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Determination of Regulated and Emerging Mycotoxins in Cereals, Nuts, Figs, and Animal Feeds Using Pass-Through SPE and UPLC-MS/MS

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Abstract

Mycotoxins can occur naturally in a variety of food products via pre- and post-harvest contamination mechanisms and are regulated worldwide. Herein we describe a multi-toxin method for 31 regulated and emerging mycotoxins using a quick and simple pass-through SPE clean-up prior to LC-MS/MS analysis for cereal-based products, ground- and tree-nuts, dried figs, and animal feeds.

The method has been developed to maximize recovery and enhance easy-of-use. The performance of the method has been validated using spiked samples and verified using reference materials, where available.

The method provides very good performance in terms of trueness and repeatability, with method LOQs as low as 0.25 µg/kg for aflatoxins and 1.0 µg/kg for ochratoxin A.

The use of Oasis PRiME HLB SPE clean-up is particularly advantageous as it involves a simple and quick pass-through protocol, thus eliminating the SPE conditioning, equilibration, and washing steps. Recoveries were excellent for all analytes (in the range 60–108%) which proves the wide scope of the clean-up protocol. This is important as it allows to expand the method to a wider range of compounds if needed.

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Benefits

· Single multi-mycotoxin method for a wide range of complex matrices

· Removal of major co-extractives using pass-through SPE clean-up

· Good method performance fulfilling regulatory requirements for all tested mycotoxins

· Possibility to scale-up the method to a wider number of analytes and different food and feed matrices

Introduction

Mycotoxins are secondary metabolites of fungi poisonous for humans or animals which can be found on a great variety of food and feed commodities. The most common mycotoxins are regulated in many countries of the world after thorough risk assessment, considering toxicity, occurrence, and consumption data as well as economic and political considerations. Regulations typically include values that are specified in various food and feed commodities, which are either maximum allowed levels or guidance values. The applied regulatory levels and standards of mycotoxins vary in the different regions of the world.[1] The maximum levels for mycotoxins in food are very low due to their severe toxicity. Furthermore, the legislation frameworks are expected to evolve in the following years; maximum levels will be updated and more emerging mycotoxins, including deoxynivalenol modified forms, enniatins, and Alternaria toxins, are expected to be included into future legislation.

In the present work, we describe the performance of a simple and quick sample preparation protocol and LC-MS/MS analytical method for 31 regulated and emerging mycotoxins in three different food types (cereals, nuts, figs) and animal feeds. The Oasis PRiME HLB is a pass-through solid-phase extraction (SPE) cartridge, which was applied to remove some major interferences, hence improving method robustness.[2] The Hydrophilic-Lipophilic Balance copolymer (HLB) does not require any solvation, equilibration or conditioning step, thus saving time and solvent expenses.

Experimental

Sample Description and Pre-treatment

Wheat flour, peanuts, figs, and animal feeds (mix of pet feeds) were purchased at a local market. About 200 g of each food commodity were ground and homogenized using an analytical mill (IKA) prior to extraction. Reference

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materials, including maize flour, hazelnuts (water/nuts slurry), dried figs (water/fruit slurry), and animal feeds (cattle) were purchased from Fera Science Ltd (FAPAS).

Sample Extraction and Clean-up

The sample preparation protocol was consistent to all matrices and is illustrated in Figure 1.

Homogenized sample + extraction solution

Mix on automatic vortex for 10 min and centrifuge

Supernatant passed through Oasis PRiME HLB in two steps

Discards the first portion and collect the second portion

Dilute the purified extract 1:5 with water

Inject onto LC-MS/MS

Xevo **TQ-S micro**

Oasis PRiME
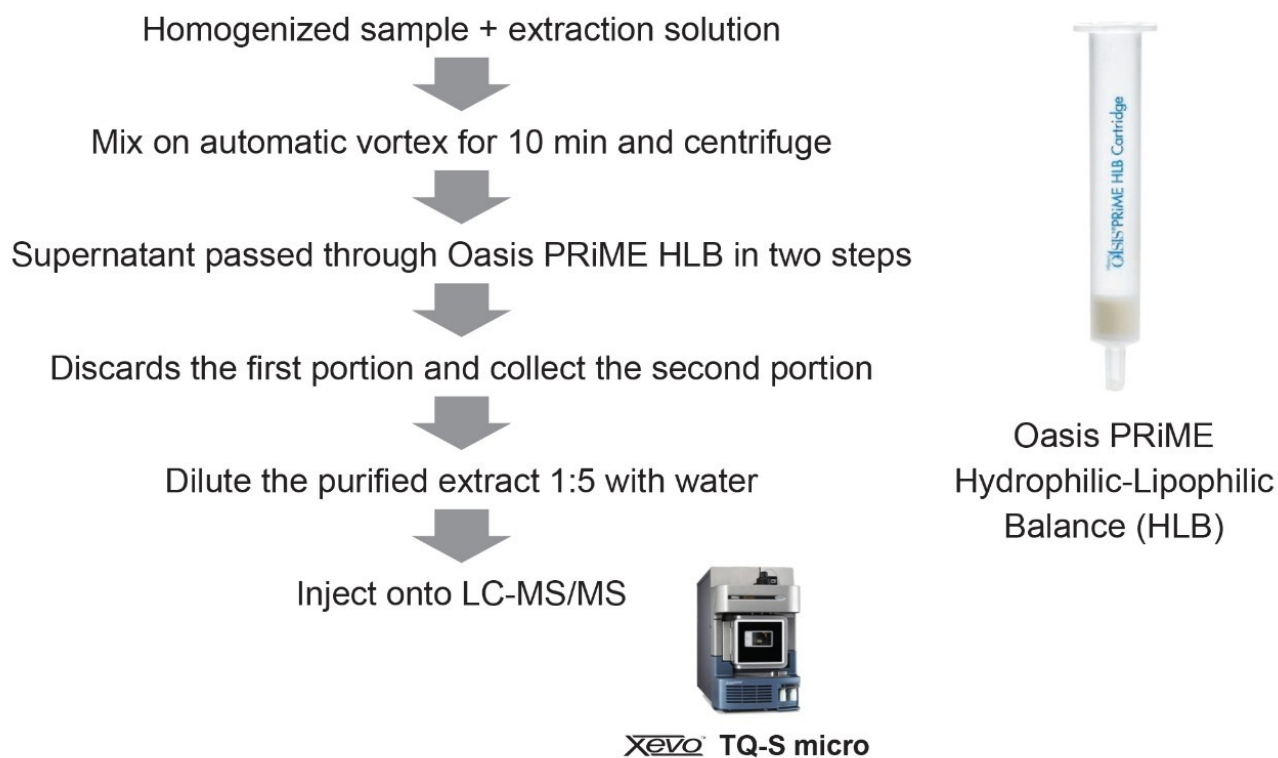Hydrophilic-Lipophilic
Balance (HLB)

Figure 1. Scheme of the sample preparation protocol.

