of OTA in wine and grape juice). The
24 levels of F-B1, F-B2 and F-B3 were largely below 800 ng/g (maximum level for the sum of
25 F-B1 and F-B2 in breakfast cereals) in all positive samples.
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41 In order to evaluate health hazard due to the intake of the contaminated food supplements, a
42 preliminary risk assessment was performed for OTA, on the sample with the highest level of
43 contamination, namely sample N° 22 (OTA, 6 ng/g). The European Food Safety Authority
44 (EFSA) estimated the Tolerable Weekly Intake (TWI) at 120 ng OTA/kg b.w. per week,
45 which corresponds to a Tolerable Daily Intake (TDI) of 17.14 ng/kg b.w. per day. This means
46 less than 1200 ng OTA per day for an adult of 70 kg. Considering the recommendations
47 provided by the supplier in terms of daily consumption (~1.5 g), OTA intake from sample N°
48 22 would be 9 ng per day, which is 0.75 % of the TDI. Consequently, the risk from the
49 occurrence of mycotoxins in the food supplements analyzed in the present study is negligible.
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3 However, the presence of mycotoxins in some food supplement samples indicates the need for
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5 quality control of these products.
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8 9 **Conclusions**

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11 An LC-MS/MS method for the simultaneous analysis of 23 mycotoxins (NIV, DON, 3-
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13 ADON, 15-ADON, NEO, F-X, DAS, HT-2, T-2, AF-B1, AF-B2, AF-G1, AF-G2, AOH,
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15 AME, ALT, F-B1, F-B2, F-B3, OTA, ZEN, BEAU and STERIG) was optimized and
16
17 validated for food supplements. The method was successfully applied to maca, soy
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19 isoflavones, St John's wort, garlic, Ginkgo biloba and black radish samples. Due to different
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21 matrix effects observed in different food supplement samples and because labelled internal
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23 standards are not available for each individual analyte, we recommend the standard addition
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25 approach for correct quantitative analysis. This study is the first report of analysis of food
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27 supplement samples for an extended list of 23 mycotoxins, and 4 of these toxins were
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29 detected.
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37 38 **Acknowledgment**

39
40 The authors thank the Belgian Federal Science Policy Office (BELSPO) for financial support
41
42 (FOODINTER SD/AF/04A).
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Table 1 MS/MS parameters for the analysis of target analytes by MRM ESI positive ionization mode

Target compounds	Retention time (min)	Precursor ion (<i>m/z</i>)	Cone voltage (V)	Product ions (<i>m/z</i>)	Collision energy (eV)
NIV	3.18	313.0 [M+H] ⁺	26	124.9 *	12
				177.2	13
DON	4.29	297.0 [M+H] ⁺	23	203.3 *	10
				249.5	15
NEO	5.39	400.1 [M+NH ₄] ⁺	20	305.3 *	13
				365.1	10
F-X	5.26	355.1 [M+H] ⁺	22	175.3 *	11
				247.3	9
3-ADON	6.47	339.2 [M+H] ⁺	23	231.2 *	10
				203.2	10
15-ADON	6.49	339.1 [M+H] ⁺	24	137.1 *	10
				321.2	8
AF-G2	6.99	331.0 [M+H] ⁺	46	313.1 *	24
				245.3	30
AF-G1	7.31	328.8 [M+H] ⁺	43	311.2 *	25
				243.4	20
AF-B2	7.68	315.0 [M+H] ⁺	50	287.3 *	25
				259.4	29
AF-B1	7.98	313.0 [M+H] ⁺	47	285.3 *	21
				241.4	34
DAS	7.97	384.2 [M+NH ₄] ⁺	19	307.3 *	11
				247.3	15
ALT	8.09	293.1 [M+H] ⁺	24	257.2 *	14
				275.2	10
FB-1	9.56	722.5 [M+H] ⁺	51	334.4 *	37
				352.3	36
HT-2	9.26	441.9 [M+NH ₄] ⁺	16	263.2 *	13
				215.3	12
AOH	10.02	259.1 [M+H] ⁺	53	185.2 *	30
				213.1	25
T-2	10.17	484.3 [M+NH ₄] ⁺	12	305.2 *	12
				245.2	12
FB-3	10.89	706.4 [M+H] ⁺	51	336.3 *	35
				354.0	29
OTA	11.27	404.0 [M+H] ⁺	24	239.1 *	22
				358.1	14
ZEN	11.66	319.2 [M+H] ⁺	25	185.4 *	25
				283.3	13
FB-2	12.30	706.4 [M+H] ⁺	51	336.3 *	35
				318.0	38
STERIG	11.73	325.0 [M+H] ⁺	44	310.2 *	35
				281.3	25
AME	12.75	273.0 [M+H] ⁺	54	199.2 *	26
				258.1	25
BEAU	13.13	801.3 [M+NH ₄] ⁺	32	244.3 *	30
				262.4	30
ZAN	11.36	321.2 [M+H] ⁺	27	303.2 *	14
				189.2	21

* Most abundant product ion

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

Compounds	Level (ng/g)	Percent recovery ((RSD %), <i>n</i> = 5)					
		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)

Table 3 Trueness and standard addition calibration data

Compound	Level of spiking (ng/g)	Recovery ((RSD %), <i>n</i> = 5)	Standard addition calibration	
			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

Compound	Intra-day precision				Inter-day precision			
	Low level		High level		Low level		High level	
	Level (ng/g)	RSD, % (n=6)	Level (ng/g)	RSD, % (n=6)	Level (ng/g)	RSD, % (n=18)	Level (ng/g)	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

Table 5 Method limits of detection and quantification

Compound	LOD (ng/g)	LOQ (ng/g)
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 N ^o	Origin	FB1 (ng/g)	FB2 (ng/g)	FB3 (ng/g)	OTA (ng/g)
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

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