A portion of homogenized representative sample (5 g) was placed in a 50-mL plastic centrifuge tube. The sample was extracted with 20 mL of 80:20 MeCN:H_2O containing 0.5% of acetic acid and 0.2% of formic acid (v/v) for 10 min on vortex. After centrifugation at >5000 g, a first portion of the supernatant (0.4 mL) was loaded onto the Oasis PRiME HLB cartridge (3 cc, 150 mg, p/n 186008717 < https://www.waters.com/nextgen/us/en/shop/sample-preparation--filtration/186008717-oasis-prime-hlb-3-cc-vac-cartridge-150-mg-sorbent-per-cartridge-.html>) and discarded by drying the cartridge bed (*i.e.* pumping 3 mL of air through the cartridge). A second portion of the supernatant (1.2 mL) was loaded and collected. The purified extract was then diluted 1:5 with water directly into a LC vial for LC-MS/MS analysis (*i.e.* 200 µL of purified extract were mixed with 800 µL of H_2O), resulting in an overall dilution factor of 20. After dilution, extracts were filtered through 13 mm I.D., 1.2 µm glass fiber syringe filter. Matrix-matched standards were used

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for quantification.

LC-MS/MS Conditions

Chromatographic system:	ACQUITY UPLC I-Class PLUS System
Autosampler and injector:	Flow-Through Needle injector (FTN) with 15-µL needle size and 50-µL extension loop (p/n: 430002012) installed between port 4 of injection valve and APH
Column:	ACQUITY UPLC BEH C$_{18}$ (2.1 × 100 mm, 1.7 µm particle size, 130 Å pore size, p/n: 186002352)
Aqueous mobile phase:	1 mM ammonium acetate in water +0.5% acetic acid +0.1% formic acid (v/v)
Organic mobile phase:	methanol + 0.5% acetic acid + 0.1% formic acid
Needle wash solvent:	Water:methanol:acetonitrile:isopropanol:acetone 20:20:20:20:20 + 1% formic acid (volumetrically)
Seal wash solvent:	Water:acetonitrile 80:20 (v/v)
Column temperature:	40 °C
Sample temperature:	15 °C
Injection volume:	8 µL
Flow rate:	0.40 mL/min

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Time (min)	% organic	% aqueous	Curve
0	5	95	–
0.7	5	95	6
6.5	50	50	6
9.5	100	0	6
12.5	100	0	6
12.6	5	95	6
14	5	95	6

Table 1. UPLC gradient.

Mass spectrometry system:	Xevo TQ-S micro
Ionization mode:	ESI+/- (polarity switching)
Acquisition mode:	Multiple Reaction Monitoring (MRM)
Capillary voltage:	+0.75/-0.3 kV
Cone gas flow:	50 L/Hr
Desolvation temperature:	600 °C
Desolvation gas flow:	1100 L/Hr
Source temperature:	150 °C
Resolution:	MS1 Unit, MS2 Unit
Software:	MassLynx v4.2 (TargetLynx XS was used for data processing)

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Analyte	RT window* (min)	Adduct	Precursor m/z	Product m/z	Cone voltage (V)	Collision energy (eV)
AFB$_1$	7.05–7.30	$[M+H]^+$	313.0	**285.0** 241.0	30	20 37
AFB$_2$	6.73–7.01	$[M+H]^+$	315.1	**287.0** 259.0	30	25 28
AFG$_1$	6.43–6.70	$[M+H]^+$	329.1	**243.0** 311.0	25	24 20
AFG$_2$	6.09–6.33	$[M+H]^+$	331.1	**313.2** 245.1	25	25 30
FB$_1$	8.07–8.55	$[M+H]^+$	722.3	**334.3** 352.3	30	40 35
FB$_2$	8.55–9.06	$[M+H]^+$	706.4	**336.4** 318.4	30	36 37
FB$_3$	8.35–8.70	$[M+H]^+$	706.4	**336.4** 94.8	10	40 60
OTA	8.80–9.08	$[M+H]^+$	404.1	**239.0** 221.0	20	25 36
ZEA	8.79–9.00	$[M+H]^+$	319.2	**185.1** 187.1	20	25 19
DON	3.33–3.59	$[M+H]^+$	297.1	**249.1** 231.1	15	10 10
DON-3-Glu	3.45–3.81	$[M-Glu+H]^+$	297.2	**249.1** 231.1	20	12 12
NIV	2.00–3.25	$[M+CH3COO]^-$	371.1	**281.0** 311.0	10	10 10
HT-2	8.17–8.42	$[M+NH4]^+$	442.3	**263.1** 215.1	20	10 10
T-2	8.55–8.76	$[M+NH4]^+$	484.2	**305.1** 215.1	20	15 22
3-Ac-DON	5.40–5.85	$[M+H]^+$	339.2	**136.8** 321.0	10	13 12
15-Ac-DON	5.40–5.85	$[M+H]^+$	339.2	**212.8** 231.1	10	18 8
DAS	7.25–7.59	$[M+NH4]^+$	384.2	**307.2** 247.0	7	10 13
NEO	4.75–5.00	$[M+NH4]^+$	400.2	**305.1** 184.9	10	12 20
FUS-X	4.35–4.62	$[M+H]^+$	355.2	**247.0** 229.0	10	15 17
Deepoxy-DON	4.43–4.67	$[M+H]^+$	281.0	**108.5** 232.9	10	16 10
Citrinin	7.95–8.47	$[M+H]^+$	251.0	**233.0** 205.0	16	17 25
Sterigmatocystin	8.89–9.22	$[M+H]^+$	325.0	**310.0** 281.0	10	25 37
Beauvericin	9.75–10.00	$[M+H]^+$	784.5	**244.1** 262.2	80	27 25
Enniatin A	9.92–10.12	$[M+NH4]^+$	699.5	**210.0** 100.0	10	32 70
Enniatin B	9.66–9.90	$[M+NH4]^+$	657.5	**196.0** 214.0	50	30 30
Enniatin A$_1$	9.84–10.07	$[M+NH4]^+$	685.5	**210.0** 228.0	10	35 35
Enniatin B$_1$	9.77–10.00	$[M+NH4]^+$	671.5	**196.0** 210.0	10	37 35
AOH-Me-Ether	8.97–9.25	$[M-H]^-$	271.0	**256.0** 228.0	20	22 30
Tentoxin	8.23–8.55	$[M+H]^+$	415.3	**57.5** 312.1	10	30 20
Tenuazonic acid	7.15–7.65	$[M+H]^+$	198.0	**124.7** 152.8	10	15 15
Altenuene	6.96–7.25	$[M+H]^+$	293.0	**257.0** 275	10	13 11

(*) Retention Time (RT) is subject to variations. Make a test injection and correct acquisition time for each MRM channel minimising overlapping, thus increasing auto dwell time (peak width = 6 seconds, #points per peak = 15).

Dwell time = Auto.

Span = 0 Da for all functions.

Table 2. Optimized MRM transitions.

Method Validation

Validation of the trueness (using recovery) and repeatability of the method was performed by spiking wheat flour,

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peanuts, figs, and animal feeds samples with a mixture of 31 mycotoxins at three concentration levels, in triplicate (concentrations are reported in Tables 4, 5, 6, and 7). Blanks and spiked samples were then extracted and analyzed as described in the previous section. According to Eurachem guidelines,[3] the performance of the method was also verified by analysing six replicates of some FAPAS reference materials: maize flour, hazelnut, dried figs, and animal feed.

Results and Discussion

Chromatography

Chromatographic performance was in agreement with SANTE/12089/2016 guidelines.[4] The first eluting compound (nivalenol) presented a retention time of 2.38 min, which resulted to be higher than twice the retention time corresponding to the void volume of the column, V_d (V_d ~0.55 min). A representative chromatogram of a wheat extract spiked with 31 mycotoxins is shown in Figure 2. The retention time of the analyte in the sample extract corresponded to that of the average of the calibration standards measured in the same sequence with a tolerance of ±0.05 min, in accordance with SANTE guidelines. The reproducibility of RT was greater than ±0.03 min within each sequence.

Critical pairs of compounds were:

-      Deoxynivalenol (DON) and deoxynivalenol-3-Glucoside (DON-3-Glu): these compounds shared the same MRM transitions as DON-3-Glu loses the glycosyl group in the ion source and it is monitored as [DON+H]$^+$. Baseline separation was achieved for these compounds in the present method.

-      3-Acetyl-deoxynivalenol (3-Ac-DON) and 15-acetyl-deoxynivalenol (15-Ac-DON): the isomers are usually monitored and quantified as the sum 3-Ac-DON + 15-Ac-DON as they usually partially coelute in the reverse-phase LC dimension. It is possible to separate these compounds by using a ACQUITY UPLC HSS T3 Column (2.1 x 150 mm, 1.8 µm, p/n 186003540 <https://www.waters.com/nextgen/us/en/shop/columns/186003540-acquity-uplc-hss-t3-column-100a-18--m-21-mm-x-150-mm-1-pk.html>) or by using a ACQUITY UPLC HSS PFP Column, (2.1 x 100 mm, 1.8 µm, p/n 186005967 <https://www.waters.com/nextgen/us/en/shop/columns/186005967-acquity-uplc-hss-pfp-fluoro-phenyl-column-100a-18--m-21-mm-x-100.html>). For more information on the separation of acetyl-DON isomer, contact Waters.
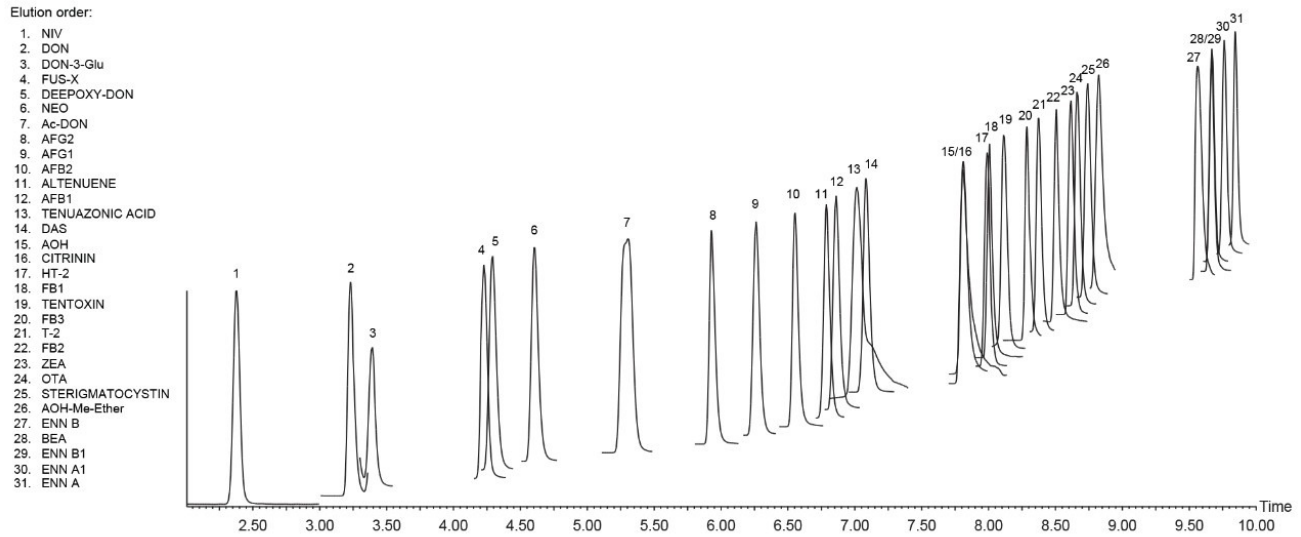

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Figure 2. Elution order and representative chromatogram of the tested mycotoxins (matrix-matched standard on wheat extract).

Linearity, Identification and Limits of Quantitation

The linearity of the method was verified across the range of concentrations tested. Coefficients of determination of the calibration curves (R^2) were >0.9900, and residuals were below ±20%. The ion ratios of all tested samples were within ±30% to the average ion ratios calculated on the calibration standards from the same sequence. Table 3 reports the instrument limit of quantitation (i-LOQ) calculated on solvent standards (signal-to-noise ratio ≥10 at the LOQ level). Table 4, 5, 6, and 7 show the method LOQ (m-LOQ)[3] and linear range in wheat flour, peanuts, figs, and animal feeds, respectively. The linear range of the method allowed for the determination of mycotoxins at typical regulatory levels for the food commodities tested.

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Analyte	i-LOQ (ng/mL)	Ion ratio	Retention time (min)
AFB$_1$	0.006	0.930	6.86
AFB$_2$	0.006	0.900	6.55
AFG$_1$	0.006	0.660	6.26
AFG$_2$	0.006	0.780	5.93
FB$_1$	0.25	0.905	8.01
FB$_2$	0.4	0.520	8.51
FB$_3$	0.4	0.700	8.28
OTA	0.025	0.380	8.66
ZEA	0.2	1.200	8.62
DON	0.15	0.383	3.22
DON-3-Glu	0.3	0.480	3.39
NIV	8.4	1.278	2.38
HT-2	0.25*	0.850	7.99
T-2	0.075	0.700	8.38
3-Ac-DON	0.15	1.020	5.34
15-Ac-DON	0.15	0.730	5.27
DAS	0.15	0.615	7.08
NEO	0.15	0.900	4.60
FUS-X	0.15	0.890	4.21
Deepoxy-DON	0.3	0.665	4.28
Citrinin	0.3	0.075	7.82
Sterigmatocystin	0.04	0.890	8.74
Beauvericin	0.07	0.610	9.67
Enniatin A	0.075	0.082	9.85
Enniatin B	0.075	0.575	9.56
Enniatin A$_1$	0.075	0.465	9.76
Enniatin B$_1$	0.075	0.720	9.67
AOH-Me-Ether	0.15	0.192	8.82
Tentoxin	0.15	0.270	8.12
Tenuazonic acid	0.9	0.552	7.02
Altenuene	0.15	0.362	6.79

Table 3. Instrument limit of quantification (i-LOQ) on the Xevo TQ-S micro, ion ratios and retention times.

Trueness and Repeatability

Recoveries were between 60% and 108% and the relative standard deviations under repeatability conditions

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(%RSD$_r$) were below 11% in all the commodity types tested, and comply with recommended values in regulations [5] (see Table 4, 5, 6, and 7). Mean recoveries are also illustrated in Figures 3, 4, 5, and 6.

Analyte	m-LOQ (µg/kg)	Method linear range (µg/kg)	Spiking level #1		Spiking level #2		Spiking level #3	
			Spiked conc (µg/kg)	Rec% (±RSD$_r$%)	Spiked conc (µg/kg)	Rec% (±RSD$_r$%)	Spiked conc (µg/kg)	Rec% (±RSD$_r$%)
AFB$_1$	0.25	0.25–100	0.5	94 (±7)	1.5	102 (±6)	10	104 (±2)
AFB$_2$	0.25	0.25–100	0.5	105 (±3)	1.5	101 (±2)	10	105 (±1)
AFG$_1$	0.25	0.25–100	0.5	100 (±3)	1.5	105 (±2)	10	104 (±3)
AFG$_2$	0.25	0.25–100	0.5	98 (±9)	1.5	100 (±3)	10	104 (±2)
FB$_1$	10	10–1000	10	108 (±2)	30	95 (±10)	200	108 (±2)
FB$_2$	10	10–1000	10	107 (±2)	30	96 (±3)	200	103 (±2)
FB$_3$	10	10–1000	10	108 (±1)	30	104 (±4)	200	108 (±1)
OTA	1	1–400	2	102 (±3)	6	100 (±3)	40	105 (±0.4)
ZEA	10	10–6000	30	104 (±1)	90	102 (±5)	600	107 (±3)
DON	6	6–2400	12	84 (±6)	36	101 (±2)	240	108 (±2)
DON-3-Glu	10	10–2400	12	90 (±10)	36	91 (±10)	240	105 (±2)
NIV	200	200–40000	200	104 (±5)	600	99 (±10)	4000	103 (±3)
HT-2	5	5–1200	6	105 (±2)	18	101 (±9)	120	105 (±2)
T-2	3	3–1200	6	104 (±1)	18	102 (±5)	120	107 (±3)
3-Ac-DON	6	6–2400	12	98 (±5)	36	99 (±3)	480	99 (±2)
15-Ac-DON	6	6–2400	12	97 (±6)	36	99 (±5)	480	99 (±6)
DAS	6	6–2400	12	80 (±4)	36	108 (±4)	240	108 (±4)
NEO	6	6–2400	12	102 (±9)	36	99 (±4)	240	105 (±2)
FUS-X	6	6–2400	12	90 (±9)	36	104 (±7)	240	102 (±8)
Deepoxy-DON	6	6–1200	12	101 (±3)	36	108 (±5)	240	107 (±2)
Citrinin	6	6–2400	12	88 (±4)	36	70 (±1)	240	63 (±5)
Sterigmatocystin	2	2–1200	6	98 (±3)	18	94 (±2)	120	93 (±3)
Beauvericin	2	2–2400	12	71 (±2)	36	90 (±1)	240	94 (±2)
Enniatin A	3	3–1200	12	86 (±2)	36	87 (±3)	240	95 (±4)
Enniatin B	3	3–1200	12	88 (±2)	36	98 (±2)	240	102 (±2)
Enniatin A$_1$	3	3–1200	12	85 (±3)	36	95 (±2)	240	95 (±4)
Enniatin B$_1$	3	3–1200	12	79 (±3)	36	93 (±2)	240	99 (±3)
AOH-Me-Ether	6	6–2400	12	70 (±9)	36	65 (±2)	240	61 (±4)
Tentoxin	3	3–2400	12	104 (±2)	36	107 (±2)	240	106 (±1)
Tenuazonic acid	15	15–5000	12	107 (±8)	36	105 (±1)	240	103 (±3)
Altenuene	6	6–2400	12	100 (±3)	36	98 (±5)	240	95 (±3)

Table 4. Method LOQ (m-LOQ), method linear range and percentage recovery (Rec%) ±percentage relative standard deviation (%RSD$_r$, n = 3) of the tested mycotoxins at three spiking levels in wheat flour.

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Analyte	m-LOQ (µg/kg)	Method linear range (µg/kg)	Spiking level #1		Spiking level #2		Spiking level #3	
			Spiked conc (µg/kg)	Rec% (±RSD$_r$%)	Spiked conc (µg/kg)	Rec% (±RSD$_r$%)	Spiked conc (µg/kg)	Rec% (±RSD$_r$%)
AFB$_1$	0.25	0.25–100	0.5	96 (±4)	1.5	103 (±2)	10	102 (±1)
AFB$_2$	0.25	0.25–100	0.5	108 (±4)	1.5	104 (±2)	10	103 (±2)
AFG$_1$	0.25	0.25–100	0.5	103 (±2)	1.5	104 (±0.4)	10	102 (±1)
AFG$_2$	0.25	0.25–100	0.5	98 (±6)	1.5	102 (±3)	10	99 (±5)
FB$_1$	10	10–1000	10	102 (±5)	30	95 (±4)	200	98 (±3)
FB$_2$	10	10–1000	10	102 (±5)	30	99 (±4)	200	97 (±4)
FB$_3$	10	10–1000	10	104 (±7)	30	99 (±1)	200	100 (±2)
OTA	1	1–400	2	98 (±2)	6	99 (±2)	40	100 (±2)
ZEA	10	10–6000	30	76 (±5)	90	88 (±3)	600	91 (±3)
DON	6	6–2400	12	95 (±3)	36	101 (±1)	240	100 (±2)
DON-3-Glu	10	10–2400	12	86 (±2)	36	109 (±6)	240	105 (±4)
NIV	200	200–40000	200	95 (±10)	600	99 (±2)	4000	98 (±3)
HT-2	5	5–1200	6	100 (±6)	18	105 (±3)	120	102 (±5)
T-2	3	3–1200	6	102 (±2)	18	106 (±4)	120	104 (±2)
3-Ac-DON	6	6–2400	12	98 (±2)	36	100 (±4)	240	98 (±3)
15-Ac-DON	6	6–2400	12	97 (±3)	36	98 (±3)	240	99 (±2)
DAS	6	6–2400	12	97 (±1)	36	106 (±3)	240	102 (±3)
NEO	6	6–2400	12	98 (±1)	36	101 (±4)	240	103 (±3)
FUS-X	6	6–2400	12	101 (±6)	36	104 (±3)	240	105 (±5)
Deepoxy-DON	6	6–1200	12	97 (±2)	36	104 (±2)	240	103 (±3)
Citrinin	6	6–2400	12	81 (±1)	36	81 (±1)	240	85 (±2)
Sterigmatocystin	2	2–1200	6	84 (±4)	18	89 (±2)	120	87 (±5)
Beauvericin	2	2–2400	12	86 (±7)	36	94 (±3)	240	95 (±5)
Enniatin A	3	3–1200	12	98 (±2)	36	101 (±2)	240	100 (±3)
Enniatin B	3	3–1200	12	108 (±2)	36	103 (±2)	240	102 (±1)
Enniatin A$_1$	3	3–1200	12	102 (±2)	36	104 (±2)	240	103 (±2)
Enniatin B$_1$	3	3–1200	12	99 (±4)	36	98 (±4)	240	99 (±2)
AOH-Me-Ether	6	6–2400	12	64 (±5)	36	65 (±3)	240	60 (±2)
Tentoxin	3	3–2400	12	96 (±5)	36	103 (±1)	240	102 (±1)
Tenuazonic acid	15	15–5000	12	97 (±8)	36	104 (±7)	240	102 (±5)
Altenuene	6	6–2400	12	102 (±7)	36	99 (±3)	240	98 (±5)

Table 5. Method LOQ (m-LOQ), method linear range and percentage recovery (Rec%) ±percentage relative standard deviation (%RSD$_r$, n = 3) of the tested mycotoxins at three spiking levels in peanuts.

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Analyte	m-LOQ (μg/kg)	Method linear range (μg/kg)	Spiking level #1		Spiking level #2		Spiking level #3	
			Spiked conc (μg/kg)	Rec% (±RSD$_r$%)	Spiked conc (μg/kg)	Rec% (±RSD$_r$%)	Spiked conc (μg/kg)	Rec% (±RSD$_r$%)
AFB$_1$	0.4	0.4–100	0.5	92 (±6)	1.5	101 (±4)	10	101 (±4)
AFB$_2$	0.25	0.25–100	0.5	99 (±7)	1.5	99 (±5)	10	99 (±4)
AFG$_1$	0.25	0.25–100	0.5	95 (±3)	1.5	101 (±10)	10	106 (±1)
AFG$_2$	0.25	0.25–100	0.5	93 (±5)	1.5	100 (±7)	10	101 (±5)
FB$_1$	10	10–1000	10	90 (±16)	30	89 (±5)	200	100 (±4)
FB$_2$	10	10–1000	10	104 (±4)	30	96 (±5)	200	101 (±3)
FB$_3$	10	10–1000	10	108 (±7)	30	104 (±4)	200	103 (±2)
OTA	1	1–400	2	91 (±3)	6	99 (±2)	40	95 (±2)
ZEA	10	10–6000	30	90 (±6)	90	94 (±7)	600	98 (±3)
DON	6	6–2400	12	97 (±5)	36	99 (±3)	240	103 (±1)
DON-3-Glu	30	30–1200	12	89 (±7)	36	102 (±4)	240	86 (±3)
NIV	200	200–40000	200	89 (±8)	600	106 (±4)	4000	93 (±1)
HT-2	5	5–1200	6	99 (±4)	18	99 (±7)	120	99 (±2)
T-2	3	3–1200	6	100 (±2)	18	101 (±2)	120	103 (±4)
3-Ac-DON	6	6–2400	12	105 (±3)	36	98 (±7)	240	102 (±3)
15-Ac-DON	6	6–2400	12	102 (±3)	36	96 (±6)	240	101 (±4)
DAS	6	6–2400	12	100 (±5)	36	105 (±3)	240	102 (±3)
NEO	6	6–2400	12	98 (±8)	36	95 (±3)	240	103 (±4)
FUS-X	6	6–2400	12	91 (±7)	36	97 (±1)	240	97 (±5)
Deepoxy-DON	6	6–1200	12	95 (±4)	36	95 (±3)	240	99 (±2)
Citrinin	6	6–2400	12	81 (±1)	36	94 (±0.1)	240	100 (±1)
Sterigmatocystin	2	2–1200	6	102 (±2)	18	100 (±1)	120	103 (±3)
Beauvericin	2	2–2400	12	70 (±4)	36	109 (±3)	240	79 (±13)
Enniatin A	3	3–1200	12	96 (±4)	36	99 (±3)	240	93 (±7)
Enniatin B	3	3–1200	12	81 (±5)	36	106 (±2)	240	106 (±3)
Enniatin A$_1$	3	3–1200	12	96 (±8)	36	100 (±5)	240	100 (±4)
Enniatin B$_1$	3	3–1200	12	96 (±6)	36	102 (±2)	240	102 (±6)
AOH-Me-Ether	6	6–2400	30	64 (±8)	90	66 (±4)	600	65 (±3)
Tentoxin	3	3–2400	12	93 (±3)	36	97 (±6)	240	101 (±4)
Tenuazonic acid	15	15–5000	12	92 (±6)	36	73 (±2)	240	73 (±2)
Altenuene	6	6–2400	12	104 (±1)	36	96 (±5)	240	95 (±4)

Table 6. Method LOQ (m-LOQ), method linear range and percentage recovery (Rec%) ±percentage relative standard deviation (%RSD$_r$, n = 3) of the tested mycotoxins at three spiking levels in figs.

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Analyte	m-LOQ (µg/kg)	Method linear range (µg/kg)	Spiking level #1		Spiking level #2		Spiking level #3	
			Spiked conc (µg/kg)	Rec% (±RSD$_r$%)	Spiked conc (µg/kg)	Rec% (±RSD$_r$%)	Spiked conc (µg/kg)	Rec% (±RSD$_r$%)
AFB$_1$	0.5	0.5–100	0.5	101 (±7)	1.5	97 (±10)	10	96 (±2)
AFB$_2$	0.5	0.5–100	0.5	98 (±9)	1.5	104 (±5)	10	100 (±5)
AFG$_1$	0.5	0.5–100	0.5	99 (±4)	1.5	101 (±1)	10	97 (±3)
AFG$_2$	0.5	0.5–100	0.5	97 (±1)	1.5	99 (±1)	10	99 (±2)
FB$_1$	10	10–1000	10	97 (±6)	30	75 (±2)	200	100 (±2)
FB$_2$	10	10–1000	10	93 (±2)	30	90 (±2)	200	97 (±1)
FB$_3$	10	10–1000	10	86 (±3)	30	87 (±3)	200	99 (±3)
OTA	3	3–400	3	95 (±4)	6	98 (±3)	40	97 (±4)
ZEA	10	10–6000	30	94 (±7)	90	99 (±7)	600	84 (±1)
DON	6	6–2400	12	100 (±9)	36	99 (±4)	240	95 (±3)
DON-3-Glu	10	10–2400	12	82 (±11)	36	93 (±8)	240	97 (±10)
NIV	200	200–40000	200	70 (±7)	600	75 (±9)	4000	85 (±10)
HT-2	5	5–1200	6	92 (±10)	18	98 (±0.3)	120	99 (±2)
T-2	3	3–1200	6	94 (±4)	18	101 (±2)	120	93 (±1)
3-Ac-DON	6	6–2400	12	95 (±10)	36	99 (±2)	240	99 (±3)
15-Ac-DON	6	6–2400	12	94 (±8)	36	98 (±1)	240	100 (±5)
DAS	6	6–2400	12	97 (±6)	36	99 (±3)	240	101 (±5)
NEO	6	6–2400	12	94 (±2)	36	100 (±1)	240	102 (±1)
FUS-X	6	6–2400	12	98 (±3)	36	100 (±1)	240	98 (±1)
Deepoxy-DON	6	6–1200	12	91 (±7)	36	99 (±0.3)	240	94 (±1)
Citrinin	6	6–2400	12	82 (±1)	36	73 (±1)	240	70 (±1)
Sterigmatocystin	2	2–1200	6	95 (±3)	18	91 (±2)	120	89 (±2)
Beauvericin	2	2–2400	12	79 (±8)	36	88 (±5)	240	82 (±6)
Enniatin A	3	3–1200	12	94 (±4)	36	99 (±3)	240	93 (±7)
Enniatin B	3	3–1200	12	89 (±1)	36	107 (±5)	240	108 (±4)
Enniatin A$_1$	3	3–1200	12	97 (±7)	36	99 (±3)	240	101 (±4)
Enniatin B$_1$	3	3–1200	12	95 (±5)	36	101 (±3)	240	103 (±5)
AOH-Me-Ether	10	10–3000	30	60 (±7)	90	63 (±0.1)	600	61 (±4)
Tentoxin	3	3–2400	12	98 (±8)	36	101 (±2)	240	96 (±6)
Tenuazonic acid	30	30–5000	12	102 (±2)	36	98 (±5)	240	101 (±3)
Altenuene	6	6–2400	12	101 (±2)	36	102 (±0.4)	240	102 (±1)

Table 7. Method LOQ (m-LOQ), method linear range and percentage recovery (Rec%) ±percentage relative standard deviation (%RSD$_r$, n = 3) of the tested mycotoxins at three spiking levels in animal feeds.

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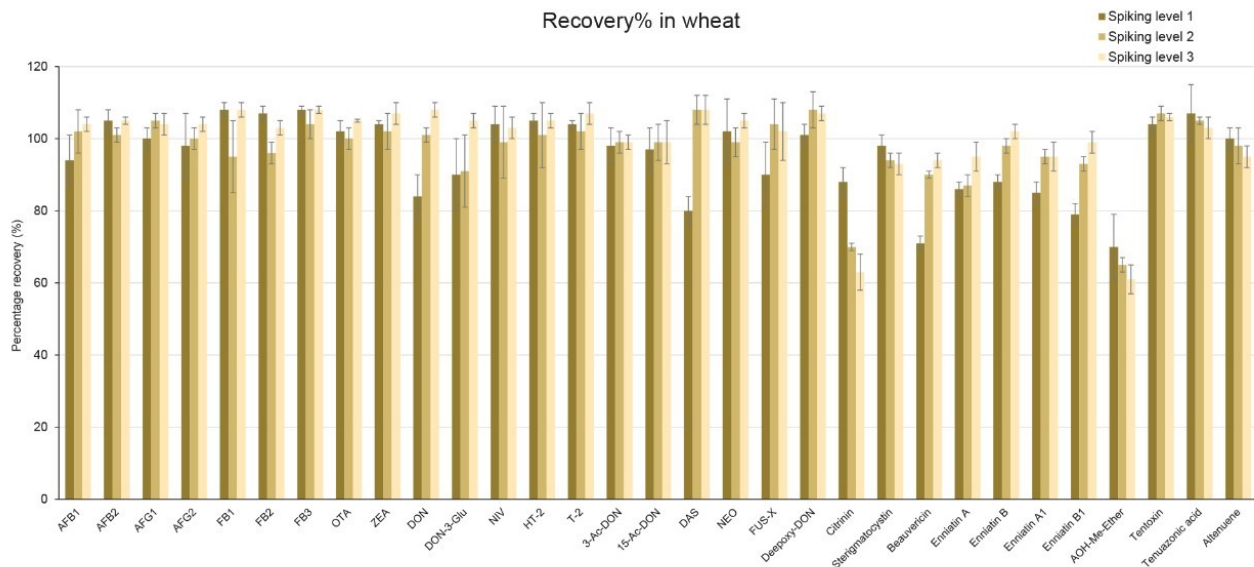
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Figure 3. Bar-plot showing mean percentage recoveries (Rec%) for the tested mycotoxins in wheat flour at three spiking levels. Error bars representing percentage relative standard deviation (%RSD$_r$, n = 3).
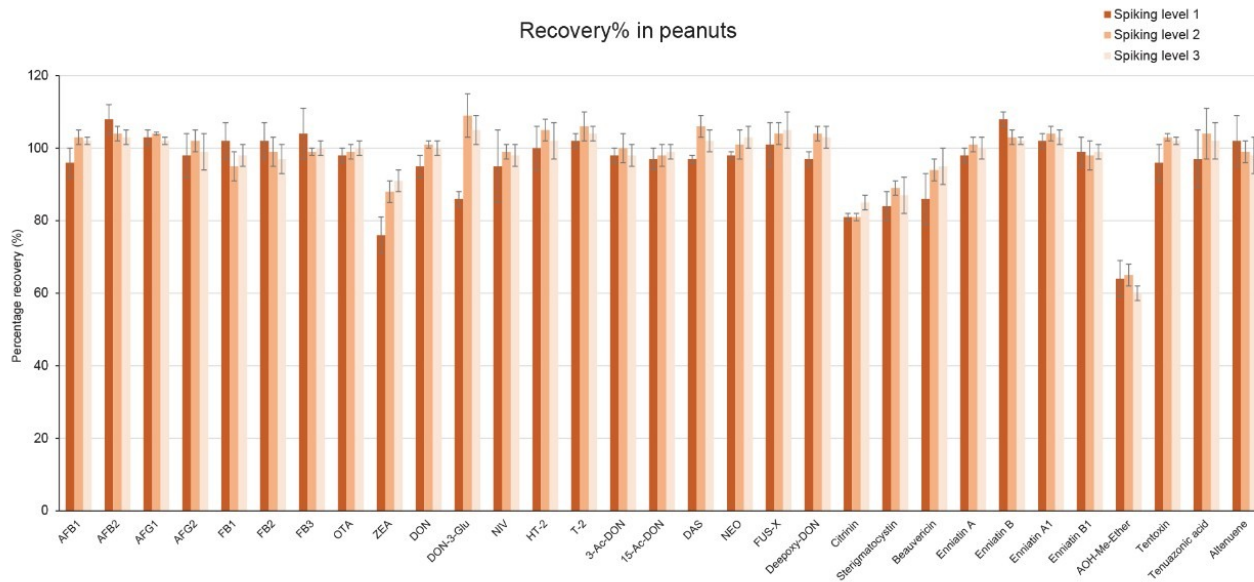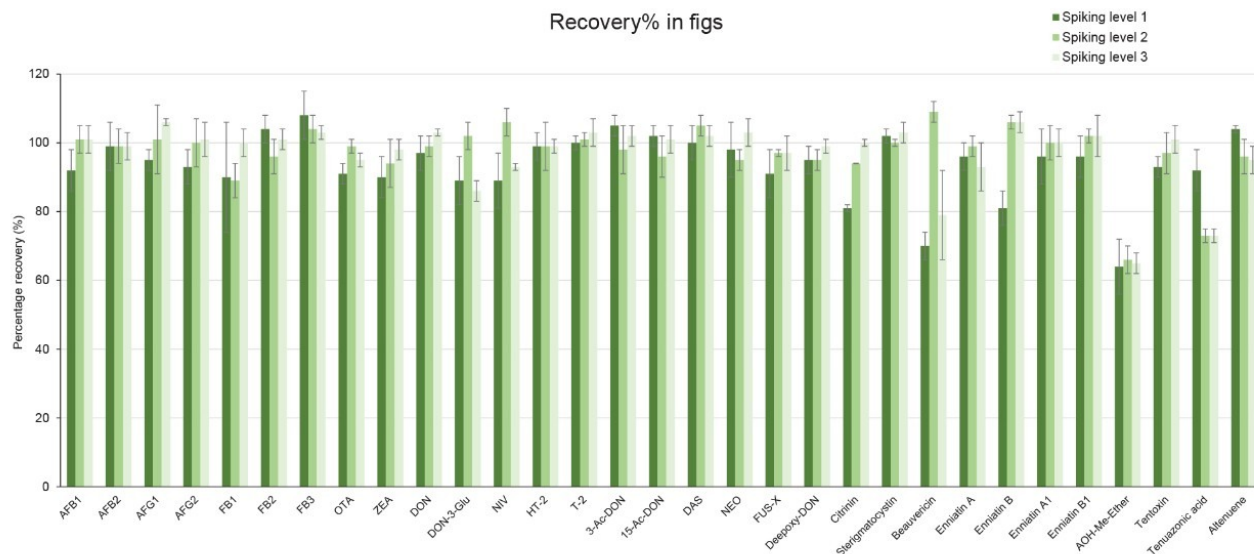

Figure 4. Bar-plot showing mean percentage recoveries (Rec%) for the tested mycotoxins in peanuts at three spiking levels. Error bars representing percentage relative standard deviation (%RSD$_r$, n = 3).

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Figure 5. Bar-plot showing mean percentage recoveries (Rec%) for the tested mycotoxins in figs at three spiking levels. Error bars representing percentage relative standard deviation (%RSD$_r$, n = 3).
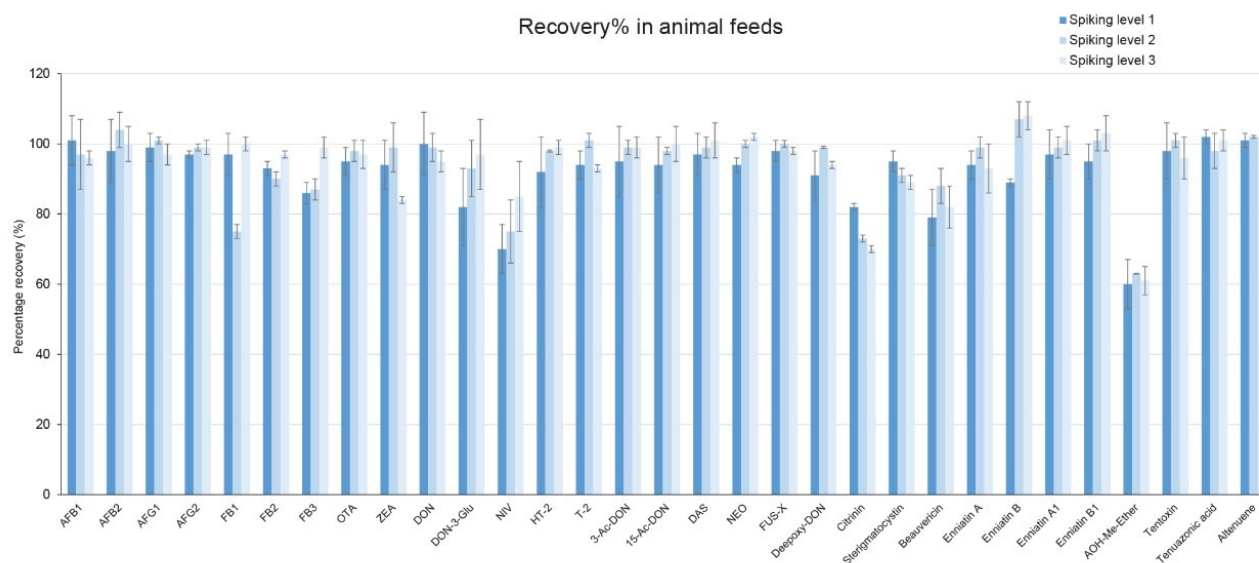

Figure 6. Bar-plot showing mean percentage recoveries (Rec%) for the tested mycotoxins in animal feeds at three spiking levels. Error bars representing percentage relative standard deviation (%RSD$_r$, n = 3). Trueness and repeatability were both also assessed using independent reference materials. Calculated concentrations in maize flour, hazelnuts/water slurry, dried figs/water slurry, and animal feeds reference material were well between ±2|z|-score. Trueness was in the range 71 and 99%, while repeatability (%RSD$_r$, n = 6) ranged between 0.5 and 7% (as shown in Tables 8, 9, 10, and 11).

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Analyte	Mean ±std. dev. (µg/kg)	RSD$_r$%	Xa (µg/kg)	Xa ±2|z| (µg/kg)	Trueness (%)
AFB$_1$	1.51 ±0.06	4	1.88	1.05–2.70	80
OTA	1.97 ±0.11	6	1.99	1.11–2.86	99
DON	181 ±1.4	1	231	139–323	78
ZEA	30.8 ±3.4	11	26.7	14.9–38.4	85
FB$_1$	575 ±2.7	0.5	526	341–711	91
FB$_2$	151 ±8.5	6	125	70–180	79
T-2	26.4 ±0.3	1	22.2	12.4–31.9	81
HT-2	27.0 ±2.0	7	22.9	12.8–33.0	82

Table 8. Mean ±standard deviation and percentage relative standard deviation (%RSD$_r$, n = 6) of the calculated concentrations in FAPAS maize reference material T04366QC. Xa = assigned value. Trueness (%) of the calculated concentration from the assigned value.

Analyte	Mean ±std. dev. (µg/kg)	RSD$_r$%	Xa (µg/kg)	Xa ±2|z| (µg/kg)	Trueness (%)
AFB$_1$	3.31 ±0.21	6	4.50	2.52–6.49	74
AFB$_2$	1.88 ±0.11	6	2.27	1.27–3.26	83
AFG$_1$	1.39 ±0.02	1	1.96	1.10–2.83	71
AFG$_2$	0.78 ±0.02	3	1.09	0.61–1.57	72
OTA	6.77 ±0.28	4	7.96	4.46–11.47	85

Table 9. Mean ±standard deviation and percentage relative standard deviation (%RSD$_r$, n = 6) of the calculated concentrations in FAPAS hazelnuts reference material T04390QC (water/nut slurry). Xa = assigned value. Trueness (%) of the calculated concentration from the assigned value.

Analyte	Mean ±std. dev. (µg/kg)	RSD$_r$%	Xa (µg/kg)	Xa ±2|z| (µg/kg)	Trueness (%)
AFB$_1$	2.26 ±0.07	3	3.01	1.68–4.33	75
AFB$_2$	1.57 ±0.05	3	1.76	0.98–2.53	89
AFG$_1$	2.11 ±0.07	3	2.8	1.57–4.03	75
AFG$_2$	1.0 ±0.02	2	1.04	0.58–1.50	95
OTA	5.17 ±0.16	3	6.36	3.56–9.16	81

Table 10. Mean ±standard deviation and percentage relative standard deviation (%RSD$_r$, n = 6) of the calculated concentrations in FAPAS dried figs reference material T04343QC (water/fruit slurry). Xa = assigned value. Trueness (%) of the calculated concentration from the assigned value.

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Analyte	Mean ±std. dev. (µg/kg)	RSD$_r$%	Xa (µg/kg)	Xa ±2|z| (µg/kg)	Trueness (%)
AFB$_1$	15.7 ±0.4	2	14.6	8.2–21.1	92
ZEA	936 ±39	4	906	612–1200	97
DON	2762 ±62	2	2702	1958–3447	98

Table 11. Mean ±standard deviation and percentage relative standard deviation (%RSD$_r$, n = 6) of the calculated concentrations in FAPAS animal feed reference material T04375QC. Xa = assigned value. Trueness (%) of the calculated concentration from the assigned value.

Conclusion

A multi-mycotoxin method has been successfully developed and validated for the 11 regulated mycotoxins and 20 emerging mycotoxins, including aflatoxins, ochratoxin A, fumonisins, zearalenone, type A and B trichothecenes, enniatins, beauvericin, sterigmatocystin, citrinin, and Alternaria toxins. The performance of the method fulfils the criteria set by the European Regulation No 401/2006 and SANTE guidelines. A simple and quick pass-through SPE clean-up was incorporated and allows the effective removal of some major interferences thus improving method robustness.

The method presented excellent trueness and repeatability, which was verified by using independent reference materials with known concentrations of mycotoxins. The method is applicable to a wide range of food commodities: wheat and maize representing cereals and high-starch-content dried foods; peanuts and hazelnuts representing ground and tree-nuts and high-lipid-content foods; figs representing fruits and high-polar-content foods; and animal feeds.

Finally, the analytical scope of the method can be extended in the future due to the remarkably wide applicability and versatility of the Oasis PRiME HLB clean-up.

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List of Abbreviations

AFB_1 = Aflatoxin B_1

AFB_2 = Aflatoxin B_2

AFG_1 = Aflatoxin G_1

AFG_2 = Aflatoxin G_2

FB_1 = Fumonisin B_1

FB_2 = Fumonisin B_2

FB_3 = Fumonisin B_3

OTA = Ochratoxin A

ZEA = Zearalenone

DON = Deoxynivalenol

DON-3-Glu = Deoxynivalenol-3-Glucoside

NIV = Nivalenol

HT-2 = HT-2 toxin

T-2 = T-2 toxin

3-Ac-DON = 3-Acetyl-deoxynivalenol

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15-Ac-DON = 15-Acetyl-deoxynivalenol

DAS = Diacetoxyscirpentol

NEO = Neosolaniol

Deepoxy-DON = Deepoxy-deoxynivalenol

FUS-X = Fusarenon X

AOH-Me-Ether = Alternariol-Methyl-Ether

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